Human Molecular Genetics, 2005, Vol. 14, Review Issue 1 **R101–R111**
doi:10.1093/hmg/ddi104

Remote control of gene transcription

Adam G. West[1,*] and Peter Fraser[2]

[1]Division of Cancer Sciences and Molecular Pathology, University of Glasgow, Western Infirmary, Glasgow G11 6NT, UK and [2]Laboratory of Chromatin and Gene Expression, The Babraham Institute, Babraham Research Campus, Cambridge CB2 4AT, UK

Received January 3, 2005; Revised and Accepted February 17, 2005

In this review, we look at the most recent studies of DNA elements that function over long genomic distances to regulate gene transcription and will discuss the mechanisms genes employ to overcome the positive and negative influences of their genomic neighbourhood in order to achieve accurate programmes of expression. Enhancer elements activate high levels of transcription of linked genes from distal locations. Recent technological advances have demonstrated chromatin loop interactions between enhancers and their target promoters. Moreover, there is increasing evidence that these dynamic interactions regulate the repositioning of genes to foci of active transcription within the nucleus. Enhancers have the potential to activate a number of neighbouring genes over a large chromosomal region, hence, their action must be restricted in order to prevent activation of non-target genes. This is achieved by specialized DNA sequences, termed enhancer blockers (or insulators), that interfere with an enhancer's ability to communicate with a target promoter when positioned between the two. Here, we summarize current models of enhancer blocking activity and discuss recent findings of how it can be dynamically regulated. It has become clear that enhancer blocking elements should not be considered only as structural elements on the periphery of gene loci, but as regulatory elements that are crucial to the outcome of gene expression. The transcription potential of a gene can also be susceptible to heterochromatic silencing originating from its chromatin environment. Insulator elements can act as barriers to the spread of heterochromatin. We discuss recent evidence supporting a number of non-exclusive mechanisms of barrier action, which mostly describe the modulation of chromatin structure or modification.

INTRODUCTION

Metazoan locus control regions (LCR) and enhancer elements activate high levels of transcription of linked genes from distal locations. Although most enhancers are located tens of kilobases away, some have been found at distances of up to a megabase from their gene target (1–3). Enhancer elements, therefore, have the potential to activate a number of neighbouring genes over a large chromosomal region. Although some enhancers in fruit flies display some preference towards core promoter or promoter proximal elements, most enhancers appear to be promiscuous (4,5). Hence, the action of enhancers must be restricted in order to prevent the activation of non-target genes within their long reach. The transcription potential of a gene is also susceptible to silencing from its chromatin environment. A major fraction of vertebrate genomes is composed of gene-poor repetitive DNA and inactive genes that can exist as extended regions of condensed chromatin (6,7). Chromatin condensation processes are self-propagating and can spread into neighbouring gene loci, potentially affecting their expression (8). This is often observed when randomly integrated genes become subjected to chromosomal position effect silencing in transgenic cells or animals. Chromosomal position effect silencing has also been observed following translocations that result in human genetic disease (9,10). Genes must, therefore, employ mechanisms to overcome the positive and negative influences of their genomic neighbourhood, if they are to achieve accurate temporal, spatial and responsive modes of expression.

DEFINING CHROMATIN BOUNDARIES

It has been proposed that genes and gene clusters may achieve regulatory autonomy by organization into chromatin domains that are maintained independently of their surroundings

*To whom correspondence should be addressed. Tel: +44 1412112974; Fax: +44 1413372494; Email: a.west@clinmed.gla.ac.uk

through the establishment of chromatin boundaries (reviewed in 11). Chromatin boundaries are observed as a physical transition from transcriptionally repressive condensed chromatin to more permissive open chromatin structures. Boundaries can be the result of a balance between countervailing chromatin opening and condensation processes that are nucleated at neighbouring gene loci. Such boundaries would not be fixed, so large intergenic regions would be required to buffer a gene from its neighbours. Alternatively, specialized DNA sequences, termed insulators, can establish boundaries of fixed location (11). Insulator elements found from yeast to man share a common ability to protect genes from inappropriate regulatory influences from their neighbours and are functionally defined as having either or both of two activities in reporter assays. An element with enhancer blocking activity interferes with the communication between a linked enhancer and promoter when positioned between the two, but it has no or little effect when positioned at either side. In contrast, an element with heterochromatin barrier activity halts the propagation of transcriptionally repressive condensed chromatin structures only when positioned between sequences that nucleate heterochromatin and the gene promoter vulnerable to silencing.

DEFINING LCRs

Examination of β-globin gene sequences in Dutch γβ-thalassemia patients in the early 1980s revealed their gene structure to be normal but found that a large deletion of upstream sequences was associated with abnormal β-globin gene expression and disease (12). Subsequent work showed that the deletion removed a number of DNase I hypersensitive sites (DHSs) (13) in a regulatory region now known as the β-globin LCR (14). LCRs were functionally defined in transgenic mice through characterization of the human β-globin and *CD2* LCRs (14,15). Since then dozens of LCRs have been described for various genes (reviewed in (16). The property that set LCRs apart from other regulatory elements was their ability to drive expression of a linked transgene at a level equivalent to its endogenous counterpart from any position in the genome (14,17,18). Thus, LCRs were hailed as the sequences both necessary and sufficient for full, position-independent expression of a transgene, suggesting a role in opening up extensive chromatin domains. Considering this broad definition, it is understandable that many genomic regions defined as LCRs have since been found to contain or to be associated with a variety of regulatory elements, often combining classical enhancers and insulators (19–21). Although the feature of position-independent transgene expression with LCRs is well established, the suggestion that LCRs controlled widespread chromatin opening was contradicted by LCR knockouts (22,23) and chromatin structural studies showing that LCRs do not necessarily control the chromatin environment of their target genes (24). Recent results imply LCR-like elements have a primary role in transcription enhancement of poised or epigenetically potentiated genes that need not be related in structure or function (24–26). For example, the human B-cell-specific gene *Igβ* is located between the pituitary-specific human growth hormone (*hGH*) gene and its LCR. High levels of seemingly non-functional *Igβ* transcripts are detected in the pituitary of humans and transgenic mice carrying the *hGH* locus, suggesting that genes can be activated as innocent bystanders if positioned within an active chromatin domain (25).

Remote control by long-range elements led to new perspectives in the genetic analysis of specific diseases in which the suspected target genes appeared to be completely normal. For example, coding regions mutations in the *SOX9* gene have been shown to be responsible for autosomal sex reversal and campomelic dysplasia (CD); however, many cases of CD were found in which the *SOX9* gene was not disrupted but associated with distal genomic lesions (27,28). Transgenic experiments support the analysis of translocation breakpoints and suggest that several *SOX9* regulatory elements are located hundreds of kilobases upstream of the gene (29,30). Similarly, disruption of long-range regulatory elements up to a megabase from the sonic hedgehog (*Shh*) gene have been implicated in *Shh* misexpression and resultant preaxial polydactyly (1,31). The fact that regulation can be achieved from considerable genomic distances has engendered lively debate on the mechanisms of long-range gene control. Technical advancements in recent years have permitted a breakthrough in elucidating the contribution of LCRs and other genomic elements to the higher-order folding of chromatin domains through long-range chromatin interactions.

BRIDGING THE GAP

The models of long-range enhancer action fall into two basic categories: those that involve contact via direct interaction with the target gene and those that propose no contact. Two recently developed techniques: capturing chromosome conformation (3C) (32,33) and tagging and recovery of associated proteins (RNA TRAP) (34) have provided compelling evidence in favour of the former hypothesis. The 3C assay involves formaldehyde crosslinking of neighbouring chromatin regions in cells, followed by restriction enzyme digestion. After dilution to very low DNA concentration, intermolecular crosslinked chromatin fragments are ligated and detected via PCR across the novel ligation junctions. Several recent reports detail the use of this technique in the analysis of higher-order folding of specific loci and two groups have used 3C to investigate LCR–gene interactions in the β-globin and T_H2 cytokine loci (32,35–38).

The RNA TRAP technique directs horseradish peroxidase (HRP) activity to the site of a transcribing gene via a modified RNA FISH procedure. The localized HRP activity then catalyses the activation and subsequent covalent attachment of a biotinyl-tyramide tag onto chromatin proteins in the immediate vicinity of the transcribing gene. Tagged chromatin components are then recovered and the associated DNA analysed by PCR. RNA TRAP analysis of the transcribing mouse β-globin genes found enrichment of specific DHSs of the β-globin LCR (50 kb upstream) implying close association with the transcribing gene, whereas inactive intervening genes and distal upstream and downstream DHSs were not enriched (34). Similar interactions between the active gene

and LCR were inferred from 3C assays but ligation products for many other DHS elements both upstream of the LCR and downstream of the β-globin genes were also detected, implying that many elements over a 150 kb region participate in the formation of what has been called an 'active chromatin hub' (ACH) (37). The additional upstream and downstream DHS elements detected by 3C appeared to associate prior to, or in the absence of, active gene transcription (36,38), suggesting that an initial chromatin hub is formed between DHSs not directly involved in transcriptional activation of the genes. Interestingly, many of the regions that associate in the chromatin hub are capable of binding the protein CTCF and have enhancer-blocking activity (19,20) (discussed subsequently). Evidence for the formation of a precursor higher-order structure was also found in the T_H2 cytokine locus (35). In this case, genomic elements in the gene promoter regions formed an initial chromatin core configuration that was evident in non-expressing cell types and naive T-cells prior to transcriptional activation of the cytokine genes. Inclusion of the LCR in the ACH complex was found in differentiated cell types that expressed the cytokine genes.

ORGANIZING THE HUB FOR TRANSCRIPTION

What is missing from studies detailing interactions among LCRs, genes and other genetic elements is the key that explains how higher-order structure activates high-level gene transcription. It has been known for some time that gene positions in the nucleus often reflect activity states (39–43). In some cases, silenced genes have been found to associate with centromeric heterochromatin or the nuclear periphery, whereas active genes were found in more internal positions in the nucleus. Gene activity has also been linked to position relative to its chromosome's territory. In the mouse *HoxB* cluster, the sequential activation of genes was related to chromatin decondensation and nuclear reorganization (44). Concurrent with activation, an early expressed gene appeared to loop out of its chromosome territory and away from other late expressed genes in the cluster, only to be followed at the appropriate stage by a late expressing gene. Although extra-territorial positioning has been correlated with the transcriptional activity of several genes, transcription can also occur inside what is loosely defined as the chromosome territory (45). What has become increasingly clear is that individual genes are moving in association with function (46). Exactly where active genes are going was addressed in a recent study, showing that potentiated genes migrate to nuclear foci enriched in RNAP II and transcriptional machinery, known as transcription factories (Fig. 1) (47). Interestingly, many active genes over a 40 Mb region of mouse chromosome 7 ended up in the same transcription factory as the LCR-driven β-globin genes, indicating that genes migrate to pre-assembled, shared sites of transcription. The available evidence suggests that transcription factories are at least meta-stable structures (48,49), possibly formed through the principles of self-organization whereby the structure is formed and stabilized by the addition of interacting components and transcription units (50).

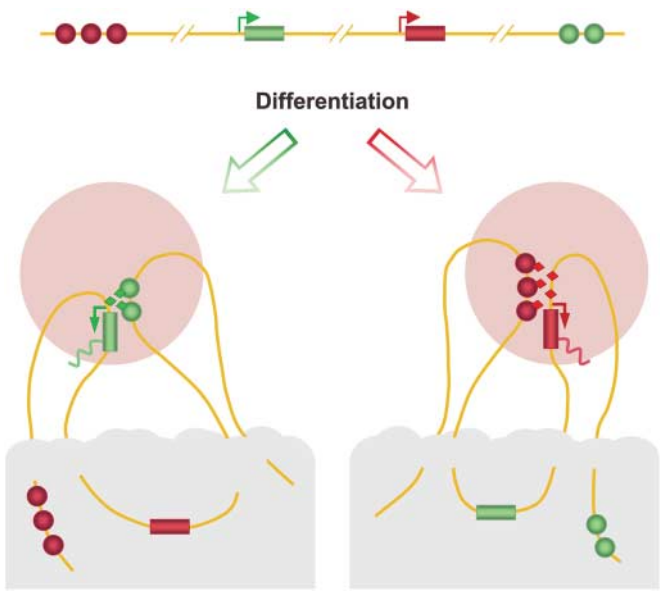

Figure 1. Long-range chromatin interactions upon gene activation. Recent studies have shown that upon their transcriptional activation, genes can migrate to the edge of, or beyond, their chromosomal territories (grey) to foci enriched in RNA polymerase II and transcriptional machinery, known as transcription factories (red disk). Gene promoters (arrows) have been observed to be in close physical proximity to linked enhancer elements (circles) upon transcription. *Trans*-acting factors (diamonds) recruited to enhancers and promoters determine interactions between looped chromatin fibres (gold). Different chromatin loops may form depending on the tissue-specific expression of *trans*-acting factors. Overlapping tissue-specific gene loci may operate independently from one another owing to the absence of their respective regulatory factors in one another's tissue. Alternatively, overlapping gene loci may share common enhancers of broad function. Promoter specificity factors or enhancer-blocking elements will be required to determine the correct promoter choice and loop interactions in such a situation.

FACILITATING ENHANCER–PROMOTER CONTACT

The ease of looping between a gene and distal enhancer is determined by the intrinsic flexibility of the chromatin fibre. By modelling the chromatin fibre as a polymer, it has been calculated that the three-dimensional juxtaposition of elements separated by several kilobases would be inefficient (51). This is supported by the finding that site-specific recombination between elements separated by several kilobases is inefficient in mammalian cells compared with interactions within a kilobase (52). It appears unlikely that unassisted looping would be either efficient or specific. Most importantly, it is hard to reconcile the position dependence of enhancer-blocking elements with a free diffusion model of enhancer looping. Rather, the position dependence of enhancer-blocking elements indicates that the initial communication between an enhancer and promoter is directly linked to the intervening chromatin fibre. We suggest that the following order of events leading to enhancer–promoter contact (Fig. 2). Step 1: LCR/enhancer-bound factors direct the assembly of, or migration to, a transcription factory. Step 2: the enhancer reels in the chromatin fibre in *cis* in search of a potentiated promoter. Step 3: interactions between enhancer- and promoter-bound factors stabilize the association of the promoter with the

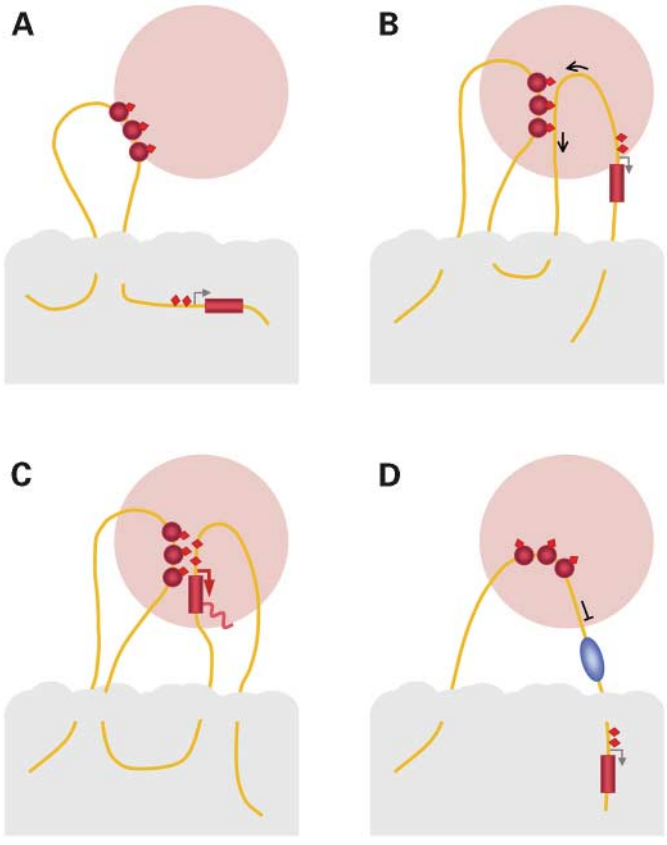

Figure 2. Events that might lead to enhancer-promoter contact. (**A**) Step 1: LCR/enhancer-bound factors direct the assembly of, or migration to, a transcription factory. (**B**) Step 2: The chromatin fibre is reeled in (black arrows) to search for a potentiated promoter (red) in *cis*. (**C**) Step 3: Interactions between enhancer- and promoter-bound factors stabilize the association of the potentiated promoter with the transcription factory to permit high levels of gene transcription (red arrow). (**D**) An enhancer-blocking element (blue) could interfere with chromatin fibre repositioning in step 2 in order to block a gene's access to a factory. Refer to text for models of enhancer blocking.

transcription factory. This model takes into account the *cis* positional dependence of enhancer-blocking elements in the context of looping interactions between genomic elements. It is quite conceivable that enhancer contact with the high concentration of immobilized RNA polymerase at a factory (49,53) in step 1 could lead to the initiation of intergenic transcription. The powerful motor protein activity of RNA polymerase (54), and associated chromatin remodelling enzymes (55), would facilitate reeling of the chromatin fibre in step 2. Enhancer-blocking elements could interfere with step 2 (56,57) by preventing gene access to a factory (Fig. 2D). In this scenario, it is likely that nuclear relocation (44) and the initial engagement of regulatory elements or their target genes with transcription factories (47) are critical steps that determine expression levels, especially in light of the finding that most 'active' genes are not continually transcribed, but appear to move in and out of factories with corresponding oscillations in transcription (47).

An alternate, though not necessarily exclusive, view is based on the finding that a number of highly conserved transcription factors have been described to facilitate long-range enhancer–promoter communication (58). For example, the conserved LIM-domain binding protein chip, which is required for maximal activation by a diverse set of enhancers in *Drosophila*, is proposed to act as a protein crosslinker. Chip can form cooperative interactions between LIM proteins nucleated at an enhancer and homeodomain proteins, whose binding sites are frequently interspersed between enhancers and promoters. Chip-assisted homeodomain binding may occur in a processive manner along the chromatin fibre, eventually bringing enhancer and promoter complexes into close proximity (59). Another enhancer facilitator protein, Nipped-B, is related to the adherins, which promote the loading and removal of cohesin (60). Remodelling of cohesin anchors may facilitate the movement of a chromatin fibre beyond its chromosomal territory.

SETTING THE BOUNDARIES OF ENHANCER ACTION

Although a number of enhancer-blocking proteins have been identified in *Drosophila* (11), CTCF so far exists as the only protein known to mediate enhancer-blocking activity in vertebrates. CTCF sites have been found at vertebrate enhancer blocking elements that function in a wide variety of contexts (20,61–74). Evidence from several systems has led to the proposal of a number of mechanisms to explain enhancer-blocking activity (Fig. 3). Enhancer blockers may mimic the characteristics of a promoter that mediate interactions with an enhancer. For example, enhancer-blocking proteins can interact with facilitator proteins to terminate enhancer-promoter communication (Fig. 3A) (75). The problem with this model is that a so-called decoy element placed outside a linked enhancer and promoter could sequester the enhancer and render it unproductive, thus behaving as a bidirectional silencer rather than a position-dependent enhancer blocker. However, position dependence may still be achievable if enhancer–decoy interactions are transient. If this is the case, a decoy element placed outside a linked enhancer and promoter may only reduce enhancer activity by a small fraction, but would repeatedly block enhancer activity when positioned between an enhancer and its target.

Another model for enhancer blocking involves the tethering to other elements or fixed structures in the nucleus. The *gypsy* element from *Drosophila* is observed to be tethered to foci near the nuclear periphery, whereas the chicken HS4 element has been found to be tethered to the nucleolar periphery in human cells (76–78). It appears unlikely that the nuclear localization resulting from tethering to a fixed structure would determine enhancer-blocking activity, as any affects of localization on enhancer activity would not be position dependent. Consistent with this view, it has been shown that different sequences within the *gypsy* element mediate enhancer blocking and localization towards the nuclear periphery (78). Alternative interpretations are based on the observation that the tethering of multiple enhancer-blocking elements to the same foci can lead to the formation of chromatin loops (79). By using DNA FISH on nuclear halo preparations, probes located between two endogenous *Drosophila* insulators revealed that the intervening chromatin

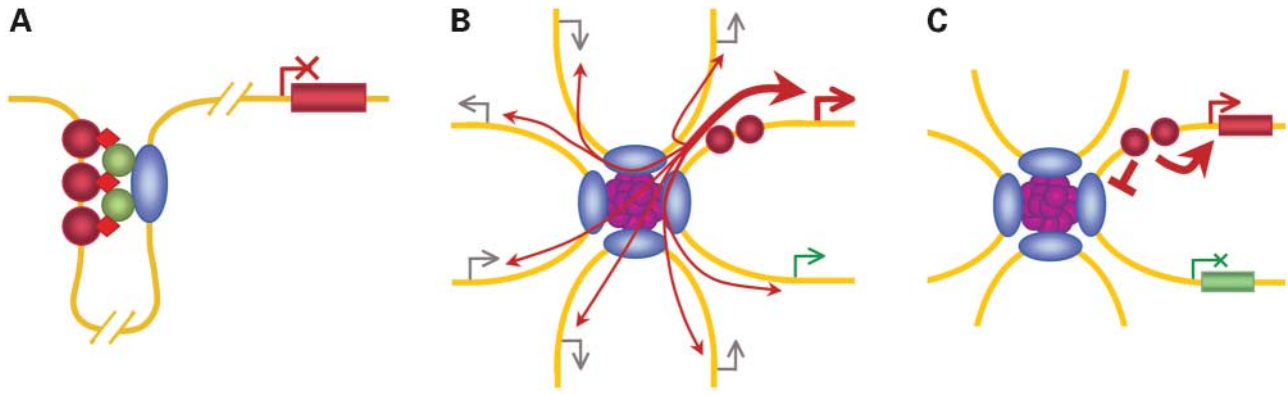

Figure 3. Potential mechanisms of enhancer-blocking. (**A**) Mimic or decoy: enhancer blockers (blue) may mimic the characteristics of a promoter that mediates interactions with enhancer factors (red). For example, enhancer-blocking proteins can interact with facilitator proteins (green) to terminate enhancer–promoter communication. (**B**) Distraction: an enhancer may be 'mis-directed' (red arrows) by the close proximity of other chromatin fibres (gold) at the base of loops tethered by enhancer blockers, resulting in a dilution of the enhancer's efforts to communicate with a particular target gene (green). (**C**) Obstruction: an enhancer may be blocked from making interactions with promoters beyond their own loop by steric interference at the base of the loop.

is arranged in a loop, with the two insulators located at the base. Furthermore, insulators were shown to cause the chromatin looping. Insertion of an additional insulator in the centre of the loop results in the formation of two smaller loops, and mutations of insulator-binding proteins lead to the disruption of the loop (79). Tethering of an enhancer blocker to foci could interfere with an enhancer's access to a gene promoter in two ways. First, an enhancer may be 'mis-directed' by the close proximity of other chromatin fibres at the base of the tethered loops, resulting in a dilution of the enhancer's efforts to communicate with the target gene (Fig. 3B) (11). There is evidence of such *trans* enhancer action in *Drosophila*, where homologous chromosomes are paired somatically (80). Secondly, enhancers may be 'obstructed' from making interactions with promoters beyond their own loop by torsional constraints or steric interference at the base of the loop (Fig. 3C). In this scenario, the choice of which neighbouring elements an enhancer blocker pairs with would be critical in determining which loop an enhancer is restricted to (reviewed in 81). This model would also depend on enhancer–promoter communication being linked to the chromatin fibre, otherwise there is little to stop enhancer–promoter communication between loops. For example, chromatin reeling driven by intergenic transcription from an enhancer might be obstructed at the base of the tethered loops. In support of this model, it has recently been demonstrated that the blocking of intergenic transcription from a β-globin enhancer can interfere with its long-range enhancement in a transgenic assay (56,57).

REGULATING ENHANCER BLOCKING

Recent studies have revealed a number of means by which enhancer-blocking elements can be regulated (Fig. 4). The binding of the vertebrate enhancer-blocking protein CTCF can be abrogated by CpG methylation, for example. The establishment of differential DNA methylation patterns can, therefore, be employed as means to regulate enhancer access, as observed at many imprinted gene loci (61,62, 64,66,67,73,74). The regulation of CTCF binding in this manner means that enhancer-blocking elements are sensitive to aberrant DNA methylation observed during disease progression, potentially resulting in deleterious enhancer cross-talk (63,82–85). It was recently shown that CTCF interacts with poly(ADP-ribose) polymerase (PARP) and that it is itself poly(ADP-ribosyl)ated (76,86). Poly(ADP-ribosyl)ation is associated with a number of enhancer-blocking elements, whose activity is dependent on PARP activity (86). Without studying modification site mutants of CTCF, it is unclear whether it is the modification of CTCF itself, or some other factor, that is required for enhancer-blocking activity of these elements. For example, the poly(ADP-ribosyl)ation of histones, associated with chromatin opening (87), may play an essential role in enhancer blocking at CTCF elements. Regardless, this finding highlights how the regulation of this post-translational modification may allow genome-wide control of insulators which may be critical during the setting of imprinted marks in germlines, for example.

Enhancer blocking has also been shown to be modulated by a number of protein co-factors. In vertebrates, the activity of CTCF-binding elements can be modulated by neighbouring binding sites for thyroid hormone receptor, TR (65). The enhancer-blocking activity of composite CTCF/TR binding elements, which are found at several gene loci, is abrogated in a hormone-dependent manner. This form of regulation can allow otherwise constitutive enhancer-blocking elements to be modulated in specific tissues during development and differentiation.

Transgenic assays in *Drosophila* point to a third way in which protein co-factors can lead to the regulation of an enhancer blocker, termed 'bypass'. The first evidence of bypass came from experiments where two copies of the *gypsy* insulator positioned in tandem neutralize one another's enhancer-blocking activity (88,89). It is postulated that closely spaced *gypsy* insulators physically pair with one another. We suggest that pairing interactions disrupt the enhancer-blocking activity of this insulator by interfering with its ability to form or tether to nuclear foci, although this remains to be tested. The enhancer-blocking activity of the *gypsy* element is dependent on the DNA-binding

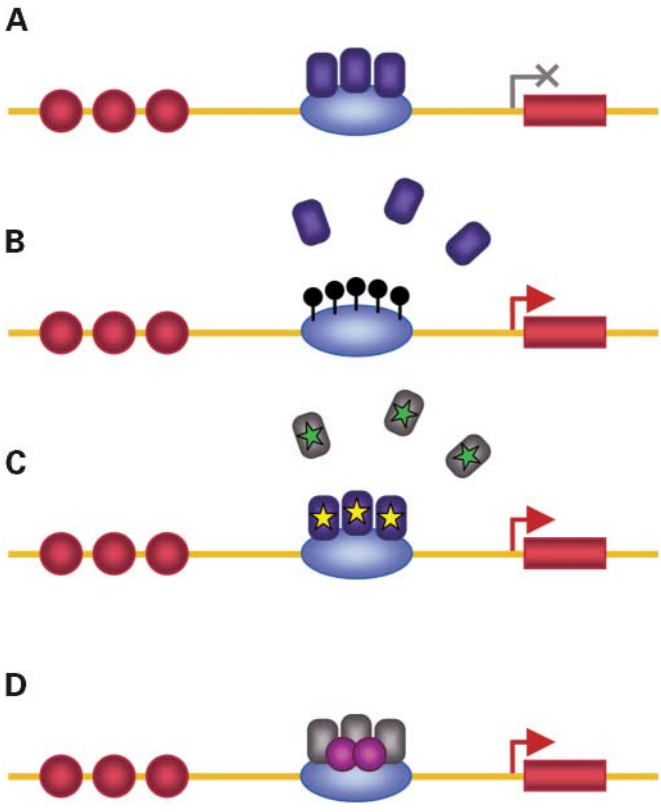

Figure 4. Regulating enhancer blocking activity. (**A**) Enhancer blocking mediated by enhancer-blocking proteins (blue). Recent evidence has shown that enhancer blocking can by regulated by a number of means. (**B**) The binding of enhancer-blocking proteins can be abrogated by DNA methylation (black lollipops) of their binding sites. (**C**) Post-translational modification of enhancer-blocking proteins (yellow stars) may be required to facilitate their activity, potentially by regulating their interactions with other proteins. Alternatively, modifications (green stars) may abrogate the activity of enhancer-blocking proteins, potentially by obstructing their interactions with proteins or their DNA sites. (**D**) Enhancer blocking has also been shown to be regulated by protein co-factors (purple) that may regulate the interactions of enhancer-blocking proteins.

protein Su(Hw) and its interaction partners mod(mdg4) and CP190 (11,90). Mod(mdg4) and CP190 mediate protein–protein interactions via their BTB domains to direct tethering to nuclear foci, and presumably mediate self-pairing. It was recently demonstrated that natural elements containing arrays of binding sites for the BTB protein GAGA protein can also allow an enhancer to bypass the *gypsy* enhancer-blocking element (91). This bypass of *gypsy* is dependent on the presence of both GAGA and mod(mdg4), which can efficiently interact with one another through their BTB domains. Pairing may be a common means of mediating bypass of the subgroup of enhancer-blocking elements that function by tethering to nuclear foci. Dynamic regulation of enhancer blocking is achievable by regulating the expression of proteins that interact with the pairing elements, such as the BTB factors, which can then in turn interfere with adjacent enhancer blockers.

It remains to be demonstrated whether pairing interactions are used to regulate the activity of enhancer blockers in their natural contexts. We suggest that the anti-insulator component of the promoter targeting sequence (PTS), which is located adjacent to the *Fab-8* insulator of the *Drosophila Abd-B* homeotic gene locus, may function as a pairing module (92). The PTS element can abrogate the enhancer-blocking activity of both the *Fab-8* and the *gypsy* insulators to permit enhancer bypass in specific-cell types (92,93). It is tempting to speculate that the PTS may pair with *Fab-8* in a cell-type specific manner. It should be cautioned that not all elements that function through BTB factors can mediate bypass of the *gypsy* element (94). Furthermore, other well characterized *Drosophila* enhancer blockers that do not use BTB factors to mediate their interactions are not sensitive to tandem pairing (94–96). Limited evidence suggests that CTCF-mediated enhancer blocking in vertebrates is not sensitive to tandem pairing. For example, two tandem copies of the chicken β-globin HS4 element display normal enhancer blocking in cell culture or when inserted into mice to substitute for the *Igf2/H19* ICR element (97,98). Furthermore, a duplicated *Igf2/H19* ICR element also retains its CTCF-mediated enhancer blocking function (99). Nevertheless, the principle of bypass of enhancer blocking has been established and may occur at vertebrate gene loci with complex patterns of regulation.

DEFINING THE BOUNDARIES OF CHROMATIN STATES

Insulator elements were first identified at defined boundaries between open and condensed chromatin domains (11). These boundary elements were found to possess both enhancer blocking and heterochromatin barrier activities, but recent dissection of insulators has revealed that these activities are separable. For example, the enhancer-blocking activity of the chicken β-globin HS4 element is dependent on a single binding site for the protein CTCF, whereas binding sites for other proteins including USF are required for its barrier activity (97,100,101). The *Drosophila* SF1 insulator also has separable enhancer blocking and barrier activities (102) (H. Cai, personal communication). Furthermore, CTCF-binding enhancer-blocking elements from several diverse gene loci do not harbour barrier activity (101). The barrier activity of the HS4 element has proved useful in protecting transgenes from chromosomal position effect silencing in a wide variety of contexts in vertebrates (103). It is necessary to flank transgenes with barrier elements to prevent silencing, suggesting that they interfere with the spread of silencing, rather than promote activation. Indeed, the removal of barrier elements or their binding proteins leads to the spread of heterochromatin markers beyond their natural boundaries (100,104). Heterochromatin from yeast to man is understood to assemble in a stepwise process, originating at a nucleation site from which it can spread onto nearby sequences (reviewed in 8). For example, the methylation of H3-lysine 9 creates a binding site for chromo-domain adaptor proteins such as heterochromatin protein 1 (HP1). HP1, in turn, can recruit SUV39H1, an H3-lysine 9-specific histone methyltransferase, in principle allowing further methylation of H3-lysine 9 and HP1 binding to extend onto successive nucleosomes in a

self-propagating fashion. Equivalent mechanisms for the propagation of silencing may involve other modifications and their associated proteins, such as histone deacetylation and Sir proteins in yeast or the methylation of H3-lysine 27 and the polycomb group of proteins in metazoa.

Studies of barrier elements from yeast to vertebrates have led to the development of several models of barrier activity (Fig. 5). One passive mechanism is the tethering of barrier elements to fixed structures, which may create a steric hindrance to propagation mechanisms. It has been shown that artificial tethering to the nuclear pore complex (NPC) can form an efficient barrier to silencing at the HML locus in budding yeast, for example (Fig. 5A) (105). Many yeast genes localize to nuclear pores upon their transcriptional activation indicating that this may be a compartment of the nucleus particularly favourable to transcription (39). Vertebrate genes, on the other hand, show no preferential association with nuclear pores when transcribed, although a comprehensive screen has not been performed. However, targeting to a compartment does not explain the position dependence of barrier elements or the necessity to flank a reporter gene with NPC barriers. Furthermore, although tethering to the NPC can form a barrier to silencing at the HML locus, it does not block silencing at HMR (106). It is possible that distinct modes of silencing exist at each of these loci, with an NPC-tethered barrier having a different level of effectiveness against each. Tethering to fixed structures has not been demonstrated at natural barriers to date. Interestingly, a mutation of the CTCF-binding site within the chicken HS4 insulator element abrogates its tethering and enhancer-blocking activity but has no affect on its barrier activity (76,101).

Another passive mechanism involves the creation of a nucleosome gap, thereby interrupting the substrate that heterochromatin factors require (Fig. 5B) (107). This can be achieved through multiple sites for DNA-binding proteins that prevent nucleosome placement. A variation on this model is nucleosome masking, where barrier-binding proteins also interact with histones (Fig. 5C). For example, the NFI transcription factor family protein CTF-1, whose binding sites can form a barrier to silencing at budding yeast telomeres, specifically interacts with the histone variant H3.3 (108). It is proposed that CTF-1 competes with the binding of co-repressor proteins such as the histone deacetylase Sir2 to block the propagation of heterochromatin. A more substantial and active approach to barrier formation involves manipulation of the histone code (Fig. 5D). The modification of histones is a common component of native chromatin boundaries in budding yeast (109). Furthermore, it has been demonstrated that the artificial tethering of histone acetyltransferases to chromatin is sufficient to form a barrier to heterochromatin in yeast (110). The barrier activity of the most well characterized vertebrate insulator element, chicken HS4, is also dependent on a number of histone modifications. Nucleosomes in the immediate vicinity of the HS4 element are acetylated at multiple sites on H3 and H4 and methylated at H3-lysine 4 (111,112). It was recently found that the ubiquitous transcription factor USF is responsible for recruiting a number of histone modifying enzymes responsible for the modification profile of the HS4 element (100). These findings have lead to a chain terminator model of barrier activity, where activating histone modifications localized at a barrier element interrupt the propagation of silencing histone modifications by chromatin condensation complexes. Consistent with this view, removal of the USF binding site or knock down of USF expression results in a loss of HS4 barrier activity, leading to an encroachment of silent chromatin marks over the barrier (100). Active histone modifications may contribute to barrier activity either by blocking the modification of the

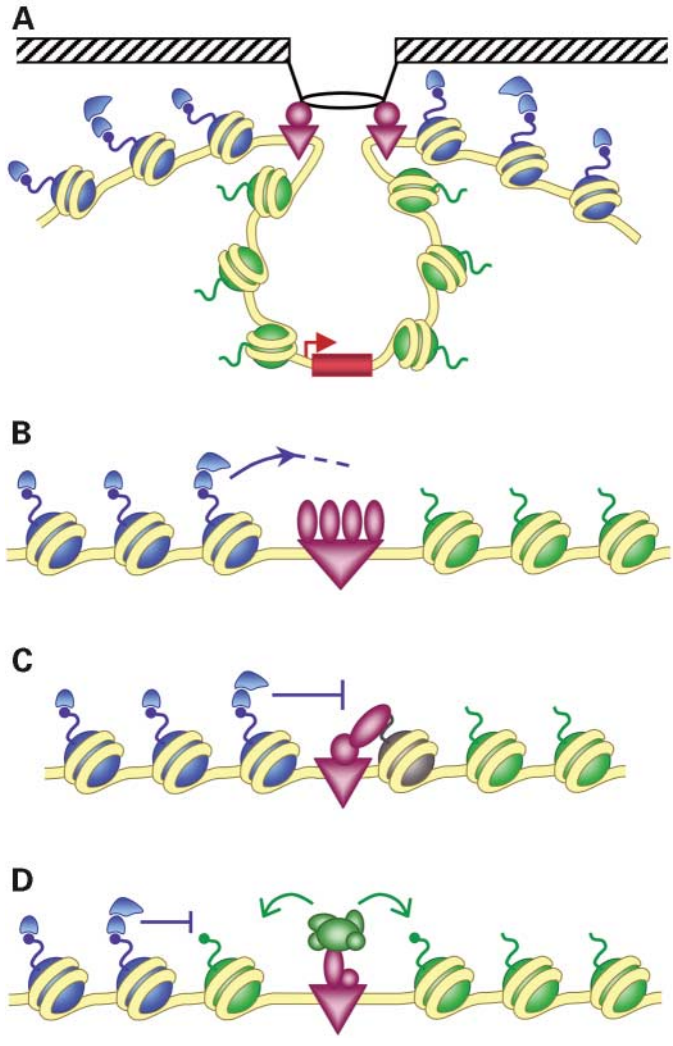

Figure 5. Potential mechanisms of barrier action. (**A**) Tethering: it was recently shown that artificial tethering to a fixed structure such as the NPC (black) can form an efficient barrier (purple) to the spread of histone modifications (blue dots) and heterochromatin factors (blue) associated with repression. Flanking with barriers (purple) can help a transgene to establish an active chromatin domain (green). (**B**) Nucleosome gap: multiple binding sites for some barrier proteins (purple) have been shown to exclude nucleosomes, thereby interrupting the substrate required for further heterochromatin factor binding. (**C**) Nucleosome masking: it was recently shown that a barrier protein can compete with heterochromatin propagation by binding directly to histones. (**D**) Histone code manipulation: evidence from several natural barrier elements has revealed that barrier proteins often recruit multiple histone modifying enzymes (green). Histone modifications associated with activation (green dots) in the immediate vicinity of the barrier can compete with the propagation of heterochromatin factor binding.

same histone residue required for silencing or by preventing the interaction of co-repressor complexes with histones. In striking similarity to HS4, peaks of H3-lysine 4 methylation also flank the active chromatin of the poised DJ regions of the murine *IgH* and *TCRβ* antigen receptor loci (113). It remains to be seen whether these regions also have barrier activity, but constitutive peaks of H3-lysine 4 methylation between gene loci may be a hallmark of barrier elements in vertebrates. Although the recruitment of active histone modifications is a necessary component of the HS4 barrier, it is not sufficient (100). This marks a clear distinction between this vertebrate barrier and those characterized in yeast. Mutagenesis of HS4 has found that three further protein binding sites, which do not contribute to the studied histone modifications at HS4, are also required for barrier activity (101). It is clear that this compound element still has stories to tell.

OUTSTANDING QUESTIONS

Recent technological advances have rekindled investigations into the long-range control of gene transcription. It has been demonstrated that enhancers are in close physical proximity to their target gene promoters in two out of two gene loci studied so far. It remains to be seen whether all enhancers mediate promoter contact, especially those that are located at distances of up to a megabase from their target promoters. Although the 3C and related methods will allow the study of enhancer–promoter contact at other well characterized gene loci, we suggest that it might also be possible to use the 3C approach to trap novel regulatory elements that are in physical proximity with a known gene promoter of interest. We have proposed a sequential model of the events that might lead to enhancer–promoter contact. It remains to be determined whether enhancers locate to, or nucleate, transcription foci prior to gene transcription. It may now be possible to study this at extended mammalian gene loci whose genes undergo considerable nuclear repositioning upon their transcription. It would be of great interest to observe what happens to their nuclear repositioning when an enhancer blocker is inserted between a distal enhancer and promoter. Will the enhancer blocker prevent repositioning of the gene? Will the enhancer blocker be in contact with the enhancer?

While insulator elements and their properties have been known for some time, we are just beginning to identify vertebrate insulators and understand their contributions to gene expression. We do not know how common insulators are in the human genome, how many have enhancer-blocking activity or how many have barrier activity. The recent identification of the first conserved vertebrate insulator proteins from studies of the chicken HS4 element now allows protein-led screens for novel elements. Only when we have detailed information from a large set of insulators can we assess their overall contribution to regulating transcription of the genome. The identification of insulator proteins also now allows a detailed investigation of their mechanism of action. More biochemistry is required to reveal their binding partners and/or the enzymatic activities associated with insulators. The most well studied vertebrate insulator HS4 has served as a paradigm constitutive element with both enhancer-blocking and barrier activities. Yet, since the identification of CTCF, none of the newly identified enhancer-blocking elements has been shown to have barrier activity. Are barrier elements uncommon or are they just rarely co-located with enhancer-blocking elements? In addition, a number of the human enhancer-blocking elements found to date are regulated. Can barrier elements also be regulated? Considerable progress has recently been made in beginning to identify and understand the elements we have discussed here. We now have the tools to address fundamental questions regarding their mechanisms and their roles in the remote control of gene transcription.

ACKNOWLEDGEMENTS

We are grateful to our colleagues, particularly Miklos Gaszner, Katherine West and the guest editors for their helpful discussions. Our laboratories are supported by the UK Biotechnology and Biological Sciences Research Council and Medical Research Council (MRC) and by the European Commission.

REFERENCES

1. Lettice, L.A., Heaney, S.J., Purdie, L.A., Li, L., de Beer, P., Oostra, B.A., Goode, D., Elgar, G., Hill, R.E. and de Graaff, E. (2003) A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. *Hum. Mol. Genet.*, **12**, 1725–1735.
2. Nobrega, M.A., Ovcharenko, I., Afzal, V. and Rubin, E.M. (2003) Scanning human gene deserts for long-range enhancers. *Science*, **302**, 413.
3. Qin, Y., Kong, L.K., Poirier, C., Truong, C., Overbeek, P.A. and Bishop, C.E. (2004) Long-range activation of Sox9 in Odd Sex (Ods) mice. *Hum. Mol. Genet.*, **13**, 1213–1218.
4. Butler, J.E. and Kadonaga, J.T. (2001) Enhancer–promoter specificity mediated by DPE or TATA core promoter motifs. *Genes Dev.*, **15**, 2515–2519.
5. Calhoun, V.C., Stathopoulos, A. and Levine, M. (2002) Promoter-proximal tethering elements regulate enhancer-promoter specificity in the Drosophila Antennapedia complex. *Proc. Natl. Acad. Sci. USA*, **99**, 9243–9247.
6. Gilbert, N., Boyle, S., Fiegler, H., Woodfine, K., Carter, N.P. and Bickmore, W.A. (2004) Chromatin architecture of the human genome: gene-rich domains are enriched in open chromatin fibers. *Cell*, **118**, 555–566.
7. Ghirlando, R., Litt, M.D., Prioleau, M.N., Recillas-Targa, F. and Felsenfeld, G. (2004) Physical properties of a genomic condensed chromatin fragment. *J. Mol. Biol.*, **336**, 597–605.
8. Grewal, S.I. and Moazed, D. (2003) Heterochromatin and epigenetic control of gene expression. *Science*, **301**, 798–802.
9. Kleinjan, D.A. and van Heyningen, V. (2005) Long-range control of gene expression: emerging mechanisms and disruption in disease. *Am. J. Hum. Genet.*, **76**, 8–32.
10. Sharp, A.J., Spotswood, H.T., Robinson, D.O., Turner, B.M. and Jacobs, P.A. (2002) Molecular and cytogenetic analysis of the spreading of X inactivation in X; autosome translocations. *Hum. Mol. Genet.*, **11**, 3145–3156.
11. West, A.G., Gaszner, M. and Felsenfeld, G. (2002) Insulators: many functions, many mechanisms. *Genes Dev.*, **16**, 271–288.
12. Kioussis, D., Vanin, E., deLange, T., Flavell, R.A. and Grosveld, F.G. (1983) Beta-globin gene inactivation by DNA translocation in gamma beta-thalassaemia. *Nature*, **306**, 662–666.
13. Tuan, D., Solomon, W., Li, Q. and London, I.M. (1985) The "beta-like-globin" gene domain in human erythroid cells. *Proc. Natl Acad. Sci. USA*, **82**, 6384–6388.
14. Grosveld, F., van Assendelft, G.B., Greaves, D.R. and Kollias, G. (1987) Position-independent, high-level expression of the human beta-globin gene in transgenic mice. *Cell*, **51**, 975–985.

15. Greaves, D.R., Wilson, F.D., Lang, G. and Kioussis, D. (1989) Human CD2 3′-flanking sequences confer high-level, T cell-specific, position-independent gene expression in transgenic mice. *Cell*, **56**, 979–986.
16. Li, Q., Peterson, K.R., Fang, X. and Stamatoyannopoulos, G. (2002) Locus control regions. *Blood*, **100**, 3077–3086.
17. Festenstein, R., Tolaini, M., Corbella, P., Mamalaki, C., Parrington, J., Fox, M., Miliou, A., Jones, M. and Kioussis, D. (1996) Locus control region function and heterochromatin-induced position effect variegation. *Science*, **271**, 1123–1125.
18. Milot, E., Strouboulis, J., Trimborn, T., Wijgerde, M., de Boer, E., Langeveld, A., Tan-Un, K., Vergeer, W., Yannoutsos, N., Grosveld, F. *et al.* (1996) Heterochromatin effects on the frequency and duration of LCR-mediated gene transcription. *Cell*, **87**, 105–114.
19. Bulger, M., Schubeler, D., Bender, M.A., Hamilton, J., Farrell, C.M., Hardison, R.C. and Groudine, M. (2003) A complex chromatin landscape revealed by patterns of nuclease sensitivity and histone modification within the mouse beta-globin locus. *Mol. Cell. Biol.*, **23**, 5234–5244.
20. Farrell, C.M., West, A.G. and Felsenfeld, G. (2002) Conserved CTCF insulator elements flank the mouse and human beta-globin loci. *Mol. Cell. Biol.*, **22**, 3820–3831.
21. Mutskov, V.J., Farrell, C.M., Wade, P.A., Wolffe, A.P. and Felsenfeld, G. (2002) The barrier function of an insulator couples high histone acetylation levels with specific protection of promoter DNA from methylation. *Genes Dev.*, **16**, 1540–1554.
22. Bender, M.A., Bulger, M., Close, J. and Groudine, M. (2000) Beta-globin gene switching and DNase I sensitivity of the endogenous beta-globin locus in mice do not require the locus control region. *Mol. Cell*, **5**, 387–393.
23. Reik, A., Telling, A., Zitnik, G., Cimbora, D., Epner, E. and Groudine, M. (1998) The locus control region is necessary for gene expression in the human beta-globin locus but not the maintenance of an open chromatin structure in erythroid cells. *Mol. Cell. Biol.*, **18**, 5992–6000.
24. Gribnau, J., Diderich, K., Pruzina, S., Calzolari, R. and Fraser, P. (2000) Intergenic transcription and developmental remodeling of chromatin subdomains in the human beta-globin locus. *Mol. Cell*, **5**, 377–386.
25. Cajiao, I., Zhang, A., Yoo, E.J., Cooke, N.E. and Liebhaber, S.A. (2004) Bystander gene activation by a locus control region. *EMBO J.*, **23**, 3854–3863.
26. Spitz, F., Gonzalez, F. and Duboule, D. (2003) A global control region defines a chromosomal regulatory landscape containing the HoxD cluster. *Cell*, **113**, 405–417.
27. Foster, J.W., Dominguez-Steglich, M.A., Guioli, S., Kowk, G., Weller, P.A., Stevanovic, M., Weissenbach, J., Mansour, S., Young, I.D., Goodfellow, P.N. *et al.* (1994) Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature*, **372**, 525–530.
28. Wagner, T., Wirth, J., Meyer, J., Zabel, B., Held, M., Zimmer, J., Pasantes, J., Bricarelli, F.D., Keutel, J., Hustert, E. *et al.* (1994) Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell*, **79**, 1111–1120.
29. Wunderle, V.M., Critcher, R., Hastie, N., Goodfellow, P.N. and Schedl, A. (1998) Deletion of long-range regulatory elements upstream of SOX9 causes campomelic dysplasia. *Proc. Natl Acad. Sci. USA*, **95**, 10649–10654.
30. Pop, R., Conz, C., Lindenberg, K.S., Blesson, S., Schmalenberger, B., Briault, S., Pfeifer, D. and Scherer, G. (2004) Screening of the 1 Mb SOX9 5′ control region by array CGH identifies a large deletion in a case of campomelic dysplasia with XY sex reversal. *J. Med. Genet.*, **41**, e47.
31. Lettice, L.A., Horikoshi, T., Heaney, S.J., van Baren, M.J., van der Linde, H.C., Breedveld, G.J., Joosse, M., Akarsu, N., Oostra, B.A., Endo, N. *et al.* (2002) Disruption of a long-range *cis*-acting regulator for Shh causes preaxial polydactyly. *Proc. Natl Acad. Sci. USA*, **99**, 7548–7553.
32. Tolhuis, B., Palstra, R.J., Splinter, E., Grosveld, F. and de Laat, W. (2002) Looping and interaction between hypersensitive sites in the active beta-globin locus. *Mol. Cell*, **10**, 1453–1465.
33. Dekker, J., Rippe, K., Dekker, M. and Kleckner, N. (2002) Capturing chromosome conformation. *Science*, **295**, 1306–1311.
34. Carter, D., Chakalova, L., Osborne, C.S., Dai, Y.F. and Fraser, P. (2002) Long-range chromatin regulatory interactions *in vivo*. *Nat. Genet.*, **32**, 623–626.
35. Spilianakis, C.G. and Flavell, R.A. (2004) Long-range intrachromosomal interactions in the T helper type 2 cytokine locus. *Nat. Immunol.*, **5**, 1017–1027.
36. Palstra, R.J., Tolhuis, B., Splinter, E., Nijmeijer, R., Grosveld, F. and de Laat, W. (2003) The beta-globin nuclear compartment in development and erythroid differentiation. *Nat. Genet.*, **35**, 190–194.
37. Patrinos, G.P., de Krom, M., de Boer, E., Langeveld, A., Imam, A.M., Strouboulis, J., de Laat, W. and Grosveld, F.G. (2004) Multiple interactions between regulatory regions are required to stabilize an active chromatin hub. *Genes Dev.*, **18**, 1495–1509.
38. Drissen, R., Palstra, R.J., Gillemans, N., Splinter, E., Grosveld, F., Philipsen, S. and de Laat, W. (2004) The active spatial organization of the beta-globin locus requires the transcription factor EKLF. *Genes Dev.*, **18**, 2485–2490.
39. Casolari, J.M., Brown, C.R., Komili, S., West, J., Hieronymus, H. and Silver, P.A. (2004) Genome-wide localization of the nuclear transport machinery couples transcriptional status and nuclear organization. *Cell*, **117**, 427–439.
40. Brown, K.E., Amoils, S., Horn, J.M., Buckle, V.J., Higgs, D.R., Merkenschlager, M. and Fisher, A.G. (2001) Expression of alpha- and beta-globin genes occurs within different nuclear domains in haemopoietic cells. *Nat. Cell Biol.*, **3**, 602–606.
41. Brown, K.E., Baxter, J., Graf, D., Merkenschlager, M. and Fisher, A.G. (1999) Dynamic repositioning of genes in the nucleus of lymphocytes preparing for cell division. *Mol. Cell*, **3**, 207–217.
42. Mahy, N.L., Perry, P.E., Gilchrist, S., Baldock, R.A. and Bickmore, W.A. (2002) Spatial organization of active and inactive genes and noncoding DNA within chromosome territories. *J. Cell Biol.*, **157**, 579–589.
43. Ragoczy, T., Telling, A., Sawado, T., Groudine, M. and Kosak, S.T. (2003) A genetic analysis of chromosome territory looping: diverse roles for distal regulatory elements. *Chromosome Res.*, **11**, 513–525.
44. Chambeyron, S. and Bickmore, W.A. (2004) Chromatin decondensation and nuclear reorganization of the HoxB locus upon induction of transcription. *Genes Dev.*, **18**, 1119–1130.
45. Verschure, P.J., van Der Kraan, I., Manders, E.M. and van Driel, R. (1999) Spatial relationship between transcription sites and chromosome territories. *J. Cell Biol.*, **147**, 13–24.
46. Zink, D., Amaral, M.D., Englmann, A., Lang, S., Clarke, L.A., Rudolph, C., Alt, F., Luther, K., Braz, C., Sadoni, N. *et al.* (2004) Transcription-dependent spatial arrangements of CFTR and adjacent genes in human cell nuclei. *J. Cell Biol.*, **166**, 815–825.
47. Osborne, C.S., Chakalova, L., Brown, K.E., Carter, D., Horton, A., Debrand, E., Goyenechea, B., Mitchell, J.A., Lopes, S., Reik, W. *et al.* (2004) Active genes dynamically colocalize to shared sites of ongoing transcription. *Nat. Genet.*, **36**, 1065–1071.
48. Iborra, F.J., Pombo, A., Jackson, D.A. and Cook, P.R. (1996) Active RNA polymerases are localized within discrete transcription "factories' in human nuclei. *J. Cell Sci.*, **109**, 1427–1436.
49. Cook, P.R. (2002) Predicting three-dimensional genome structure from transcriptional activity. *Nat. Genet.*, **32**, 347–352.
50. Misteli, T. (2001) The concept of self-organization in cellular architecture. *J. Cell Biol.*, **155**, 181–185.
51. Rippe, K. (2001) Making contacts on a nucleic acid polymer. *Trends Biochem. Sci.*, **26**, 733–740.
52. Ringrose, L., Chabanis, S., Angrand, P.O., Woodroofe, C. and Stewart, A.F. (1999) Quantitative comparison of DNA looping *in vitro* and *in vivo*: chromatin increases effective DNA flexibility at short distances. *EMBO J.*, **18**, 6630–6641.
53. Cook, P.R. (2003) Nongenic transcription, gene regulation and action at a distance. *J. Cell Sci.*, **116**, 4483–4491.
54. Yin, H., Wang, M.D., Svoboda, K., Landick, R., Block, S.M. and Gelles, J. (1995) Transcription against an applied force. *Science*, **270**, 1653–1657.
55. Sims, R.J., III, Belotserkovskaya, R. and Reinberg, D. (2004) Elongation by RNA polymerase II: the short and long of it. *Genes Dev.*, **18**, 2437–2468.
56. Ling, J., Ainol, L., Zhang, L., Yu, X., Pi, W. and Tuan, D. (2004) HS2 enhancer function is blocked by a transcriptional terminator inserted between the enhancer and the promoter. *J. Biol. Chem.*, **279**, 51704–51713.

57. Zhao, H. and Dean, A. (2004) An insulator blocks spreading of histone acetylation and interferes with RNA polymerase II transfer between an enhancer and gene. *Nucleic Acids Res.*, **32**, 4903–4919.
58. Dorsett, D. (1999) Distant liaisons: long-range enhancer–promoter interactions in Drosophila. *Curr. Opin. Genet. Dev.*, **9**, 505–514.
59. Bulger, M. and Groudine, M. (1999) Looping versus linking: toward a model for long-distance gene activation. *Genes Dev.*, **13**, 2465–2477.
60. Rollins, R.A., Korom, M., Aulner, N., Martens, A. and Dorsett, D. (2004) Drosophila nipped-B protein supports sister chromatid cohesion and opposes the stromalin/Scc3 cohesion factor to facilitate long-range activation of the cut gene. *Mol. Cell. Biol.*, **24**, 3100–3111.
61. Hark, A.T., Schoenherr, C.J., Katz, D.J., Ingram, R.S., Levorse, J.M. and Tilghman, S.M. (2000) CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature*, **405**, 486–489.
62. Bell, A.C. and Felsenfeld, G. (2000) Methylation of a CTCF-dependent boundary controls imprinted expression of the *Igf2* gene. *Nature*, **405**, 482–485.
63. Filippova, G.N., Thienes, C.P., Penn, B.H., Cho, D.H., Hu, Y.J., Moore, J.M., Klesert, T.R., Lobanenkov, V.V. and Tapscott, S.J. (2001) CTCF-binding sites flank CTG/CAG repeats and form a methylation-sensitive insulator at the DM1 locus. *Nat. Genet.*, **28**, 335–343.
64. Takada, S., Paulsen, M., Tevendale, M., Tsai, C.E., Kelsey, G., Cattanach, B.M. and Ferguson-Smith, A.C. (2002) Epigenetic analysis of the Dlk1-Gtl2 imprinted domain on mouse chromosome 12: implications for imprinting control from comparison with Igf2–H19. *Hum. Mol. Genet.*, **11**, 77–86.
65. Lutz, M., Burke, L.J., LeFevre, P., Myers, F.A., Thorne, A.W., Crane-Robinson, C., Bonifer, C., Filippova, G.N., Lobanenkov, V. and Renkawitz, R. (2003) Thyroid hormone-regulated enhancer blocking: cooperation of CTCF and thyroid hormone receptor. *EMBO J.*, **22**, 1579–1587.
66. Hikichi, T., Kohda, T., Kaneko-Ishino, T. and Ishino, F. (2003) Imprinting regulation of the murine *Meg1*/*Grb10* and human *GRB10* genes; roles of brain-specific promoters and mouse-specific CTCF-binding sites. *Nucleic Acids Res.*, **31**, 1398–1406.
67. Ishihara, K. and Sasaki, H. (2002) An evolutionarily conserved putative insulator element near the 3′ boundary of the imprinted Igf2/H19 domain. *Hum. Mol. Genet.*, **11**, 1627–1636.
68. Fillipova, G.N., Cheng, M.K., Moore, J.M., Truong, J.P., Hu, Y.J., Nguyen, D.K., Tsuchiya, K.D. and Disteche, C.M. (2005) Boundaries between chromosomal domains of X inactivation and escape bind CTCF and lack CpG methylation during early development. *Dev. Cell*, **8**, 31–42.
69. Magdinier, F., Yusufzai, T.M. and Felsenfeld, G. (2004) Both CTCF-dependent and -independent insulators are found between the mouse T cell receptor alpha and *Dad1* genes. *J. Biol. Chem.*, **279**, 25381–25389.
70. Valadez-Graham, V., Razin, S.V. and Recillas-Targa, F. (2004) CTCF-dependent enhancer blockers at the upstream region of the chicken alpha-globin gene domain. *Nucleic Acids Res.*, **32**, 1354–1362.
71. Gombert, W.M., Farris, S.D., Rubio, E.D., Morey-Rosler, K.M., Schubach, W.H. and Krumm, A. (2003) The c-myc insulator element and matrix attachment regions define the c-myc chromosomal domain. *Mol. Cell. Biol.*, **23**, 9338–9348.
72. Chao, W., Huynh, K.D., Spencer, R.J., Davidow, L.S. and Lee, J.T. (2002) CTCF, a candidate *trans*-acting factor for X-inactivation choice. *Science*, **295**, 345–347.
73. Kanduri, C., Pant, V., Loukinov, D., Pugacheva, E., Qi, C.F., Wolffe, A., Ohlsson, R. and Lobanenkov, V.V. (2000) Functional association of CTCF with the insulator upstream of the *H19* gene is parent of origin-specific and methylation-sensitive. *Curr. Biol.*, **10**, 853–856.
74. Kanduri, C., Fitzpatrick, G., Mukhopadhyay, R., Kanduri, M., Lobanenkov, V., Higgins, M. and Ohlsson, R. (2002) A differentially methylated imprinting control region within the Kcnq1 locus harbors a methylation-sensitive chromatin insulator. *J. Biol. Chem.*, **277**, 18106–18110.
75. Gause, M., Morcillo, P. and Dorsett, D. (2001) Insulation of enhancer–promoter communication by a gypsy transposon insert in the *Drosophila* cut gene: cooperation between suppressor of hairy-wing and modifier of mdg4 proteins. *Mol. Cell. Biol.*, **21**, 4807–4817.
76. Yusufzai, T.M. and Felsenfeld, G. (2004) The 5′-HS4 chicken beta-globin insulator is a CTCF-dependent nuclear matrix-associated element. *Proc. Natl Acad. Sci. USA*, **101**, 8620–8624.
77. Gerasimova, T.I., Byrd, K. and Corces, V.G. (2000) A chromatin insulator determines the nuclear localization of DNA. *Mol. Cell*, **6**, 1025–1035.
78. Xu, Q., Li, M., Adams, J. and Cai, H.N. (2004) Nuclear location of a chromatin insulator in *Drosophila melanogaster*. *J. Cell Sci.*, **117**, 1025–1032.
79. Byrd, K. and Corces, V.G. (2003) Visualization of chromatin domains created by the gypsy insulator of *Drosophila*.*J. Cell Biol.*, **162**, 565–574.
80. Chen, J.L., Huisinga, K.L., Viering, M.M., Ou, S.A., Wu, C.T. and Geyer, P.K. (2002) Enhancer action in *trans* is permitted throughout the *Drosophila* genome. *Proc. Natl Acad. Sci. USA*, **99**, 3723–3728.
81. Schedl, P. and Broach, J.R. (2003) Making good neighbors: the right fence for the right job. *Nat. Struct. Biol.*, **10**, 241–243.
82. Ulaner, G.A., Vu, T.H., Li, T., Hu, J.F., Yao, X.M., Yang, Y., Gorlick, R., Meyers, P., Healey, J., Ladanyi, M. *et al.* (2003) Loss of imprinting of IGF2 and H19 in osteosarcoma is accompanied by reciprocal methylation changes of a CTCF-binding site. *Hum. Mol. Genet.*, **12**, 535–549.
83. Takai, D., Gonzales, F.A., Tsai, Y.C., Thayer, M.J. and Jones, P.A. (2001) Large scale mapping of methylcytosines in CTCF-binding sites in the human H19 promoter and aberrant hypomethylation in human bladder cancer. *Hum. Mol. Genet.*, **10**, 2619–2626.
84. Cui, H., Niemitz, E.L., Ravenel, J.D., Onyango, P., Brandenburg, S.A., Lobanenkov, V.V. and Feinberg, A.P. (2001) Loss of imprinting of insulin-like growth factor-II in Wilms' tumor commonly involves altered methylation but not mutations of CTCF or its binding site. *Cancer Res.*, **61**, 4947–4950.
85. Nakagawa, H., Chadwick, R.B., Peltomaki, P., Plass, C., Nakamura, Y. and de La Chapelle, A. (2001) Loss of imprinting of the insulin-like growth factor II gene occurs by biallelic methylation in a core region of H19-associated CTCF-binding sites in colorectal cancer. *Proc. Natl Acad. Sci. USA*, **98**, 591–596.
86. Yu, W., Ginjala, V., Pant, V., Chernukhin, I., Whitehead, J., Docquier, F., Farrar, D., Tavoosidana, G., Mukhopadhyay, R., Kanduri, C. *et al.* (2004) Poly(ADP-ribosyl)ation regulates CTCF-dependent chromatin insulation. *Nat. Genet.*, **36**, 1105–1110.
87. Tulin, A. and Spradling, A. (2003) Chromatin loosening by poly(ADP)-ribose polymerase (PARP) at *Drosophila* puff loci. *Science*, **299**, 560–562.
88. Muravyova, E., Golovnin, A., Gracheva, E., Parshikov, A., Belenkaya, T., Pirrotta, V. and Georgiev, P. (2001) Loss of insulator activity by paired Su(Hw) chromatin insulators. *Science*, **291**, 495–498.
89. Cai, H.N. and Shen, P. (2001) Effects of *cis* arrangement of chromatin insulators on enhancer-blocking activity. *Science*, **291**, 493–495.
90. Pai, C.Y., Lei, E.P., Ghosh, D. and Corces, V.G. (2004) The centrosomal protein CP190 is a component of the gypsy chromatin insulator. *Mol. Cell*, **16**, 737–748.
91. Melnikova, L., Juge, F., Gruzdeva, N., Mazur, A., Cavalli, G. and Georgiev, P. (2004) Interaction between the GAGA factor and Mod(mdg4) proteins promotes insulator bypass in *Drosophila. Proc. Natl Acad. Sci. USA*, **101**, 14806–14811.
92. Zhou, J. and Levine, M. (1999) A novel cis-regulatory element, the PTS, mediates an anti-insulator activity in the *Drosophila* embryo. *Cell*, **99**, 567–575.
93. Lin, Q., Wu, D. and Zhou, J. (2003) The promoter targeting sequence facilitates and restricts a distant enhancer to a single promoter in the *Drosophila* embryo. *Development*, **130**, 519–526.
94. Majumder, P. and Cai, H.N. (2003) The functional analysis of insulator interactions in the *Drosophila* embryo. *Proc. Natl Acad. Sci. USA*, **100**, 5223–5228.
95. Kuhn, E.J., Viering, M.M., Rhodes, K.M. and Geyer, P.K. (2003) A test of insulator interactions in *Drosophila.EMBO J.*, **22**, 2463–2471.
96. Blanton, J., Gaszner, M. and Schedl, P. (2003) Protein:protein interactions and the pairing of boundary elements *in vivo. Genes Dev.*, **17**, 664–675.
97. Bell, A.C., West, A.G. and Felsenfeld, G. (1999) The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. *Cell*, **98**, 387–396.
98. Szabo, P.E., Tang, S.H., Reed, M.R., Silva, F.J., Tsark, W.M. and Mann, J.R. (2002) The chicken beta-globin insulator element conveys chromatin boundary activity but not imprinting at the mouse Igf2/H19 domain. *Development*, **129**, 897–904.

99. Kaffer, C.R., Srivastava, M., Park, K.Y., Ives, E., Hsieh, S., Batlle, J., Grinberg, A., Huang, S.P. and Pfeifer, K. (2000) A transcriptional insulator at the imprinted H19/Igf2 locus. *Genes Dev.*, **14**, 1908–1919.
100. West, A.G., Huang, S., Gaszner, M., Litt, M.D. and Felsenfeld, G. (2004) Recruitment of histone modifications by USF proteins at a vertebrate barrier element. *Mol. Cell*, **16**, 453–463.
101. Recillas-Targa, F., Pikaart, M.J., Burgess-Beusse, B., Bell, A.C., Litt, M.D., West, A.G., Gaszner, M. and Felsenfeld, G. (2002) Position-effect protection and enhancer blocking by the chicken beta-globin insulator are separable activities. *Proc. Natl Acad. Sci. USA*, **99**, 6883–6888.
102. Belozerov, V.E., Majumder, P., Shen, P. and Cai, H.N. (2003) A novel boundary element may facilitate independent gene regulation in the Antennapedia complex of *Drosophila. EMBO J.*, **22**, 3113–3121.
103. Recillas-Targa, F., Valadez-Graham, V. and Farrell, C.M. (2004) Prospects and implications of using chromatin insulators in gene therapy and transgenesis. *Bioessays*, **26**, 796–807.
104. Noma, K., Allis, C.D. and Grewal, S.I. (2001) Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. *Science*, **293**, 1150–1155.
105. Ishii, K., Arib, G., Lin, C., Van Houwe, G. and Laemmli, U.K. (2002) Chromatin boundaries in budding yeast: the nuclear pore connection. *Cell*, **109**, 551–562.
106. Oki, M., Valenzuela, L., Chiba, T., Ito, T. and Kamakaka, R.T. (2004) Barrier proteins remodel and modify chromatin to restrict silenced domains. *Mol. Cell. Biol.*, **24**, 1956–1967.
107. Bi, X. and Broach, J.R. (2001) Chromosomal boundaries in *S. cerevisiae. Curr. Opin. Genet. Dev.*, **11**, 199–204.
108. Ferrari, S., Carmine-Simmen, K., Dusserre, Y., Muller, K., Fourel, G., Gilson, E. and Mermod, N. (2004) Chromatin domain boundaries delimited by a histone-binding protein in yeast. *J. Biol. Chem.*, **279**, 55520–55530.
109. Donze, D. and Kamakaka, R.T. (2002) Braking the silence: how heterochromatic gene repression is stopped in its tracks. *Bioessays*, **24**, 344–349.
110. Chiu, Y.H., Yu, Q., Sandmeier, J.J. and Bi, X. (2003) A targeted histone acetyltransferase can create a sizable region of hyperacetylated chromatin and counteract the propagation of transcriptionally silent chromatin. *Genetics*, **165**, 115–125.
111. Litt, M.D., Simpson, M., Gaszner, M., Allis, C.D. and Felsenfeld, G. (2001) Correlation between histone lysine methylation and developmental changes at the chicken beta-globin locus. *Science*, **293**, 2453–2455.
112. Litt, M.D., Simpson, M., Recillas-Targa, F., Prioleau, M.N. and Felsenfeld, G. (2001) Transitions in histone acetylation reveal boundaries of three separately regulated neighboring loci. *EMBO J.*, **20**, 2224–2235.
113. Morshead, K.B., Ciccone, D.N., Taverna, S.D., Allis, C.D. and Oettinger, M.A. (2003) Antigen receptor loci poised for V(D)J rearrangement are broadly associated with BRG1 and flanked by peaks of histone H3 dimethylated at lysine 4. *Proc. Natl Acad. Sci. USA*, **100**, 11577–11582.