Transcriptional interference: an unexpected layer of complexity in gene regulation

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Summary

Much of the genome is transcribed into long untranslated RNAs, mostly of unknown function. Growing evidence suggests that transcription of sense and antisense untranslated RNAs in eukaryotes can repress a neighboring gene by a phenomenon termed transcriptional interference. Transcriptional interference by the untranslated RNA may prevent recruitment of the initiation complex or prevent transcriptional elongation. Recent work in yeast, mammals, and *Drosophila* highlights the diverse roles that untranslated RNAs play in development. Previously, untranslated RNAs of the bithorax complex of *Drosophila* were proposed to be required for its activation. Recent studies show that these untranslated RNAs in fact silence *Ultrabithorax* in early embryos, probably by transcriptional interference.

Key words: *Drosophila*, Hox, cis-acting, Repression, Untranslated RNA

Introduction

Much of the genome in higher eukaryotes (70% in humans) is transcribed on one or both strands (Carninci et al., 2005). A question of increasing importance for cell biologists is the function of the large number of untranslated RNAs. Infrastructural RNAs depend on their sequence and/or structure and include rRNAs, tRNAs, and spliceosomal small nuclear RNAs (snRNAs) (Gesteland et al., 2006). Others have roles in chromosome maintenance, epigenesis and chromatin architecture (Cam et al., 2005). The function of some untranslated RNAs depends on sequence complementarity with their targets, and these act in trans to regulate gene activity (microRNAs and small interfering RNAs) (Massirer and Pasquinelli, 2006; Sen and Blau, 2006).

Transcription of one gene can act directly to prevent transcription of a neighboring gene in cis by a mechanism originally termed 'promoter occlusion' in prokaryotes (Adhya and Gottesman, 1982; Frankham, 1988) and later termed 'transcriptional interference' (TI) (for a review, see Shearwin et al., 2005). The startling implication of TI is that the act of transcription itself rather than the sequence of the transcribed RNA is important. TI can occur when the genes concerned are on the same DNA strand (sense-TI) (Martens et al., 2004) or opposite strands (antisense-TI) (Callen et al., 2004; Timmons and Good, 2006) (see Fig. 1A). This is consistent with the idea that the RNA polymerase II (Pol II) machinery transcribing one gene interferes with transcriptional initiation, elongation or termination at the neighbouring gene.

TI is best characterized in prokaryotes and their extrachromosomal elements, such as phages, transposable elements and plasmids. Many studies have established that both sense-TI and antisense-TI are important in prokaryotes (for a review, see Shearwin et al., 2005) (see Fig. 1). Nevertheless, there are surprisingly few reports that addresses mechanisms of TI (Ward and Murray, 1979; Horowitz and Platt, 1982). Observations of Elledge and Davis, recently confirmed by Callen et al. indicate that TI at the target gene increases as the strength of the promoter for the regulatory RNA increases (Elledge and Davis, 1989; Callen et al., 2004). Recent studies of the convergent promoters used in the lysis-lysogeny switch from coliphage 186 show that insertion of a transcriptional terminator between the promoters strongly reduces TI. In vitro studies show that transcription by RNA polymerase at the regulatory gene does not prevent binding of RNA polymerase at the target gene. Instead, transcription of the regulatory RNA reduces the ability of the open form of polymerase bound at the target gene to clear the promoter (Callen et al., 2004).

In this Commentary, we highlight recent results from eukaryotes that suggest that endogenous cis-acting RNAs that participate in TI are important in gene regulation. We then focus on recent results from *Drosophila* showing that cis-acting RNAs are necessary for developmental regulation of *Hox* genes.

Yeast

Untranslated RNAs are present in *Saccharomyces cerevisiae* (David et al., 2006; Samanta et al., 2006), but until recently their function had not been characterized. An early report suggested a role for TI in repression of a cryptic promoter for the actin locus but it is not clear whether this mechanism is functional in vivo (Irniger et al., 1992). More recent work, however indicates that TI does indeed operate in yeast (Martens et al., 2004). The *SER3* gene in *S. cerevisiae* encodes an enzyme that catalyzes a step in serine biosynthesis in nutrient-poor medium. Expression of the upstream, tandemly oriented *SRG1* gene represses *SER3* in rich medium to prevent unnecessary production of serine. Martens et al. eliminated the possibility that the *SRG1* RNA works in trans, or that promoter competition occurs, leaving TI as the likely possibility

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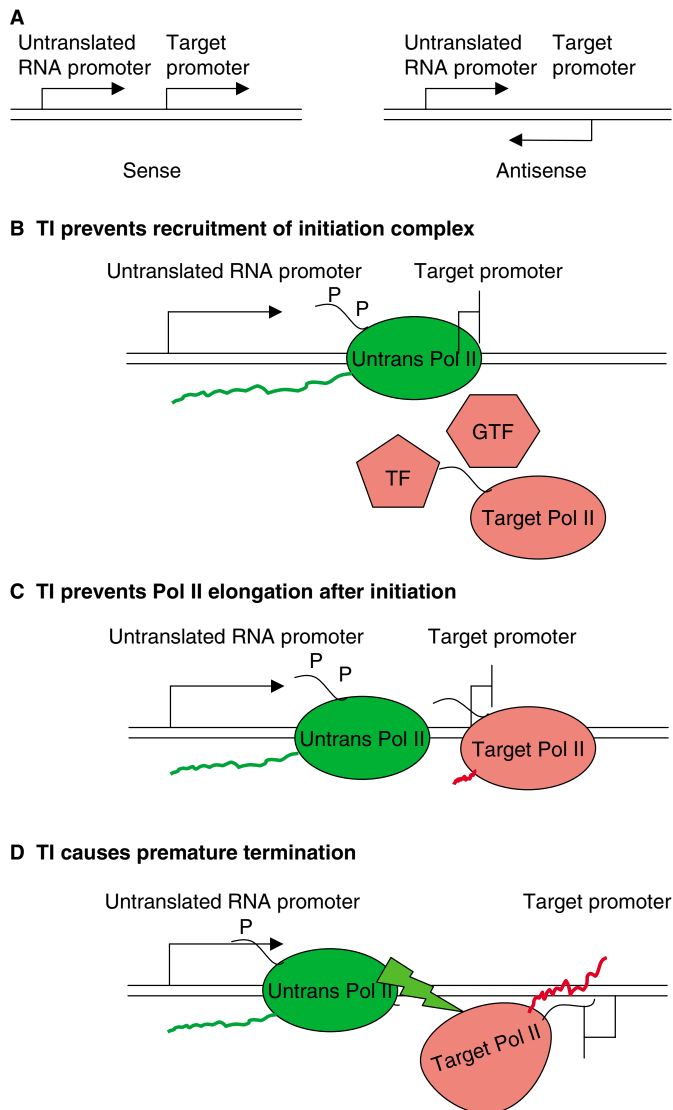

Fig. 1. Mechanisms of TI. (A) Tandem and convergent orientation of genes that permit sense and antisense TI. (B) Transcription of the untranslated RNA through the promoter could prevent recruitment of Pol II (shown with an unphosphorylated C-terminal domain in red), general transcription factors (GTFs) or specific transcription factors (TF) to the target. The transcribing Pol II from the ncRNA promoter is show in green and with a phosphorylated C-terminal domain. (C) Transcription of ncRNA could prevent promoter clearance or elongation. (D) Transcription of ncRNA could cause premature termination, shown here as resulting from a collision between converging polymerases that dislodges the Pol II transcribing the target gene.

(Martens et al., 2004). They showed that transcription of *SRG1* allows recruitment of TATA-binding protein and Pol II to *SER3* (although interpretation of these results is complicated by the presence of Pol II transcribing *SRG1*). Because the endogenous activating factors for *SER3* are not known, they replaced the *SER3* upstream activating sequence by Gal4 binding sites. *SRG1* represses *SER3* even under conditions in which Gal4 is induced, which demonstrated that TI is still occurring. Chromatin immunoprecipitation experiments show that transcription of *SRG1* reduces binding of Gal4to *SER3*. TI in this system could therefore operate at the level of transcription factor recruitment.

TI resulting from antisense expression of untranslated RNA has also been described recently in *S. cerevisiae* (Hongay et al., 2006). Haploid cells of opposite mating type (*Mat a* and *MAT α*) mate to produce *MATa/α* diploid cells (Hongay et al., 2006). Diploid cells cease vegetative growth when nutrients are limiting and enter meiosis to produce four haploid daughter cells (Hongay et al., 2006). Entry into meiosis is regulated in part by initiator of meiosis 4 (*IME4),* a putative RNA methyltransferease that is expressed in *MATa/α* diploids (Hongay et al., 2006). Expression of the *IME4* sense transcription is regulated by an *IME4* antisense transcript that spans the entire sense transcript of *IME4*. The *IME4* antisense transcript is present in haploid yeast and in *MATa/a* and *MATα/α* diploid homozygotes but absent in *MATa/α* diploid heterozygotes. The *IME4* antisense transcript might, therefore, repress *IME4* transcription in haploid cells and in *MAT a/a* and *α/α* diploid cells. Indeed, expression of the *IME4* antisense transcript is repressed by a1-α2 heterodimers to enable expression of the *IME4* sense transcript (Fig. 2). If expression of the *IME4* antisense transcript is forced in diploid cells, these mutants acquire haploid characteristics. Transcription of the antisense *IME4* transcript in trans fails to repress *IME4*, ruling out RNA-mediated repression mechanisms such as RNA interference; only *IME4* antisense transcription in cis reduces transcription of the *IME4* sense transcript. As previously shown in coliphage (Callen et al., 2004), the strength of TI at *IME4* depends on the relative strength of the opposing promoters. Overexpression of the *IME4* sense transcript reduces TI mediated by the wild-type *IME4* anti-sense promoter.

Mammals

There have been several indications that TI also operates in mammals. For example, in mice, the tandemly arranged upstream of N-ras (*unr*) and *N-ras* genes are coordinately expressed. Promoter deletion experiments in embryonic stem cells show that abolishing *unr* expression upregulates *N-ras*, which would be consistent with TI with *N-ras* by *unr.* However, it is not clear whether *unr* regulates *N-ras* in vivo at the endogenous locus (Boussadia et al., 1997). The sphingosine kinase gene (*sphk1*) is another candidate. It is transcribed in both the sense and antisense directions. Interestingly, the sense and antisense transcripts of *sphk1* are mutually exclusive at the single cell level, which again is consistent with the possibility that TI occurs (Imamura et al., 2004). Furthermore, a recent report indicates a role for TI at the β-globin locus of mice. The *βh0* gene encodes an adult β-like globin that pairs with α-globin. *βh0* lies immediately downstream of the embryonic β-globin gene *Ey*. Deletion of the *Ey* promoter upregulates transcription of the downstream *βh0* gene (Hu et al., 2007). This interaction is a developmental switch that prevents simultaneous expression of embryonic and adult β-globins in embryos, but allows expression of *βh0* when expression of *Ey* ceases.

An important recent paper reported that the human dihydrofolate reductase (*DHFR)* gene is regulated by TI (Martianov et al., 2007). *DHFR* is expressed under the control of the downstream major promoter in cycling cells because it

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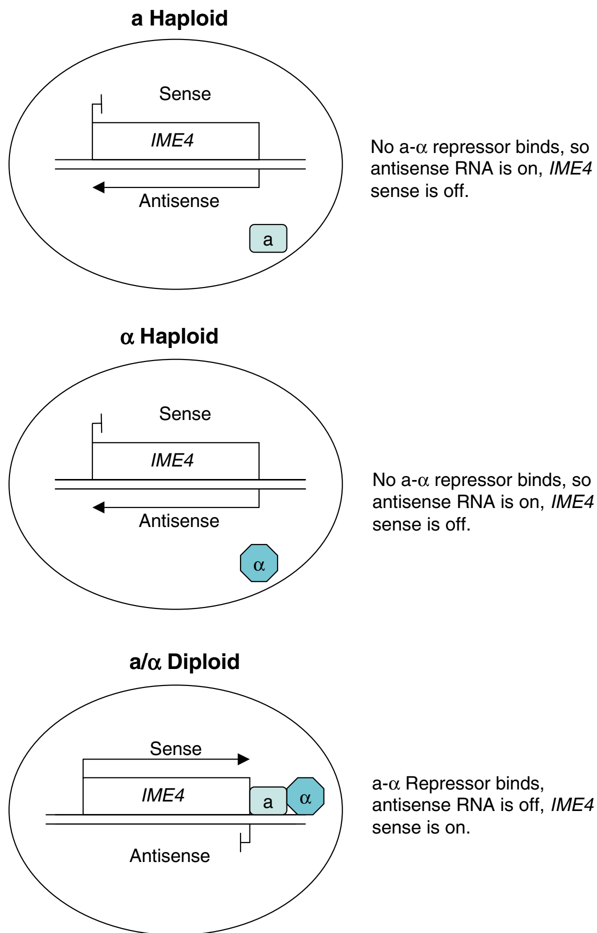

Fig. 2. Schematic diagram showing mechanism of TI at the *IME4* locus in yeast. The *IME4* locus is transcribed by a sense and an antisense transcript in haploid cells of the a or α mating type because the MATa-α repressor that represses the antisense transcript is not present. As a result, IME4 protein is not produced. In diploid *MATa/α* cells, MATa-α represses transcription of the *IME4* antisense transcript. This allows production of IME4 and the initiation of meiosis.

is necessary for DNA synthesis. *DHFR* is repressed to prevent unnecessary DNA synthesis in quiescent cells by expression of an untranslated RNA produced from a minor upstream promoter that terminates in the second intron of *DHFR*. Expression of the untranslated RNA reduces binding of TATA-binding protein (TBP) and TFIIB in vitro, and of TBP, TFIIB, and Pol II in vivo, to the major *DHFR* promoter. Reduction in recruitment of the general transcription factors does not occur in cells in which the regulatory upstream transcript is degraded by RNA interference. Thus, competition between the promoter for the regulatory transcript and the bona fide *DHFR* promoter for transcription factors cannot explain the repression. Indeed, insertion of a transcriptional termination signal upstream of the *DHFR* major promoter permits recruitment of TFIIB and gene activation in quiescent cells, where *DHFR* is normally repressed. Together, these results suggest that the recruitment of the pre-initiation complex still occurs, albeit less efficiently, and that the block occurs at the step of promoter clearance (see Callen et al., 2004).

Intriguingly, the regulatory transcript in this system forms an RNA-DNA purine-purine-pyrimidine (H-form) triplex (Letai et al., 1988) in the region of the *DHFR* promoter in vitro. Martianov et al. propose that stable triplex formation between the sense-strand regulatory RNA and the *DHFR* gene at the promoter contribute to TI and promoter targeting in vivo (Martianov et al., 2007). However, a previous report suggested that triplex formation is not important for preventing recruitment of transcription factors in vitro at the *DHFR* locus (Blume et al., 2003). It would be interesting to know whether triplex formation is necessary for TI at the *DHFR* gene in vivo, since >95% of genes in mice and humans have triplex-forming ability in their promoters or transcribed regions (Wu et al., 2007).

Drosophila

TI also operates in developmental gene regulation in *Drosophila*, where recent work has provided significant insight into the mechanisms involved. The activities of the tandem promoters of the alcohol dehydrogenase (*Adh*) locus provide the first reported example (Corbin and Maniatis, 1989). *Adh* is expressed tissue specifically and at different levels at different times in development. The proximal promoter is active from embryogenesis up to the mid-third instar larva, whereas the distal promoter is active from mid-third instar onwards. In the absence of the distal promoter, or if the proximal promoter is placed upstream of the distal promoter, expression from the proximal promoter continues throughout development. These results are consistent with repression of the proximal promoter by TI from the distal promoter. Significantly, they are inconsistent with promoter competition. Thus TI at the *Adh* locus results in a developmental switch, because the early acting *Adh* promoter represses the later-acting downstream promoter. In the mid-third instar larva the early acting promoter is repressed by developmentally regulated transcription factors, which in turn allows derepression of the later promoter.

The bithorax complex

The *Drosophila* bithorax complex (BX-C) provides the most complex known example of TI known in eukaryotes. It is one of the two clusters of homeotic (*Hox*) genes in the genome and specifies the identity of the fly thoracic and abdominal segments. We refer readers to excellent recent reviews of the BX-C elsewhere (Akbari et al., 2006; Maeda and Karch, 2006).

The BX-C contains three *Hox* genes: *Ultrabithorax* (*Ubx*), which is expressed in parasegments 5-12; *abdominal A* (*abd-A*), which is expressed in parasegments 7-13; and *Abdominal B* (*Abd-B*), which is expressed in parasegments 10-14 (Fig. 3A). The spatial position of genes expressed and levels of *Hox* gene expression regulate embryonic parasegment identity along the anteroposterior axis, which ultimately controls segment identity in the adult (Beachy et al., 1985; Celniker et al., 1990; Duncan, 1987; Karch et al., 1990; Lewis, 1978; White and Wilcox, 1985). Each *Hox* gene is controlled by long (up to 55 kb) complex regulatory regions that govern expression in specific parasegments (see Fig. 3B). These regulatory regions contain developmental-stage-specific enhancers, tissue- and germ-layer-specific enhancers, and boundary elements that prevent cross-talk between regions (Akbari et al., 2006). In addition, each regulatory region also contains a DNA sequence called a

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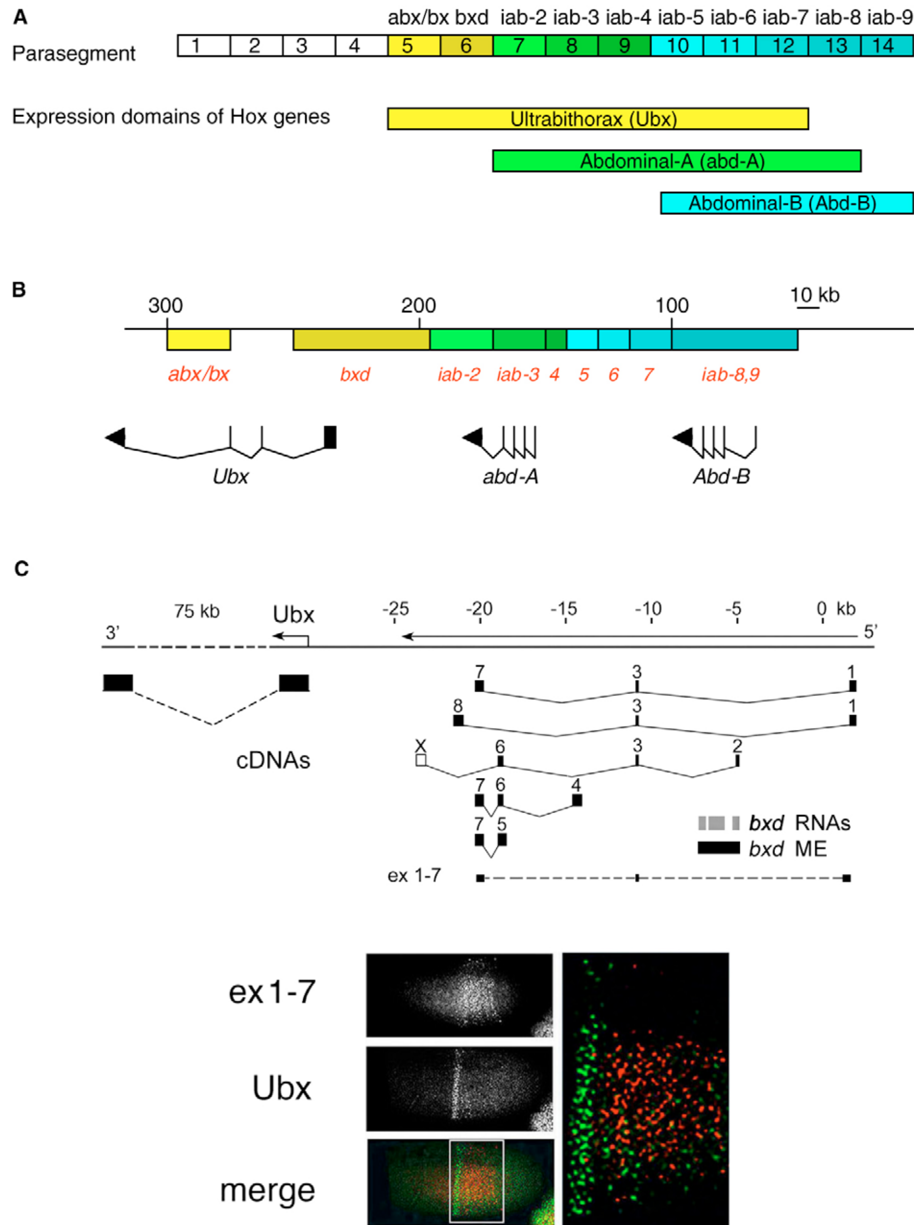

Fig. 3. The BX-C and untranslated RNAs. (A) Relationship between embryonic parasegments, cis-regulatory domains of the BX-C, and expression domains of *Hox* genes in embryos. The names of the regulatory regions are given above, and the embryonic expression domains of *Hox* genes are shown below. Regulatory domains set the anterior borders of the parasegments indicated but also function in posterior parasegments. (B) Molecular map of BX-C. Cis-regulatory domains are colored as in A, and *Hox* mRNA structure is shown (simplified for *Abd-B*) below. Regulatory domains marked in red are transcribed to give untranslated RNAs. (C) Detailed map of untranslated RNAs of the *bxd* regulatory region [after Lipshitz et al. (Lipshitz et al., 1987)]. Grey bars show additional transcripts identified recently (Sanchez-Elsner et al., 2006). The locations of the maintenance element (ME) and the transcription start site for *Ubx* are indicated. The bottom panel shows that *Ubx* (green) and *bxd* ncRNA (red) are expressed in different cells of the blastoderm embryo [adapted from Petruk et al. (Petruk et al., 2006)].

maintenance element that is the binding site for Polycomb and trithorax group (PcG and trxG) proteins that respectively maintain the repressed and activated states of *Hox* genes in different parasegments (Breiling et al., 2007; Grimaud et al., 2006; Ringrose and Paro, 2007).

Intergenic untranslated RNAs of the BX-C

The potential importance of untranslated RNAs at the BX-C was recognized very early. More than 20 years ago, the *bxd* region, which regulates *Ubx*, was shown to generate untranslated RNAs from about 2-6 hours of embryogenesis (see Fig. 3C), and a later transcript is detected in larvae and adults (Akam et al., 1985; Hogness et al., 1985; Lipshitz et al., 1987). Shortly afterwards, other untranslated intergenic transcripts were discovered in the *abd-A* and *Abd-B* domains (Cumberledge et al., 1990; Sanchez-Herrero and Akam, 1989). Later studies characterized these transcripts in more detail (Bae et al., 2002; Casares and Sanchez-Herrero, 1995; Drewell et

al., 2002). A common feature of these RNAs is that their expression is developmental stage specific and spatially regulated. Their expression within parasegments seems to correspond with that of the *Hox* gene controlled by the regulatory region that transcribes them in early embryogenesis (Akam et al., 1985; Bae et al., 2002; Cumberledge et al., 1990; Drewell et al., 2002). Thus the *bxd* region regulates *Ubx* expression in parasegments 6-12, and *bxd* untranslated RNAs are expressed in the same parasegments.

These pioneering studies did not ascribe a function to these untranslated RNAs. One of the reasons is that analysis of mutants that affect untranslated RNAs is difficult. The complexity of the regulatory regions means that most available mutations or mobile element insertions have the potential to disrupt multiple functions of regulatory regions in addition to affecting production of untranslated RNAs. For example, the pbx^1 and pbx^2 deletions of *bxd* eliminate transcription of *bxd* untranslated RNAs but also eliminate embryonic and imaginal enhancers, and in the case of pbx^2 they eliminate the *bxd* maintenance element. Another difficulty is that most analyses of homeotic phenotypes depend on segment-specific changes in embryonic or adult cuticle. These analyses of cuticle probably will not detect phenotypes caused by misexpression of untranslated RNAs in 2-hour- to 6-hour-old cells of specific tissues or germ layers in embryos (Hogness et al., 1985). Detecting homeotic phenotypes caused by mutations in untranslated RNAs is therefore likely to require markers specific for the cells of interest. Despite these difficulties confirming an in vivo role of BX-C untranslated RNAs, it was recognized that their tight regulation argued for a functional role (Bae et al., 2002; Drewell et al., 2002). Indeed, some five years ago, one group postulated that transcription itself might be more important than the sequence of the untranslated RNA, although they did not suggest TI as a potential mechanism (Drewell et al., 2002).

Functions of untranslated RNAs in the BX-C

One widely accepted idea is that the untranslated RNAs from the BX-C prevent repression of *Hox* genes by PcG proteins (Akbari et al., 2006; Ringrose and Paro, 2007; Schmitt and Paro, 2006). This model is based in part on in situ hybridization experiments showing that the expression domains of *Hox* genes and neighbouring untranslated RNAs are similar (Bae et al., 2002; Drewell et al., 2002; Rank et al., 2002), which would be consistent with a role in activation. Moreover, other experiments have shown that transcription through maintenance elements correlates with loss of silencing (Bender and Fitzgerald, 2002; Hogga and Karch, 2002; Schmitt et al., 2005). Therefore, transcription of untranslated RNA through the maintenance elements in BX-C regulatory regions has been proposed to prevent recruitment of PcG proteins. Chromatin immunoprecipitation experiments show that binding of PcG proteins to maintenance elements is constitutive in imaginal disks, which suggests that PcG proteins are not displaced even in cells where *Ubx* is active. This evidence has been used to argue against an activatory role for untranslated RNA (Muller and Kassis, 2006). However, given that untranslated RNAs are not expressed in imaginal disks, and PcG binding in cells expressing *Ubx* from early embryos has not been examined, data from studies of the imaginal disks do not exclude the possibility that untranslated RNAs prevent PcG binding.

More recent evidence, however, indicates that *bxd* untranslated RNAs in fact repress *Ubx* expression in vivo (Petruk et al., 2006). First, high-resolution fluorescent in situ hybridization (FISH) experiments with *bxd* untranslated RNAs and *Ubx* show that *Ubx* is not expressed in cells expressing *bxd* untranslated RNAs and vice versa within parasegments 6-12 (see Fig. 3C). Second, introduction of transgenes lacking promoters for *bxd* untranslated RNAs causes *Ubx* expression from the transgene to expand into domains formerly occupied by *bxd* untranslated RNAs within the parasegment. Additional experiments suggest that untranslated RNAs from *iab-3* and *iab-4* are not expressed in cells that express *abd-A*, which supports the idea these untranslated RNAs similarly repress *abd-A* (Petruk et al., 2007). Interestingly, different *bxd* untranslated RNAs are expressed in different germ layers, and in specific cells. Spatial regulation of the synthesis of untranslated RNAs in the BX-C may therefore account for the observed mosaic expression patterns of *Hox* genes in parasegments in early embryonic development (Petruk et al., 2007; Petruk et al., 2006).

Note that the results of the genetic experiments showing that transcription through the maintenance element inhibits silencing of *Hox* genes could be interpreted as evidence of TI. Transcription from strong mobile elements (Whitelaw and Martin, 2001) or experimentally inducible promoters could prevent transcription of the untranslated RNAs, which in turn would cause the observed derepression of *Hox* targets (Bender and Fitzgerald, 2002; Hogga and Karch, 2002; Schmitt et al., 2005). Derepression of *Hox* genes caused by transcription through maintenance elements disrupts silencing in anterior parasegments mediated by PcG proteins, whereas deletion of untranslated RNA promoters disrupts expression of *Ubx* in posterior parasegments. This argues that TI by non-coding RNAs and the induced TI through maintenance elements affect different processes.

Mechanisms of transcriptional interference

How might TI operate? There are several possibilities (see Shearwin et al., 2005) (see Fig. 1). If transcription of the regulatory gene occurs earlier or more often than that of the target gene, recruitment of Pol II or other factors required for initiation of the target gene may be suppressed (Adhya and Gottesman, 1982; Martens et al., 2004; Martianov et al., 2007). This would depend on the firing rate of the regulatory promoter, the size and strength of the target promoter, and the elongation rate. Prevention of factor recruitment to the target could occur directly if passage of the Pol II elongation complex over the target promoter prevents binding. Alternatively, it could be indirect if transcription of the untranslated RNA gene induces alterations in DNA topology or chromatin structure of the target gene promoter (Fig. 1B).

A second model (Fig. 1C) assumes that recruitment of the pre-initiation complex to the target promoter occurs normally, but that the recruited Pol II is subsequently dislodged by the elongating Pol II complex from the regulatory gene, which has been experimentally demonstrated in coliphage (Callen et al., 2004). The converse possibility, that occupation of an interior promoter by Pol II prevents transcriptional elongation in the other gene, has not been confirmed experimentally.

Another possibility for which there is experimental support is that interacting elongation complexes from the sense and

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antisense promoters cause premature termination of one, or more likely, both complexes (Prescott and Proudfoot, 2002) (Fig. 1D). It is intriguing that in yeast, loops form between sites of transcriptional initiation and termination, and that some components are shared by transcriptional initiation and termination complexes (O'Sullivan et al., 2004). Perhaps in TI interaction of the termination complex for the untranslated RNA gene prevents interaction of the promoter and transcription termination site for the repressed gene. The termination complex of the transcribed gene may interfere with changes in chromatin structure needed for transcription of the repressed gene (Proudfoot, 2004). These possibilities could explain results showing that TI can occur even in genes in which promoter competition does not occur and where transcripts do not overlap (Eszterhas et al., 2002).

Promoter competition for a limiting transcription factor has also been classified as a mechanism of TI (Shearwin et al., 2005) but is considered by many to be a separate phenomenon (Martens et al., 2004). The mechanisms that establish conditions for the regulatory promoter to fire earlier or more strongly than the target promoter probably occur before transcription begins, which is consistent with promoter competition being different from TI. However, initial conditions for each promoter may be similar. A positive feedback mechanism resulting from transcription of the regulatory promoter may allow it to outcompete the target promoter, which would be consistent with TI. Distinguishing these possibilities will require further experiments.

Concluding remarks

Recent discoveries in yeast, *Drosophila* and mammals suggest that gene regulation by transcription of cis-acting untranslated RNAs could be more prevalent and important than previously realized. Some attempts have been made to address TI in vivo in eukaryotes by using transgenes (Eszterhas et al., 2002; Prescott and Proudfoot, 2002; Proudfoot, 1986). In the future, it will be important to understand the mechanisms of TI at endogenous loci. There are several other questions that remain to be answered. For example, some untranslated RNAs that regulate the BX-C are produced upstream of their target *Hox* loci (e.g. in the case of *bxd* and *iab-4*), whereas others are produced downstream (e.g *abx/bx*, *iab-2* and *iab-3*, and *iab-5* to *iab-8* inclusive). If all untranslated RNAs regulate *Hox* genes by TI in cis, then both sense and antisense TI must be possible. Both the *iab-4* and *iab-6* regulatory regions have both sense and antisense transcripts (Bae et al., 2002). Intriguingly, the expression of the sense and antisense transcripts from *iab-4* and *iab-6* is mutually exclusive, which is consistent with the possibility that untranslated RNAs themselves are regulated by TI. We predict that further studies of untranslated RNAs in the BX-C will reveal roles for many of these in repression of *Hox* genes. It is unlikely that untranslated RNAs explain all aspects of regulation of *Hox* genes of the BX-C. It will therefore be interesting to determine how TI is integrated with longer-lasting repression by PcG proteins or with regulation by posterior *Hox* genes.

Alternative promoters are common in eukaryotes. If, as shown by recent analysis of *DHFR* (Martianov et al., 2007), usage of alternative promoters reflects TI, then it would be worthwhile to examine genes that have multiple promoters to see whether TI occurs in these cases. It has been speculated that transcription of untranslated RNAs in eukaryotes provides an additional layer of genetic regulation that accounts for the increased complexity of higher eukaryotes that cannot be explained by the small increase in the number of translated RNAs in higher eukaryotes versus bacteria (Mattick, 2004; Mattick and Makunin, 2006). A systematic examination of the role of long untranslated RNAs in gene regulation in development is an important goal for the future.

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