An activation-independent role of transcription factors in insulator function

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Chromatin insulators are defined as transcriptionally neutral elements that prevent negative or positive influence from extending across chromatin to a promoter. Here we show that yeast subtelomeric anti-silencing regions behave as boundaries to telomere-driven silencing and also allow discontinuous propagation of silent chromatin. These two facets of insulator activity, boundary and silencing discontinuity, can be recapitulated by tethering various transcription activation domains to tandem sites on DNA. Importantly, we show that these insulator activities do not involve direct transcriptional activation of the reporter promoter. These findings predict that certain promoters behave as insulators and partition genomes in functionally independent domains.

INTRODUCTION

Silencing is a form of repression that extends to large chromosomal segments and affects many types of DNA transactions, including transcription, recombination, repair and transposition. In *Saccharomyces cerevisiae*, it is found at silent mating-type loci (*HM*), within rDNA repeats and in telomere proximity (TPE, telomere position effect). Silencing is generally associated with an overall loss of DNA accessibility, histone hypoacetylation, a regular spacing of the nucleosomal array and a shift in DNA topology (Lustig, 1998). All of these properties suggest that silent chromatin is organized into a 'closed-up', relatively uniform nucleosomal array. Silent chromatin appears to nucleate at *cis*-acting elements known as silencers and to propagate along the

chromosome from this initiation site (Locke *et al.*, 1988; Renauld *et al.*, 1993). However, recent observations demonstrate discontinuity in the 'spreading' of silencing, which probably involves relay elements (Fourel *et al.*, 1999; Talbert and Henikoff, 2000). This rather argues in favour of a 'coalescence' model of silencing in which silencers and relay elements form complex interactions to lead to an apparent spreading of silent chromatin (Talbert and Henikoff, 2000).

Chromosomal regions can be protected from silencing, and also from the influence of positive DNA elements such as enhancers, by *cis*-acting elements called insulators (Sun and Elgin, 1999). They have been described in a variety of higher eukaryotes, and more recently in yeast (Bi and Broach, 1999; Donze *et al.*, 1999; Fourel *et al.*, 1999). Insulators have been uncovered at the borders of both active and inactive chromosome segments, in agreement with a role in delimiting independent chromatin domains (Donze *et al.*, 1999; Saitoh *et al.*, 2000). Abutting the telomeric arrays of *S. cerevisiae* are short sequences that can protect a reporter gene placed on their centromere side from TPE (Fourel *et al.*, 1999). Accordingly, they have been considered as insulators, although their enhancer-blocking capacity was not addressed. They are called STARs (for subtelomeric anti-silencing regions) and are part of longer repeated elements belonging either to the X- or Y'-family (Louis, 1995). STARs have been proposed to delimit a very terminal telomeric compartment protected from influences of the rest of the genome (Fourel *et al.*, 1999). However, STARs do

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not affect the interaction of telomeres with subtelomeric silencing elements naturally found on their centromere side, which relay silent chromatin propagation into more internal regions of the chromosome (Fourel *et al.*, 1999; Pryde and Louis, 1999). The Tbf1p and Reb1p proteins probably mediate STAR activity. Indeed, both X- and Y'-STAR harbour multiple binding sites for Tbf1p and Reb1p, and multimers of oligonucleotides containing either Tbf1p- or Reb1p-binding sites display anti-silencing properties (Fourel *et al.*, 1999).

Amazingly little is known about the molecular mechanism(s) by which insulators function, and no single model satisfies all observations (Sun and Elgin, 1999). Yeast offers unique possibilities as an amenable system to screen for either the expression or the silencing of a variety of reporter genes. We therefore characterized STARs more thoroughly and devised two assays that define the insulator function in *S. cerevisiae*. Tbf1p and Reb1p were found to recapitulate these properties, as well as different transcription activation domains upon tethering to DNA. However, insulator activity clearly did not involve transcriptional activation of reporter genes.

RESULTS AND DISCUSSION

STAR as boundary elements

A strain was first generated that carries a STAR derived from a subtelomeric Y' element (Y'-STAR) (Fourel *et al.*, 1999) placed between two reporter genes, *URA3* and *TRP1*, at a fragmented telomere VII-L as shown in Figure 1A (GF98). A 120 bp fragment containing four tandem binding sites for the transcription factor Gal4p (4xUASg) was also interposed between the two genes, and a strain was constructed that carried only this fragment, as a control (Figure 1A, GF97). The 4xUASg fragment behaves neutrally with regard to silencing in this setting (not shown). The fraction of *URA3* OFF colonies, as assessed by growth on synthetic medium containing 5-fluoro-otic acid (5-FOA) (Boeke *et al.*, 1984), barely decreased upon Y'-STAR insertion (Figure 1A, compare lines 1 and 4). In contrast, *TRP1* was efficiently protected from silencing, as revealed by an increased number of colonies on a synthetic complete medium lacking tryptophan (SC-W) (Figure 1A, compare lines 2 and 5). These results have two important implications. First, telomere silencing capacity *per se* is not altered by a neighbouring Y'-STAR. Secondly, only a gene located in a distal position, outside the STAR/telomere path, is efficiently protected from silencing. Furthermore, not a single colony emerged on SC-W+5-FOA from GF97 cultures (Figure 1A, line 3), showing that the 0.1% fraction of colonies in which *TRP1* is ON corresponds to colonies in which *URA3* is also expressed. In contrast, the presence of a Y'-STAR between *TRP1* and *URA3* in strain GF98 allowed expression of *TRP1* in ~1% of the *URA3* OFF cells (Figure 1A, compare lines 4 and 6). Thus, a Y'-STAR can apparently constrain the propagation of silent chromatin to a telomere-proximal domain and decouple the silencing status of adjacent genes. It thus phenotypically behaves as a boundary to the spreading of silencing, as schematized in Figure 1B, like insulators described in higher eukaryotes. Similar results were obtained at the silent mating type locus *HML* (see Supplementary data available at *EMBO reports* Online).

STARs permit discontinuous silencing

We showed previously that when two STARs bracket a reporter gene, its expression is no longer influenced by surrounding silencing elements (Fourel *et al.*, 1999). For instance, in strain GF61 (Figure 1C), *TRP1* is protected by STARs not only from telomere-driven silencing, but also from the influence of the subtelomeric silencing element core X, which induces downstream *URA3* silencing in 50% of cells (Figure 1C, lines 7 and 8). In addition, expression of the two genes appears decoupled as all the *URA3* OFF cells have the *TRP1* gene ON (Figure 1C, lines 8 and 9). Strikingly, this decoupling was also observed when only a STAR, but not a 4xUASg fragment was inserted between *URA3* and *TRP1* (Figure 1C, lines 3 and 6). In addition, TPE strengthening conferred by deletion of the *RIF1* gene (Hardy *et al.*, 1992) emphasized this effect (Figure 1C, lines 13–15). Thus, synergy between the two STARs bracketing *TRP1* in strain GF60 Δ *rif1* essentially maintained its expression while allowing predominant repression of *URA3*. Analogous discontinuity in the 'spreading' of silencing was recently reported in *Drosophila* (Talbert and Henikoff, 2000).

Altogether, these experiments strongly suggest that two distant STARs cannot only organize an insulated domain, as previously demonstrated for boundary elements in different organisms (Pikaart *et al.*, 1998; Sun and Elgin, 1999), but can do so without seriously affecting efficient propagation of silent chromatin beyond this domain, as summarized in Figure 1D. The capacity to generate an apparent discontinuity in silent chromatin propagation, together with the boundary activity, operationally define the STAR insulator functions.

Tbf1p and Reb1p fulfil insulator functions

We then devised Gal4-based tethering assays in order to identify proteins that can recapitulate the insulator functions of STARs (strains GF97 and GF100 Δ *rif1*; Figure 2). The Tbf1p and Reb1p proteins probably mediate the activities of natural STAR elements (Fourel *et al.*, 1999). Chimeras were therefore constructed in which the DNA binding domains of Reb1p and Tbf1p are replaced by that of Gal4p (Gal4p DNA binding domain or GBD). Expression of GBD alone had very little influence on the overall silencing pattern when four binding sites for Gal4p (4xUASg) are located in the silent region (strain GF97; compare Figures 1A and Figure 2A, lines 1–3). In contrast, expression of the Tbf1p- or Reb1p-GBD chimeras imparted a prominent boundary activity to the 4xUASg fragment, but had no effect on reporter gene expression in the absence of UASg. It is even more pronounced than that of Y'-STAR in the case of Reb1p with 20% of the *URA3* OFF colonies expressing the distal *TRP1* gene (compare Figure 2A, lines 4, 6, 7 and 9 with Figure 1A, lines 4 and 6). Only a very modest decrease in the fraction of *URA3* OFF cells occurred upon expression of the Tbf1p and Reb1p chimeras in GF97, which is comparable to that observed in the presence of an intervening STAR in GF98. Thus, tethering the N-terminal moiety of Tbf1p and Reb1p mimics the boundary effects of a Y'-STAR. In addition, expression of the Tbf1p and Reb1p chimeras in a strain deleted for *RIF1* (GF100 Δ *rif1*) induced an apparent discontinuity in the propagation of silent chromatin (Figure 2B). While expressing the Tbf1p-GBD protein did not significantly modify the ratio of *TRP1* ON and *URA3*

Fig. 1. STAR elements can both protect a reporter gene from silencing (A and B) and allow a gap in silent chromatin propagation (C and D). Experiments are shown in (A) and (C), and the corresponding interpretative schemes in (B) and (D), respectively. (A and C) Constructs introduced by telomere fragmentation at telomere VII-L are shown on the left. Arrows indicate the 5' to 3' direction of transcription of reporter genes. STAR elements (X-STAR from telomere II-R, in natural orientation, and Y'-STAR from telomere XII-L, in reverse orientation) are represented as a stippled box, and the silencing relay element core X (from telomere II-R, in natural orientation) as a hatched box. Four high-affinity binding sites for the transcription factor Gal4p are depicted as ovals (4xUASg). Tandem arrowheads represent telomere repeats. Cultures were serially diluted and grown on distinct medium: synthetic complete (SC), and SC+5-FOA, which only allows growth of cells that do not express URA3, SC-W cells that express TRP1, and SC-W+5-FOA cells that display both URA3 OFF and TRP1 ON. Each diamond indicates the ratio of colonies growing on a given medium versus SC for a single culture and the histogram bar the average for a strain. When not a single colony grew out on selective medium from 10 μ l of undiluted culture, the ratio was estimated as $<2 \times 10^{-5}$. (B and D) Silent chromatin is represented as a gradient to indicate that silencing decreases with increasing distance from the telomere. STARs (box) can both behave as a boundary limiting the spreading of silent chromatin (B) and delimit an insulated domain while allowing efficient propagation of silent chromatin beyond this domain (D).

OFF cells as compared with the expression of GBD alone, this thoroughly decoupled the two reporter genes as all the TRP1 ON cells are now also URA3 OFF (Figure 2B, compare lanes 1–3 and 4–6). In addition, expression of the Reb1p–GBD protein induced

a silencing pattern superposable on that of a strain carrying an X-STAR instead of the UASg sequences (Figure 2B, compare lanes 7–9 and Figure 1C, lanes 19–21). This shows that the tethering of Reb1p is also able to provoke a discontinuous

Fig. 2. Targeted Tbf1p or Reb1p N-terminal domains recapitulate insulator activities. Tbf1p and Reb1p N-terminal portions, which do not overlap with their Myb-related, C-terminal DNA binding domains, were fused to GBD. These plasmids were introduced in strains GF97 (A) and GF100 Δ *rif1* (B). Plasmids were selected through histidine deprivation (SC-H). Details are otherwise the same as in the legend of Figure 1.

silencing effect. Altogether, these results confirm the capacity of Tbf1p and Reb1p to recapitulate STAR activities, and demonstrate that their Myb-related DNA binding domains are not directly involved or at least can be swapped with that of Gal4p. They further validate the use of the boundary and discontinuity tethering assays to address the capacity of a variety of GBD chimera to recapitulate insulator activities.

Transcription factors as potent insulators

Because Reb1p is thus far known as a transcription factor and Tbf1p binding sites are found in the upstream regions of several yeast genes (Koering *et al.*, 2000 and references therein), we wondered whether independently characterized transcriptional activation domains would behave as STAR insulators. A chimera containing the full-length proline-rich domain of CTF displayed

a strong boundary activity upon expression in GF97, allowing ~10% of the URA OFF colonies to grow on SC-W+5-FOA while only mildly affecting *URA3* silencing (Figure 3, lines 10–12). This figure decreased to 2% upon expression of derivatives carrying either only the very short, 13 amino acid, transforming growth factor- β -responsive domain (TRD) (Alevizopoulos *et al.*, 1995) or its complementary deletion mutant (Figure 3, lines 15 and 18). In contrast, essentially all the *URA3* OFF colonies also had the *TRP1* gene repressed upon expression of the GBD control proteins (Figure 3, lanes 3 and 30) or of the fusions carrying the glutamine-rich activation domains of Oct1, Oct2 and Sp1 (Figure 3, lanes 6 and 9, and not shown). We also assayed the acidic transcriptional activation domain of the breast cancer protein BRCA1. This chimera displayed the strongest boundary activity among all those assayed (Figure 3, lines 31–33), whereas derivatives having partially or totally lost

Fig. 3. Boundary activity of mammalian transcription factors and herpes simplex VP16. The chimeras represented on the left part were expressed using two different expression systems (GBD' and GBD°; see Methods). Numbers above boxes indicate protein domain coordinates that were incorporated. An asterisk represents an A to E substitution in BRCA1 that naturally occurs in breast cancer (GBD°-BRCA1AE). Details are otherwise the same as in the legends of Figures 1 and 2.

BRCA1 chromatin remodelling capacity (Hu *et al.*, 1999) still proved active, but less impressively (Figure 3, lines 36 and 39, and not shown). In addition, expression of a hybrid containing the acidic activation domain of VP16 essentially abolished any silencing of both *URA3* and *TRP1* (Figure 3, lines 19 and 20). We therefore could not really address decoupling capacity in this context. However, truncated versions harbouring either the

N- or C-terminal portion of VP16 activation domain, although still largely affecting *URA3* silencing, allowed 5 and 1% of the remaining 5-FOA-resistant colonies to grow on SC-W+5-FOA, respectively. Of note, none of these chimeras had any effect on reporter gene expression in the absence of UASg. The principal conclusion from these experiments is that the boundary activity of STARs can be recapitulated by tethering transcription

activation domains to tandem UASg sites. In addition, proline-rich and acidic activation domains, but not glutamine-rich activation domains, function efficiently.

We also tested the ability of the same GBD chimera to induce a discontinuous silencing effect as assayed for Tbf1p and Reb1p chimeras in strain GF100 Δ rif1 (Figure 2B). Chimeras that displayed boundary capacity (Figure 3) also recapitulated silencing discontinuity and the order of efficiency was similar in both assays (see Supplementary data). These data strongly support the view that the boundary and discontinuity assays of insulator functions actually reflect related molecular phenomena.

Insulator function does not correlate with promoter transactivation

We then addressed whether the activities of reconstituted STAR sequences may in fact rely on direct transcriptional activation of the reporter genes. In a first approach, the capacity of the GBD chimeras to directly activate the *GAL1* promoter was measured. There was seemingly no general correlation between the insulator activities and the *cis*-activation capacity displayed by the various chimeras (not shown). In particular, the Tbf1p and Reb1p chimeras had no effect.

We further reasoned that if the boundary property imparted to UASg by tethered transcription activation domains is specific to silent chromatin, then reporter genes should not be influenced by these elements in any manner upon abrogation of silencing. Reporter transcript levels were therefore analysed in GF97' (which has a modified telomere VII-L identical to GF97, but in a different genetic background; see Methods) upon deletion of the *SIR4* gene (Figure 4A). The levels of *TRP1* transcripts normalized to *CDC28* used as an internal loading control are represented in Figure 4B. In *SIR4* cells, the relative *TRP1* mRNA levels paralleled the proportion of colonies growing out from the same culture on SC-W medium, although RNA analysis appears less sensitive (Figure 4B, odd lines, compare with Figures 2 and 3). In contrast, in the absence of silencing, the steady-state level of *TRP1* mRNA appeared elevated and approximately uniform irrespective of the GBD chimera expressed (Figure 4B, even lines). Therefore, none of these proteins seems capable of directly activating the *TRP1* promoter from the distant UASg tethering sites in GF97', in the absence of silencing. We conducted the same type of analysis in a strain in which *URA3* is separated from telomere repeats by UASg sites (GF95; Figure 4C and D). The *URA3* mRNA level was raised by less than one log upon expression of Reb1N and CTF chimeras, and by one and a half log upon expression of VP16 chimera, confirming the anti-silencing properties of these fusion proteins. Importantly, no significant variation in *URA3* mRNA levels was observed upon expression of GBD or of the various chimeras in cells deleted for *SIR4* (Figure 4D, compare even lines and line 9). Thus, the various transcriptional activation domains here tethered through GBD appear unable to activate either *TRP1* or *URA3* promoter in these experimental settings. Of note, analysis of *URA3* mRNA levels in the absence of silencing in GF97' confirmed that the VP16 chimera can activate the *URA3* promoter from a proximal targeting site (not shown), which probably accounts for the apparent retrograde anti-silencing effect observed with VP16 chimera and its derivatives (see Figure 3).

One may object that a low, hardly observable increase in transcription might suffice to account for apparent boundary effects. We therefore decided to analyse strains in which a prominent anti-silencing effect could be observed. *URA3* appeared fully protected from E silencer-driven silencing at the *HML* locus upon interposing natural STARs or reconstituted, particularly potent STARs made of Tbf1p- and Reb1p-binding sites (Fourel *et al.*, 1999; see also Supplementary data). When yeasts were grown on synthetic complete medium (SC), *URA3* levels were approximately the same for all the strains analysed, except for the GF20 control strain in which *URA3* expression was one log below (Figure 5A and the right part of B, odd lines). This strain does not contain any STAR sequence at *HML*, and is indeed the only one that displayed significant silencing of *URA3* (Figure 5B, left part, line 5). In the absence of silencer, even potent STARs did not alter the levels of *URA3* transcripts. Of note, the position of the *URA3* transcription initiation site was not modified either by proximal STARs (see Supplementary data).

One may further object that in this experiment, a putative transcription activation capacity of STARs may have been conceivably masked by limitations on the maximal transcription initiation rate at the *URA3* promoter. We therefore grew exactly the same strains on a medium lacking uracil, which activates the *URA3* promoter ~2-fold (Figure 5, compare SC and SC-U). Again, stimulated *URA3* levels were uniform among all the strains analysed, except for GF20, for which growth on SC-U seemingly resulted in a partial alleviation of silencing and/or the selection of cells expressing a threshold *URA3* level (Figure 5A and B right panel, compare SC and SC-U, lines 17 and 18). Overall, we conclude that STARs capable of fully protecting reporter genes from silencing at *HML* had absolutely no influence on the transcription of the reporter gene in the absence of silencing.

Altogether, these findings are in agreement with a model in which the insulator effects cannot be accounted for by direct, classical stimulation of the reporter promoters, but rather by phenomena specifically affecting silent chromatin. Alternatively, direct transcriptional activation of reporter genes through direct contacts between the promoter and remote transactivators may be envisaged to require functional silent chromatin and would thus not be observed in Δ *sir4* strains or at the *HML* locus deprived of silencers. Silent chromatin was indeed shown previously to bring together distant sequences and may thus facilitate the formation of an intervening loop (Strahl-Bolsinger *et al.*, 1997). However, the establishment and/or the maintenance of the loop owing to silent chromatin is not expected to be compatible with the efficient transcription of the underlying gene in subtelomeric constructs, and this model thus appears rather unlikely.

Collectively, these results demonstrate that a variety of transcription activation domains tethered to tandem DNA sites can reconstitute bona fide insulators in *S. cerevisiae*. Thus, the structure of insulators does not follow strict organization, nor do they necessarily involve specialized proteins. The fact that many different transcription factors operate implies that distinct, possibly redundant mechanisms can underlie insulator activity. Candidates are all the modifications of chromatin and/or of the higher order organization of the nucleus that are incompatible with silencing. Our results further suggest that promoters may

Fig. 4. The GBD chimeras do not directly activate reporter transcription. Chimeras were expressed in GF97' or in GF95 and in derivatives that bear a deletion in the *SIR4* ORF ($\Delta sir4$). RNase protection experiments using *CDC28*- and *TRP1*- (A) or *URA3*- (C) internal probes were quantified using a phosphorimager, and the ratios of reporter to *CDC28* transcript levels are presented in (B) and (D), respectively. MW, molecular weight markers; probe, unprocessed probes that were hybridized to total cellular RNAs of strains indicated above each lane. Details are otherwise the same as in the legends of Figures 1–3.

Fig. 5. Even potent STARs do not directly activate transcription. An RNase protection experiment using *CDC28* and *URA3* internal probes (**A**) was performed with yeast strains grown either in synthetic medium (SC) or the same medium lacking uracil (SC-U), as indicated above the lanes. (**B**) Left, estimation of *URA3* silencing in the corresponding strains after pre-growth in SC. The *HML*-E and -I silencers are drawn as boxes, black when intact and white when carrying the e1 and i (*IA*²⁴²) deletions (Fourel *et al.*, 1999). Fragments were inserted between *HML*-E and *URA3*, the 120 bp 4xUASg fragment used here as a stuffer, the X-STAR derived from telomere XI-L, or multimerized oligonucleotides corresponding to 31 bp portions of X-STAR and containing Tbf1p- or Reb1p-binding sites, respectively (STR-A and STR-d) (Fourel *et al.*, 1999). Details are otherwise the same as in the legends of Figures 1–4.

behave as insulators and partition genomes in functionally independent domains.

METHODS

Yeast strains. Most of the yeast strains described in this study contain a modified VII-L telomere, are derivatives of W303-1a and were obtained following standard genetic manipulations as described in Fourel *et al.* (1999). The variegated expression of *URA3* and *TRP1* was monitored as described in Fourel *et al.* (1999). GF95 and GF97' were constructed in an FYBL2-5D background (*MATα URA3-Δ851 TRP1Δ63 leu2Δ1*) in order to allow RNase protection analysis of *URA3* and *TRP1* reporter transcripts, and further carried a *HIS3* deletion. Deletions in the *RIF1* and *HIS3* genes were obtained by replacement of the full-length open reading frames (ORFs) by the KanMX cassette. The *SIR4* gene disruption used pJR276 (Kimmerly and Rine, 1987). Yeast strains harbouring a modified *HML* locus have an S150-2B background (*Mata, leu2-3-112, ura3-52 trp1289 his3Δ gal2 gal4:LEU2*) and were obtained as described in Fourel *et al.* (1999).

Plasmid constructions. All the DNA fragments assembled to generate telomeric constructs are described in Fourel *et al.* (1999). Fragments obtained by HiFi PCR (Boehringer Mannheim) of *S. cerevisiae* genomic DNA and corresponding to the 405 N-terminal residues of Tbf1p and Reb1p were introduced in GBTh

to generate Tbf1p and Reb1p chimeras. GBTh itself was constructed by replacing the *TRP1* selection marker of GBT9 (Stratagene) by *HIS3* and harbours Gal4p residues 1–147 expressed from the ADH1 promoter. Chimeras labeled GBD' contain Gal4p residues 1–93 fused to the indicated protein domains and are expressed from the ADH1 promoter on a pBRHAC-derived low-copy vector (Künzler *et al.*, 1994). They were kindly provided by W. Schaffner, except for GBD'-TRD and GBD'-CTFN, which were PCR generated using GBD'-CTF as a template. The BRCA1 chimeras harbour Gal4p residues 1–94 (GBD⁹) and are expressed from the *CUP1* promoter of constructs stably integrated at the *LEU2* chromosomal locus, in the presence of 100 μM CuSO₄ (Hu *et al.*, 1999). Expression of the Gal4p chimera was checked by immunoblotting using an anti-Gal4 antibody (Santa Cruz).

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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