Molecular Cell, Vol. 1, 301–308, January, 1998, Copyright ©1998 by Cell Press

The scs and scs′ Insulator Elements Impart a *cis* Requirement on Enhancer–Promoter Interactions

Jocelyn E. Krebs and Marietta Dunaway*
Department of Molecular and Cell Biology
University of California, Berkeley
Berkeley, California 94720-3202

Summary

The Xenopus rRNA enhancer activates its cognate promoter when the two elements are placed on opposite rings of dimeric catenanes. Here we show that when scs elements flank either the enhancer or promoter in catenanes, the enhancer cannot activate the promoter on the ring in *trans*. A series of catenanes containing different permutations of the insulators, enhancer, and promoters shows that when insulators are present, the enhancer is permitted to activate the promoter only when both elements are on the same piece of DNA with no intervening insulator. These results suggest that insulators have the potential to block enhancer–promoter interactions between chromosomes and between independent topological domains within a chromosome.

Introduction

An unresolved question in eukaryotic transcriptional regulation is how transcription units are organized in the chromosome so that promoters are activated by their cognate enhancers, but not by inappropriate enhancers. Experiments using either dimeric catenanes or two linear DNAs linked by a streptavidin bridge suggest that enhancers activate promoters through a random collision mechanism (Dunaway and Dröge, 1989; Müller et al., 1989; Wedel et al., 1990), resulting in formation of a DNA loop (Su et al., 1990, 1991). If this is generally true, enhancers and promoters on different chromosomes or in separate loops of a model chromosome with sequential radial loops could freely interact.

Insulator elements, also called boundary elements, may have the role of defining a transcriptional domain in vivo. One set of insulator elements, the scs and scs′ elements, was identified first through nuclease sensitivity studies of the *Drosophila melanogaster* 87A7 heat shock locus (Udvardy et al., 1985) and has been shown to protect an integrated reporter gene from negative position effects and from activation by endogenous enhancers (Kellum and Schedl, 1991). The observation that the scs and scs′ elements protect the promoter from activation by endogenous enhancers provided the basis for the enhancer-block assay, the most commonly used and well-defined assay available for these elements. In the enhancer-block assay, the insulator element is placed between an enhancer and promoter, and enhancer-activated transcription is measured (Kellum and Schedl, 1992). The scs and scs′ insulators block enhancer-activated transcription only when positioned between the enhancer and promoter but are not orientation-dependent (Kellum and Schedl, 1992; Cai and Levine, 1995; Dunaway et al., 1997). Insulator activity therefore depends on the position of the element with respect to the enhancer and promoter, yet the element itself has no inherent functional asymmetry.

Several models for the scs and for other insulator elements, such as the *suppressor of Hairy wing* binding sites from the *Drosophila* gypsy transposon and the chicken β-globin insulator, have been suggested. These include higher order chromatin structure (Udvardy et al., 1985; Kellum and Schedl, 1991; Vasquez and Schedl, 1994), tethering to restrict DNA movement (Chung et al., 1993), blocking a process that facilitates enhancer activation (Chung et al., 1993; Morcillo et al., 1996), and acting as a decoy or trap for the enhancer (Geyer, 1997). Although the available data do not unambiguously distinguish among these models, it is clear that the insulator elements do not form a repressive chromatin structure that prevents accessibility of transcription factors or recombinases to the DNA (Cai and Levine, 1995; Scott and Geyer, 1995; Dunaway et al., 1997).

Dimeric catenane analysis addresses whether a continuous path of DNA is required between two DNA elements or whether the DNA between two elements is simply a means of holding the two elements in high local concentration. This method has been used to determine the mechanism of sensing the orientation of sites for site-specific recombination (Benjamin et al., 1985; Craigie and Mizuuchi, 1986), to demonstrate the necessity for DNA tracking for type I restriction endonucleases (Szczelkun et al., 1996), and to distinguish between tracking and random collision mechanisms for transcriptional and recombinational enhancers (Dunaway and Dröge, 1989; Kanaar et al., 1989; Wedel et al., 1990; Herendeen et al., 1992). Since the *Xenopus laevis* rRNA enhancer activates the rRNA promoter when the two elements are placed on separate rings of dimeric catenanes (Dunaway and Dröge, 1989), and the scs and scs′ elements block enhancer-activated transcription of this enhancer–promoter pair in a *Xenopus* oocyte microinjection assay (Dunaway et al., 1997), we were in a position to use dimeric catenanes to probe the mechanism of the enhancer-blocking activity of the scs and scs′ insulator.

We show here that the scs and scs′ elements have the remarkable ability to block enhancer-activated transcription in dimeric catenanes where the scs elements flank the promoter or the enhancer. When the scs is isolated on a catenane ring, however, it has no effect on enhancer-activated transcription. The ability of the insulator elements to block the enhancer on a separate catenane ring suggests that the insulator confers a requirement on the enhancer to activate a promoter in *cis*. In the presence of insulators, therefore, an enhancer may activate a promoter only when both elements are on the same DNA segment with no intervening insulator elements. The ability of these insulators to prevent communication between an enhancer and promoter on separate rings of DNA suggests that scs elements have the

*To whom correspondence should be addressed.

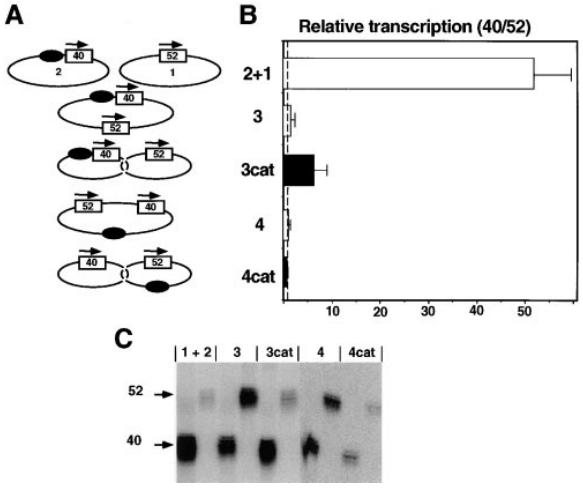

Figure 1. The rRNA Enhancer Has No Preference for Promoters in *cis*

(A) Plasmid constructs. The plasmid constructs used in this paper are numbered sequentially and plasmid maps are shown in Figure 6. The catenated form of the plasmid is designated by the construct number and cat. The cartoons show the composition of the catenane rings and the relative positions of the key elements within the rings. Neither the rings nor the parent plasmids are drawn to scale. A closed ellipse represents the rRNA enhancer, open boxes labeled 40 and 52 indicate the two rRNA reporters, and arrows indicate the direction of transcription.

(B) Quantitative transcription analysis. The ratio of transcription from the 40/52 promoter was determined by quantitation of phosphorimager data as described in the Experimental Procedures. The average of three independent experiments and standard error is shown. The transcription ratios from the unrecombined plasmids are shown by open bars, and closed bars are used to graph relative transcription of the catenanes. The dashed line on the graph marks a transcriptional ratio of 1 (i.e., the point at which transcription from both reporters is equal). This is indicated on all subsequent graphs as well.

(C) S1 nuclease assay.

potential to prevent interactions between enhancers and promoters on different chromosomes.

Results

The rRNA Enhancer Has No Significant Preference for a Promoter in *cis*

The enhancer-block assay for insulator elements is inherently complicated because it assays interference in the complex process of enhancer-activated transcription. Therefore, the interpretations of the insulator experiments are at least partly dependent on how well we understand enhancer-activated transcription itself. The assay that we use for enhancer-activated transcription is a competition assay in which two plasmid constructs are coinjected into the nucleus of *Xenopus* oocytes (Reeder et al., 1983; Labhart and Reeder, 1984). The two plasmids contain identical *Xenopus* rRNA promoters driving marked transcripts, but one plasmid also contains an rRNA enhancer. When the two plasmids are coinjected, the enhancer-bearing plasmid has a transcriptional advantage of 10- to 50-fold, depending on the batch of oocytes and the distance between the enhancer and promoter (Figure 1, constructs 1 and 2). Enhancer-activated transcription has now been reproduced in vitro, and these results strongly suggest that the two templates compete for the transcription factor xUBF, which competes in turn for Rib1 (SL1) (McStay et al., 1997).

We have previously shown that efficient enhancer-activated transcription occurs when the *Xenopus* rRNA enhancer and promoter are placed on separate rings of a dimeric catenane (Dunaway and Dröge, 1989). This experiment showed that a continuous piece of DNA is not necessary for enhancer-activated transcription, suggesting that the enhancer interacts with the promoter through random collisions. Although these experiments showed that enhancer-mediated activation can occur in dimeric catenanes, the question of whether random collision is the preferred or only mechanism of enhancer-activated transcription for this enhancer–promoter pair remains. To further address this possibility, we designed dimeric catenanes where two identical promoters compete for a single enhancer. In these catenanes, one promoter is located on the same ring with the enhancer while the second promoter is on the opposite ring. We will use the term *cis* to mean that two DNA elements are on one catenane ring and *trans* to indicate that the elements are on opposite rings.

In these experiments, coinjection of plasmids 1 and 2, where only plasmid 2 contains an enhancer, resulted in enhancer-activated transcription of more than 50-fold (Figure 1). However, when both reporters are placed on the same plasmid, the ratio of transcription levels is 1, indicating that the enhancer activates both promoters equally (Figure 1, plasmid 3). When plasmid 3 is recombined in vitro to yield catenane (cat) 3 and assayed by microinjection, the enhancer shows a 5-fold preference for the promoter in *cis*, rRNA 40. Although there is a preference for the promoter in *cis* in the dimeric catenane, the preference is reduced 10-fold compared to the case where separate plasmids are coinjected (compare 2 + 1 to 3cat).

We reasoned that the higher transcription level of the 40 reporter in catenane 3 might be due to the proximity of the enhancer to this promoter, not to a true preference for a promoter in *cis*. To test this possibility, the enhancer was placed approximately equidistant to both reporters (Figure 1, plasmid 4). Again, the transcription ratio for the promoters in the unrecombined plasmid is 1, indicating that the enhancer activates the two promoters equally. When construct 4 is recombined to yield catenane 4, the enhancer activates the two promoters almost equally, indicating that there is no significant preference for the promoter in *cis* with respect to the enhancer. The simplest interpretation of these results is that enhancer-activated transcription occurs by random collision in this enhancer–promoter pair in the oocyte.

Stable Transcription Complexes Are Formed within Minutes after Injection

The time that transcriptional activation of microinjected templates occurs in the oocyte bears upon the models for insulator activity and on the catenane experiments in general. If chromatin plays an important role in the scs mechanism, then the relative times of transcription

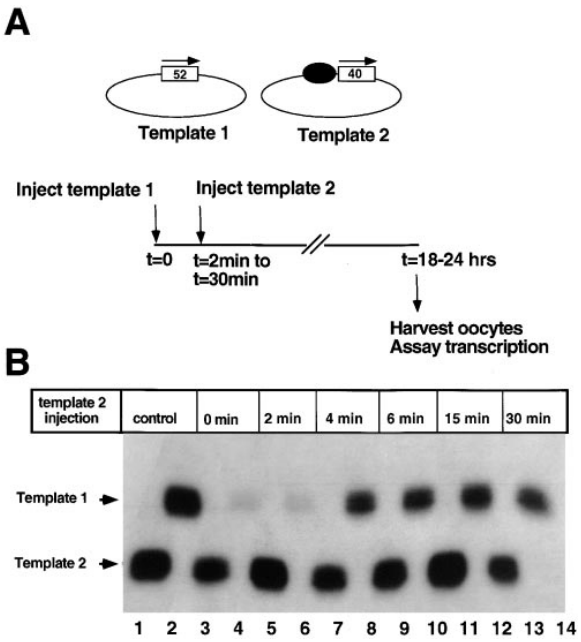

Figure 2. Stable Transcription Complexes Are Formed within Minutes after Injection

(A) Experimental strategy. Because of the short time points being done in an oocyte injection experiment, two people injected oocytes. Injector 1 injected template 1 while injector 2 injected template 2 at the times specified in the figure. At the shortest time points, it was necessary to use small pools of oocytes. This plus the speed at which each injector was required to work resulted in considerable variation in the amounts of template injected. Therefore, the transcriptional ratio between the two templates is not as accurate as in the other experiments in this paper.

(B) Transcription analysis. Lanes 1 and 2, identical promoters driving marked transcripts injected as a control; lanes 3 and 4, template 1 and 2 coinjected; lanes 5–14, template 1 injected at t_0 and template 2 injected at the indicated times.

activation and chromatin assembly are critical. Further, catenanes are rapidly decatenated after injection into the oocyte (Dunaway, 1990). Therefore, the efficiency of enhancer-activated transcription in dimeric catenanes suggests that transcriptional activation occurs very rapidly after injection (Dunaway and Dröge, 1989; Figure 1).

By measuring accumulated transcripts, we have previously shown that the scs insulator blocks enhancer-activated transcription in the oocyte before injected plasmids are fully assembled into nucleosomes (Dunaway et al., 1997). To further define the time frame in which transcription is established, we assayed the assembly of stable transcription complexes at short times after injection in a template-commitment experiment (Labhart and Reeder, 1984; Figure 2). In these experiments, an enhancerless promoter is injected to initiate the time course (t_0), and a second plasmid containing an enhancer is injected at increasing time intervals after the first injection. This second plasmid has a 10- to 50-fold transcriptional advantage when the two templates are coinjected. When stable transcription complexes are assembled on the enhancerless template, however, it escapes competition.

When plasmids 1 and 2 are coinjected, the transcription ratio in this experiment is about 20-fold (Figure 2). When only 2 min elapse before the enhancer-containing plasmid is injected, the transcription ratios are the same as when the plasmids are coinjected (Figure 2B, lanes 5 and 6), indicating that stable transcription complexes have not yet formed. However, when injection of the second plasmid is delayed for 4 min, the transcription ratio for the two plasmids is approximately 1. This indicates that template 1, containing only a promoter, has assembled stable transcription complexes prior to injection of template 2, thus allowing the promoter in template 1 to escape competition with the enhancer-containing template. Similar results to the 4 min time point are obtained for injection of the second template after longer time intervals, showing that the transcription complexes are stable during the remainder of their incubation in the oocyte, about 18 hr. These results show that stable transcription complexes form on the injected templates within 4 min after injection, while complete chromatin assembly requires 4 hr (Dunaway, 1990; Dunaway et al., 1997). Further, comparison of the time course of template commitment with previous experiments that monitored relaxation of injected plasmids or decatenation of injected plasmids (Dunaway, 1990) shows that stable transcription complexes are formed before either relaxation or decatenation is completed. Since the scs and scs′ insulators efficiently block enhancer-activated transcription, these results suggest that the scs acts within minutes after injection, long before bulk chromatin assembly is completed. Therefore, unless the transcriptionally active templates have a special chromatin assembly mechanism that occurs concomitantly with assembly of the transcription complex, it is unlikely that the enhancer-blocking activity of the scs is dependent on chromatin structure.

The scs and scs′ Elements Prevent Enhancer-Activated Transcription on Dimeric Catenanes

The experiments above lay the groundwork for analysis of the scs and scs′ insulator elements on dimeric catenanes. We constructed plasmids that can be recombined in vitro to produce catenanes which contain the rRNA promoter and enhancer plus the scs and scs′ elements (Figure 3). Plasmid 5, pPD40E, was used in the previous study to demonstrate that the rRNA enhancer efficiently activates transcription in dimeric catenanes (Dunaway and Dröge, 1989). Transcription in the unrecombined plasmid is enhanced 15-fold compared to the reference promoter, and this level of activation is typical for the rRNA enhancer and promoter separated by this distance (about 4 kb). Transcription from the recombined plasmid 5, 5cat, is enhanced 8-fold compared to the enhancerless reference, confirming that the catenanes hold the enhancer in sufficiently high local concentration to activate transcription.

We next confirmed previous observations that catenation per se does not affect transcription (Dunaway and Dröge, 1989). Both the unrecombined and the catenated forms of plasmid 6 are transcribed at the same level as the reference promoter (Figure 3), indicating that the recombination and purification procedures do not adversely affect transcription from the reporter gene. Similar results were obtained for a construct containing both

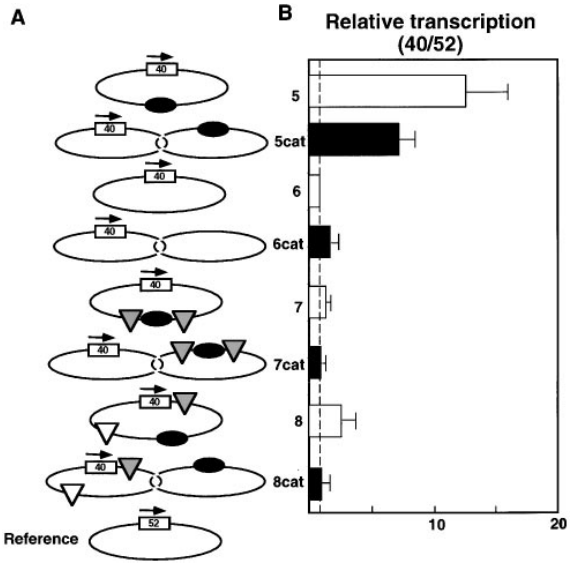

Figure 3. scs and scs′ Block Activation of the rRNA Promoter by the rRNA Enhancer in Dimeric Catenanes

(A) Plasmid constructs. The scs element is indicated by an open triangle, and the scs′ element is indicated by a closed triangle. Other symbols are described in the legend to Figure 1. (B) Transcription analysis. All plasmids and catenanes were coinjected with the indicated reference plasmid. Quantitated transcription data for the constructs shown in (A) is expressed as a ratio of the transcription levels of rRNA 40:rRNA 52. As in Figure 1 and subsequent figures, values represent the average of at least three independent injection experiments and the standard errors are shown.

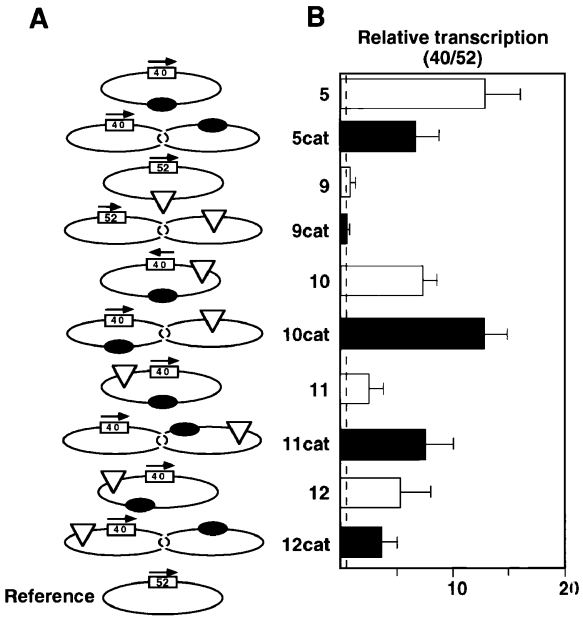

Figure 4. Analysis of Dimeric Catenanes Containing a Single scs Element

(A) Plasmid constructs. Symbols are described in the legend to Figure 1.

(B) Quantitated transcription data. Data analysis methods are described in the Experimental Procedures and in Figure 1. Note that plasmid 9 and 9cat were coinjected with a reference plasmid containing the 40 reporter.

the enhancer and promoter on one ring of the catenane, showing that catenation does not affect enhancer-activated transcription (2cat, data not shown).

With these controls in hand, we tested the effect of the scs and scs′ insulator elements on enhancer-activated transcription in plasmids 7 and 8. These enhancer-block constructs are larger plasmids than we have previously tested, and the enhancers in these plasmids are more than 4 kb from the promoter, a distance greater than either the rotational or torsional persistence length of naked DNA measured in vitro (Shore et al., 1981). Enhancer-activated transcription in each of these unrecombined plasmids is blocked, resulting in transcription levels no more than 2-fold greater than the enhancerless reference promoter. This suggests that the scs and scs′ insulators do not act by sterically hindering random collisions between the enhancer and promoter, in agreement with our previous results (Dunaway et al., 1997).

When plasmids 7 and 8 are recombined in vitro, the products are dimeric catenanes with the enhancer or the promoter flanked by scs and/or scs′ elements, respectively. The opposite catenane ring contains the second transcriptional element, the promoter or enhancer. Enhancer-activated transcription is blocked in both these dimeric catenanes, whether the insulators flank the enhancer (7cat) or the promoter (8cat). The insulators therefore interfere with the ability of the enhancer to activate transcription when it is on a separate catenane ring. This is surprising, given the previous observations that the enhancer-block activity of the insulator is position dependent. Apparently, the insulator imposes considerable constraints on the activity of the enhancer. In the presence of insulators, an enhancer can activate the promoter only when both elements are on the same continuous DNA molecule with no interposed insulator.

Analysis of Catenanes Containing a Single scs Element

To gain more insight into the constraints on enhancer-activated transcription in the presence of the scs element, we made a series of plasmid constructs that contain a single scs element (Figure 4). We first tested the effect of the scs on both promoter activity and enhancer-activated transcription when the insulator element was isolated on the opposite catenane ring (Figure 4, plasmids 9 and 10). There is no effect on promoter activity when the scs is catenated with the 52 reporter gene (9cat), indicating that the scs element has no effect on promoter activity in *trans*. When plasmid 10 is recombined in vitro, both the enhancer and promoter are on one ring with the scs on the opposite ring. Enhancer-activated transcription actually increases in 10cat, presumably because the scs element is removed from a partial blocking position on the unrecombined plasmid.

In plasmids 11 and 12, a single scs is interposed between the enhancer and the promoter, and in the catenanes a single scs is paired on one ring with the enhancer or promoter, respectively. In each case, the scs in the unrecombined plasmid partially blocks enhancer activation; construct 11 shows 20% enhancer activity of the control and construct 12 shows 40%. When construct 11 is catenated, the scs shares a ring with the enhancer. In contrast to the case where the enhancer is flanked by the scs′ elements, enhancer activation is

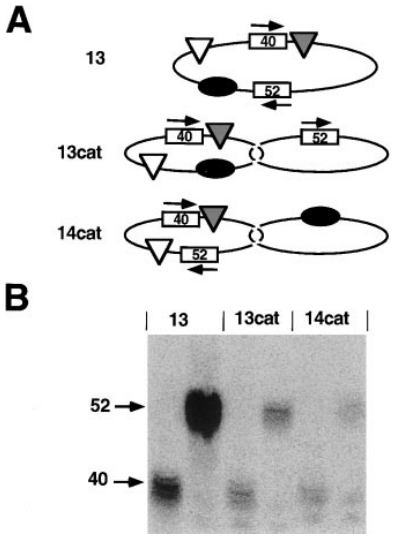

Figure 5. The scs Can Modulate Enhancer Interactions with Multiple Promoters

(A) Plasmid constructs.

(B) Transcription assay. The S1 nuclease assay of transcription from one injection experiment is shown. This experiment was also done in triplicate and quantitative results are reported in the text.

greater in the catenane than in the unrecombined plasmid and equals enhancer-activated transcription in the control catenane with no scs (5cat). This indicates that a single scs on the same ring as the enhancer does not detectably affect enhancer activity. However, when the scs shares the ring containing the promoter, enhancer-activated transcription is further reduced in the catenane compared to the unrecombined plasmid, showing that a single scs in *cis* with the promoter can partially block the enhancer on the opposite ring. These results clearly show that two insulators are necessary for full enhancer-block function either on the same DNA circle or on dimeric catenanes.

Analysis of the scs Insulator on Plasmids and Dimeric Catenanes Containing Two Promoters

The results above examine the ability of the insulators to block enhancer-activated transcription but do not demonstrate that the blocked enhancer is competent to activate transcription. We addressed this problem by testing whether an enhancer that is prevented from activating one promoter by scs insulators is still competent to activate a second promoter on the same plasmid. In constructs 13 and 14, the rRNA 40 reporter is separated from a single enhancer by scs and scs′ elements (Figure 5). A second promoter, rRNA 52 reporter, is also present in these constructs on the same side of the plasmid with the enhancer. Therefore, the enhancer is separated from the promoter in the 40 reporter by the insulator elements but is not separated from the promoter of the 52 reporter. Transcription data for the unrecombined plasmids shows that transcription from the rRNA 52 reporter is about 6-fold higher than from the rRNA 40 reporter in plasmid 13 (Figure 5B) and also in plasmid 14 (data not shown). The enhancer is competent to activate transcription in the presence of the scs and scs′ elements, consistent with the data from constructs containing a single promoter in the oocyte assay and with results from constructs containing two promoters in flies (Cai and Levine, 1995; Scott and Geyer, 1995; Dunaway et al., 1997).

When constructs 13 and 14 are recombined in vitro and assayed, the results are dramatically different from the unrecombined plasmids. In each catenane, the enhancer and rRNA 52 promoter are on different rings. In 13cat, the enhancer is isolated on a ring, and in 14cat the 52 reporter is isolated on a ring. In both catenanes, the two reporter genes are transcribed equally, indicating that neither promoter is preferentially enhanced. That is, the 52 promoter can be activated by the enhancer on the unrecombined plasmid, but not in the catenane. We consistently observe lower overall transcription levels in 13cat and 14cat. This is likely to be because the enhancer binds xUBF, and consequently SL1, but is unable to communicate with either promoter. This therefore sets up the same sort of competition between the enhancer and promoter as when the two elements are on separate, unlinked plasmids.

Discussion

Dimeric catenane analysis addresses the mechanism by which distant sequences come together by asking whether a continuous DNA path between two elements is required. The experiments presented here show that the scs and scs′ insulators prevent enhancer-activated transcription on dimeric catenanes when either element is flanked by the insulators. Analysis on dimeric catenanes therefore indicates that insulators can block the enhancer in *trans*, an activity that was previously unknown. When a single insulator element is placed on a catenane ring opposite either a promoter or enhancer–promoter pair, transcription is not affected, indicating that the scs itself cannot act in *trans*. Results from catenanes containing two promoters and two insulators further show that the enhancer is not inactivated by the insulators, and analysis of additional catenanes containing a single insulator element suggests that two insulators are required in the catenanes for effective blocking. These results show that insulators block an enhancer from activating a promoter on a ring that is topologically independent.

How can these results be reconciled with previous observations that the enhancer-block activity of the scs insulator is position dependent? We find it useful to consider the circumstances when the enhancer is permitted to activate rather than focusing on blocking. When insulators are present, an enhancer may activate a promoter only when the enhancer and promoter are on the same DNA segment. Furthermore, the insulators must not be placed between the enhancer and promoter. Considered from this point of view, blocking in the catenanes may indicate that the insulators impose a *cis* obligation on the enhancer. Because both the enhancer and the promoter must be on a continuous DNA segment with the insulators, this implies that there is a process involved that requires the DNA contour between these elements. Since the enhancer has no detectable inherent *cis* preference, the insulator is necessary for this

process. Further, the analysis of catenanes containing a single scs element shows that two insulator elements are required for full blocking activity, suggesting that the DNA path from insulator to insulator is important. Our results therefore suggest that the insulators have a mechanism for checking DNA continuity or impart this activity on the enhancer so that a promoter in *cis* can be distinguished from a promoter in *trans*. This interpretation implicates a tracking mechanism.

The plasmid-based microinjection assay used in these experiments necessarily focuses only on the enhancer-blocking activity of the insulators and may not reflect all the activities of these sequence elements in the chromosome. Given this limitation, the oocyte assay nevertheless shows that some aspects of chromosomal structure are not necessary for the enhancer-blocking activity of these elements. First, the idea that insulators function by tethering or physically constraining the DNA is unlikely given the results from the dimeric catenane experiments. We find that when either the enhancer or the promoter is flanked by the scs and/or the scs′ insulator elements on one ring of the dimeric catenane, enhancer-activated transcription does not occur. If tethering occurred through the scs element, the transcriptional element on the opposite catenane ring without insulator elements would not be constrained. We would expect then that enhancer-activated transcription would occur, unless there is additional steric hindrance on the scs catenanes that is not detected in the enhancer–promoter catenanes. The existence of additional steric barriers seems unlikely given that enhancer activity is not blocked when a single scs is on the same ring as the enhancer. Previous experiments showing that FLP recombinase can recombine a plasmid containing scs elements in the oocyte also suggest that the insulator elements do not prevent interaction between distant DNA sites by physically constraining or sequestering the DNA.

The results from these experiments also argue against chromatin structure as a predominant feature in insulator activity. We showed that transcriptional activation occurs within 4 min after injection, before separation of the catenanes into product rings by topoisomerase II and long before chromatin assembly is complete. Further, we measured enhancer-activated transcription from plasmids that contained one promoter separated from a second promoter and enhancer by scs and scs′ elements. In this unrecombined plasmid, the enhancer preferentially activates the promoter that is not separated by scs elements. Therefore, the enhancer is competent to activate the promoter. In dimeric catenanes of these plasmids, the enhancer could activate neither promoter. Although we cannot formally exclude the participation of chromatin structure in scs activity, a model proposing that changes in the chromatin structure between the scs and other regulatory elements play a dominant role in scs function would necessarily invoke a specialized chromatin assembly process concomitant with transcription to be consistent with our results.

The effect of distance between the insulator elements and either the enhancer or promoter has not been systematically studied for any known insulator, although the su(Hw) binding sites can clearly block enhancer-activated transcription from sites that are very distant from the enhancer or promoter (Dorsett, 1993). Although we have not specifically tested the importance of distance between all the regulatory elements involved in these experiments, the large number of plasmid constructs used here utilize distances between the insulator and enhancer or promoter as small as 500 bp and as large as 4.5 kb. When we plot the distances between the enhancer and insulator, enhancer and promoter, and promoter and insulator versus blocking activity, no correlation emerges with respect to blocking and distance between elements (data not shown). Therefore, at least in the size range studied here, the relative positions of the elements and not their respective distances are crucial for enhancer blocking activity.

We have previously suggested that the insulator could be viewed as a positive regulatory element that participates in pairing the correct enhancer and promoter, rather than solely as an element that blocks inappropriate enhancer-activated transcription (Dunaway et al., 1997). Although our idea of direct interaction between the enhancer and insulator giving directionality to the latter is not well supported by the dimeric catenane analysis, the catenane data is consistent with the idea that the insulator plays an active role in specifying the activity of the enhancer, rather than acting as a barrier to the it.

Experimental Procedures

Template Construction

Simplified maps of the plasmids used in this study are shown in Figure 6. Plasmids 3, 4, 6, 7, 9, 10, 11, 12, and 14 are derived from the plasmid 5, pPD40E (Dunaway and Dröge, 1989). This plasmid contains direct repeats of the Tn3 resolvase recombination site and direct repeats of the phage λ int recombinase sites (not shown in the figure). Briefly, one EcoRI site in parent plasmid pAB7.0D was removed by digestion with EcoRI, trimming with T4 DNA polymerase and ligation. One of the NdeI sites was replaced by a KpnI linker. The resulting plasmid has a single EcoRI site and a single KpnI site. Constructs 8 and 13 are based on the plasmid pMA2350 (Stark et al., 1989). The 760 bp rRNA reporters 40 and 52 and the rRNA enhancer have been previously described (Labhart and Reeder, 1984; Krebs and Dunaway, 1996). These reporter genes have a full promoter extending to −240. The scs′ fragment used is the EcoRI-BamHI fragment used in previous studies (Kellum and Schedl, 1991; Dunaway et al., 1997). The scs fragment used in these constructs is a 680 bp fragment containing nucleotides 850–1530 of the original 1.8 kb scs (Kellum and Schedl, 1991; Dunaway et al., 1997). This deletion contains the DNase I–hypersensitive regions and resistant core, and has enhancer-blocking activity equal to that of the entire scs in the oocyte assay (Dunaway et al., 1997). All plasmids were maintained in either HWB189 or HB101, strains of E. coli that do not contain endogenous Tn3 or γδ resolvase.

Preparation of Catenanes

All catenanes used in this study are singly interlinked catenanes generated by in vitro recombination using purified Tn3 resolvase. Tn3 resolvase was purified essentially as described (Liu and Wang, 1987; Hatfull et al., 1989). Preparative recombination reactions contained 20 μg of DNA in 10 mM $MgCl_2$, 20 mM HEPES (pH 7.5), 175 mM NaCl, 5 mM EDTA, and Tn3 resolvase at an approximate ratio of 175 ng resolvase per μg of DNA, in a final volume of 200 μl. After 15 min on ice, reactions were incubated at 37°C for 1 hr. Because this reaction does not scale up well, 5–10 reactions with 20 μg of plasmid each were performed for each plasmid. Resolvase was inactivated by incubating at 70°C for 10 min.

In vitro recombination yields a mixture of recombined and parental products. Since Tn3 resolvase requires a supercoiled substrate, any

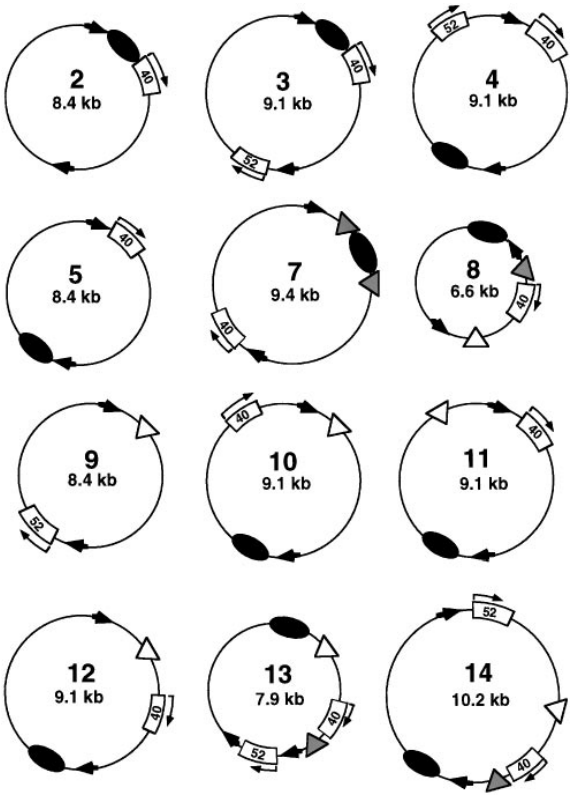

Figure 6. Plasmid Constructs

Simplified maps of the constructs used in this study are shown. Plasmid sizes are drawn to scale, and symbols for the enhancer, reporters, and insulators are approximately to scale. Symbols are those used in Figures 1 and 3. Bold arrows lying on the circle indicate sites for recombination by Tn3 resolvase. The components of each ring in the catenated products can be visualized by drawing a line between the two recombination sites in each plasmid.

nicked or relaxed molecules in the plasmid preparation will not be recombined. The unrecombined plasmids and the catenanes are the same size but must be separated for the experiments. Both product rings of Tn3 recombination are supercoiled, but catenanes in which only one ring is supercoiled can be separated from the other products and the starting parent plasmid on an ethidium bromide-CsCl gradient. To make a population of catenanes having one nicked ring, the recombination reaction is nicked with small amounts of DNase I in the presence of ethidium bromide. After the recombination reaction, 20 μg samples of DNA were precipitated and resuspended in 180 μl of 1X nicking buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 100 mM NaCl). Next, 2 μl of 10 mg/ml BSA, 8 μl of 10 mg/ml EtBr, and 1 μl of 20 μg/ml DNase I were added, and reactions were incubated at 30°C for 30 min and stopped by phenol extraction. This material was then resuspended and loaded on an ethidium bromide/CsCl gradient. The band between the nicked plasmid and supercoiled plasmid was harvested and purified.

Unrecombined parent plasmids, catenanes, and nicked catenanes were analyzed by electrophoretic separation on 0.8% TAE agarose gels (80 mM Tris-HCl [pH 7.5], 5 mM sodium acetate, 1 mM EDTA) containing 0.5% SDS (Sundin and Varshavsky, 1981). Gels were run at 2V/cm for 18–24 hr with buffer recirculation. After electrophoresis, the gels were washed extensively to remove SDS and then stained with ethidium bromide.

The purified catenanes having one nicked ring were used directly in injection experiments. Controls in which the nicked catenanes are coinjected with a supercoiled reference (Figures 3 and 4) indicate that neither the nicked template nor the purification method compromises the transcriptional activity of the catenated templates. The approximately 2-fold lower activation seen in singly interlinked catenanes compared to unrecombined parent or to multiply intertwined catenanes is consistently observed, and is probably due to the higher rate of decatenation of the singly linked catenanes in the oocyte (Dunaway, 1990). This level of activation is sufficient to allow us to perform enhancer-blocking experiments, and singly interlinked catenanes were used in all experiments reported here because of the ease of preparation and purity of the catenanes. We detect no difference in decatenation rate for plasmids containing scs or scs′ elements (data not shown).

Oocyte Injection and Transcription Analysis

Xenopus oocytes were microinjected with a reference and experimental plasmid in their circular forms. Approximately 20 nl of a solution containing 1 ng of each competing template and 50 μg/ml α-amanitin was injected into each oocyte nucleus. Between 50 and 60 oocytes were injected for each sample. Transcripts were allowed to accumulate overnight, except as noted, and the surviving oocytes (80% or greater) were collected and pooled the following day. The oocytes were homogenized in a buffer containing 1% SDS, 100 mM NaCl, 50 mM Tris-HCl (pH 7.8), 20 mM EDTA, and 0.1 mg/ml proteinase K. The mixture was incubated at 37° for 30 min and phenol extracted, and the nucleic acids were precipitated with ethanol. The precipitate was resuspended in 10 μl sterile TE buffer/oocyte and used in all subsequent assays without further purification.

Single-stranded, end-labeled probes that are specific for each of the two competing templates were prepared and hybridized with an equivalent of 5 oocytes from each injection sample. After hybridization, the samples were treated with S1 nuclease, and the reaction was stopped by addition of excess EDTA. DNAs were ethanol precipitated and electrophoresed on an 8% acrylamide, Tris-borate/urea gel. Template ratios were confirmed by Southern blots for each oocyte injection experiment.

To determine the transcriptional ratios from competing templates, both S1 nuclease protection assays and Southern blots were quantitated on a Molecular Dynamics Phosphorimager. Transcription values were corrected for both the template concentrations and for the specific activity of the individual S1 probes. Relative transcription is given by the ratio of experimental to reference transcription levels.

Acknowledgments

We thank Mengli Xiong for technical assistance, the Cozzarelli lab for gifts of reagents for recombination, and Mike Levine and members of the Dunaway lab for comments on the manuscript. This work was supported by a NIH grant GM49944.

Received May 16, 1997; revised October 6, 1997.

References

Benjamin, H.W., Matzuk, M.M., Krasnow, M.A., and Cozzarelli, N.R. (1985). Recombination site selection by Tn3 resolvase: topological tests of a tracking mechanism. Cell *40*, 147–158.

Cai, H., and Levine, M. (1995). Modulation of enhancer–promoter interactions by insulators in the *Drosophila* embryo. Nature *376*, 533–536.

Chung, J.H., Whiteley, M., and Felsenfeld, G. (1993). A 5′ element of the chicken β-globin domain serves as an insulator in human erythroid cells and protects against position effect in *Drosophila*. Cell *74*, 505–514.

Craigie, R., and Mizuuchi, K. (1986). Role of DNA topology in Mu transposition: mechanism of sensing the relative orientation of two DNA segments. Cell *45*, 793–800.

Dorsett, D. (1993). Distance-independent inactivation of an enhancer by the *suppressor of Hairy-wing* DNA-binding protein of *Drosophila*. Genetics *134*, 1135–1144.

Dunaway, M. (1990). Inhibition of topoisomerase II does not inhibit transcription of RNA polymerase I and II genes. Mol. Cell. Biol. *10*, 2893–2900.

Dunaway, M., and Dröge, P. (1989). Trans activation of the *Xenopus* rRNA promoter by its enhancer. Nature *341*, 657–659.

Dunaway, M., Hwang, J.Y., Xiong, M., and Yuen, H.-L. (1997). The activity of the scs and scs′ insulator elements is not dependent on chromosomal context. Mol. Cell. Biol. *17*, 182–189.

Geyer, P.K. (1997). The role of insulator elements in defining domains of gene expression. Curr. Opin. Genet. Dev. *7*, 242–248.

Hatfull, G.F., Sanderson, M.R., Freemont, P.S., Raccuia, P.R., Grindley, N.D., and Steitz, T.A. (1989). Preparation of heavy-atom derivatives using site-directed mutagenesis. Introduction of cysteine residues into γδ resolvase. J. Mol. Biol. *208*, 661–667.

Herendeen, D.R., Kassavetis, G.A., and Geiduschek, E.P. (1992). A transcriptional enhancer whose function imposes a requirement that proteins track along DNA. Science *256*, 1298–1303.

Kanaar, R., van de Putte, P., and Cozzarelli, N.R. (1989). Gin-mediated recombination of catenated and knotted DNA substrates: implications for the mechanism of interaction between *cis*-acting sites. Cell *58*, 147–159.

Kellum, R., and Schedl, P. (1991). A position-effect assay for boundaries of higher order chromosomal domains. Cell *64*, 941–950.

Kellum, R., and Schedl, P. (1992). A group of scs elements function as domain boundaries in an enhancer-blocking assay. Mol. Cell. Biol. *12*, 2424–2431.

Krebs, J.E., and Dunaway, M. (1996). DNA length is a critical parameter for eukaryotic transcription in vivo. Mol. Cell. Biol. *16*, 5821–5829.

Labhart, P., and Reeder, R.H. (1984). Enhancer-like properties of the 60/81 bp elements in the ribosomal gene spacer of *Xenopus laevis*. Cell *37*, 285–289.

Liu, L.F., and Wang, J.C. (1987). Supercoiling of the DNA template during transcription. Proc. Natl. Acad. Sci. USA *84*, 7024–7027.

McStay, B., Sullivan, G.J., and Cairns, C. (1997). The *Xenopus* RNA polymerase I transcription factor, UBF, has a role in transcriptional enhancement distinct from that at the promoter. EMBO J. *16*, 396–405.

Morcillo, P., Rosen, C., and Dorsett, D. (1996). Genes regulating the remote wing margin enhancer in the *Drosophila* cut locus. Genetics *144*, 1143–1154.

Müller, H.-P., Sogo, J.M., and Schaffner, W. (1989). An enhancer stimulates transcription in *trans* when attached to the promoter via a protein bridge. Cell *58*, 767–777.

Reeder, R.H., Roan, J., and Dunaway, M. (1983). Spacer regulation of *Xenopus* ribosomal gene transcription: competition in oocytes. Cell *35*, 449–456.

Scott, K.S., and Geyer, P.K. (1995). Effects of the su(Hw) insulator protein on the expression of the divergently transcribed *Drosophila* yolk protein genes. EMBO J. *14*, 6258–6267.

Shore, D., Langowske, J., and Baldwin, R.L. (1981). DNA flexibility studied by covalent closure of short fragments into circles. Proc. Natl. Acad. Sci. USA *78*, 4833–4837.

Stark, W.M., Sherratt, D.J., and Boocock, M.R. (1989). Site-specific recombination by Tn3 resolvase: topological changes in the forward and reverse reactions. Cell *58*, 779–790.

Su, W., Porter, S., Kustu, S., and Echols, H. (1990). DNA-looping and enhancer activity: association between DNA-bound NtrC activator and RNA polymerase at the bacterial glnA promoter. Proc. Natl. Acad. Sci. USA *87*, 5504–5508.

Su, W., Jackson, S., Tjian, R., and Echols, H. (1991). DNA looping between sites for transcriptional activation: self-association of DNA-bound Sp1. Genes Dev. *5*, 820–826.

Sundin, O., and Varshavsky, A. (1981). Arrest of segregation leads to accumulation of highly intertwined catenated dimers: dissection of the final stages of SV40 DNA replication. Cell *25*, 659–669.

Szczelkun, M.D., Dillingham, M.S., Janscak, P., Firman, K., and Halford, S.E. (1996). Repercussions of DNA tracking by the type IC restriction endonuclease EcoR124I on linear, circular, and catenated substrates. EMBO J. *15*, 6335–6347.

Udvardy, A., Maine, E., and Schedl, P. (1985). The 87A7 chromomere: identification of novel chromatin structures flanking the heat shock locus that may define the boundaries of higher order domains. J. Mol. Biol. *185*, 341–358.

Vasquez, J., and Schedl, P. (1994). Sequences required for enhancer blocking activity of scs are located within two nuclease-hypersensitive regions. EMBO J. *13*, 5984–5993.

Wedel, A., Weiss, D.S., Popham, D., Dröge, P., and Kustu, S. (1990). A bacterial enhancer functions to tether a transcriptional activator near a promoter. Science *248*, 487–490.