An insulator blocks access to enhancers by an illegitimate promoter, preventing repression by transcriptional interference

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Abstract

Several distinct activities and functions have been described for chromatin insulators, which separate genes along chromosomes into functional units. Here, we describe a novel mechanism of functional separation whereby an insulator prevents gene repression. When the homie insulator is deleted from the end of a Drosophila even skipped (eve) locus, a flanking P-element promoter is activated in a partial eve pattern, causing expression driven by enhancers in the 3′ region to be repressed. The mechanism involves transcriptional read-through from the flanking promoter. This conclusion is based on the following. Read-through driven by a heterologous enhancer is sufficient to repress, even when homie is in place. Furthermore, when the flanking promoter is turned around, repression is minimal. Transcriptional read-through that does not produce anti-sense RNA can still repress expression, ruling out RNAi as the mechanism in this case. Thus, transcriptional interference, caused by enhancer capture and read-through when the insulator is removed, represses eve promoter-driven expression. We also show that enhancer-promoter specificity and processivity of transcription can have decisive effects on the consequences of insulator removal. First, a core heat shock 70 promoter that is not activated well by eve enhancers did not cause read-through sufficient to repress the eve promoter. Second, these transcripts are less processive than those initiated at the P-promoter, measured by how far they extend through the eve locus, and so are less disruptive. These results highlight the importance of considering transcriptional read-through when assessing the effects of insulators on gene expression.

Author summary

Several distinct activities and functions have been described for chromatin insulators, which are regulatory DNA elements that separate genes along chromosomes into functional units. Here, we describe how insulators can prevent repression of one gene by preventing inappropriate transcription of another gene, without blocking read-through of transcription per se. When the insulator homie is deleted from the end of a transgenic eve locus, a flanking transposable element promoter is activated by eve enhancers, causing repression of the eve promoter. The mechanism involves transcriptional read-through...
Insulator deletion allows read-through-induced transcriptional interference

from the flanking promoter, which disrupts normal eve enhancer-promoter activities. When the flanking promoter is turned around, repression of eve is minimal. Thus, transcriptional interference, caused by enhancer capture and read-through when the insulator is removed, represses the eve promoter. These results show a novel role for transcriptional read-through in the effects of insulators on gene expression.

Introduction
Recent studies have shown that insulators are multi-functional elements, performing several important tasks [1]. They block both enhancer-promoter (E-P) interactions [2–7] and the spreading of repressive histone modifications along the chromatin fiber [8–12]. At the same time, they form relatively stable interactions with other insulators, sometimes nearby and sometimes at great distances, thus contributing to chromosome organization [7,13–20]. When distant regions are brought together, novel E-P interactions can occur that contribute significantly to patterns of gene expression.

In Drosophila, insulators (a.k.a. boundaries) that separate adjacent cis-regulatory domains in the bithorax complex (BX-C) have been well studied [21–25]. For example, the Fab-7 boundary separates the iab-6 and iab-7 cis-regulatory elements of the Abdominal-B (Abd-B) gene that control phenotypes of parasegments (PSs) 11 and 12, respectively. Mutations that remove Fab-7 cause transformation of PS11 to PS12, explained by ectopic activation of iab-7 in PS11 due to a loss of enhancer blocking activity in early embryos. Later studies found that Fab-7 is also required for insulating the repressive effects of a Polycomb Response Element (PRE) that prevents inappropriate activation of Abd-B in specific tissues [26,27]. Genome-wide analysis found these insulators to be enriched in insulator binding proteins [28].

Insulators are also involved in defining TADs (topologically associating domains) [1,29,30]. A recent study showed that removing the Fub boundary from the BX-C caused the fusion of two adjacent TADs [31], resulting in ectopic expression of abdominal-A (abd-A) in segment A1 [32,33]. Similarly, in a mammalian Hox complex, CTCF was shown to be required for insulating adjacent chromatin domains from each other [34,35]. While the majority of TADs in mammals are flanked by CTCF binding sites [36,37], CTCF is only one of a group of proteins that contribute to insulator function in Drosophila, and not all Drosophila insulators have CTCF binding sites [38]. Another difference between insulators in mammals and Drosophila is that mammalian TADs often contain several transcription units [39], while most transcription units in Drosophila, along with their regulatory DNA, constitute a separate TAD [40]. Despite these distinctions, mammalian insulators probably perform the same range of functions as those in Drosophila.

Roles for nuclear RNAs in regulating chromatin structure and gene expression have been uncovered in recent years [41–43]. Transcription itself, proceeding through a gene, may result in transcriptional interference. Such read-through may disrupt either enhancer or core promoter function, or disrupt their ability to interact over long distances. Mechanistically, read-through may disrupt transcription factor binding or function [44]. Read-through transcription can underlie disruptions in development. For example, insertion into the BX-C of promoters contained within either the 5’ P-element end [45] or the scs insulator [46] have been shown to produce read-through transcripts driven by nearby enhancers that cause disruptions of gene expression, and consequent developmental defects. Conversely, a 92 kb non-coding RNA, which is normally initiated within the iab-8 regulatory domain of the BX-C and reads through
several insulators, is required for repression of abd-A in parts of the nervous system, so that disrupting it causes defects [47].

Segmentation is very sensitive to the levels of eve expression, so that even minor changes cause developmental defects, making manipulations of the endogenous locus problematic [48,49]. In order to circumvent this limitation, we previously created a pseudo-locus transgene [12], which contains the entire eve locus and the 5' end of the 3'-neighboring gene, TER94. In this pseudo-locus, the eve protein coding region is replaced by that of lacZ, while that of TER94 (an essential, ubiquitously expressed gene) [50–52] is replaced by EGFP. Using the φC31 integrase system [53], this pseudo-locus was inserted at several attP docking sites, which had been introduced into the genome by P-element transformation [12]. Removing the 3' eve insulator homie from the pseudo-locus reduced eve-lacZ activity, particularly that of enhancers located 3' of the transcription unit [54,55]. This was seen as reduced expression of early stripes 1, 4, 5, and 6, as well as reduction of later-stage, tissue-specific expression [12]. Early stripes 2, 3, and 7 are driven by enhancers upstream of the start site [56–59], and they were repressed less, giving rise to a clear imbalance in early stripe expression. This kind of early stripe imbalance, when it occurs at a functional eve locus, causes severe, lethal disruptions in segmentation, illustrating the value of using the pseudo-locus to study eve regulation [48,49].

Here, we focus on the two insulators that flank the eve locus, homie and nhomie [7,12,19]. We show that the stripe imbalance seen at the pseudo-locus when an insulator is removed is caused by the capturing of eve enhancer activity by a flanking P-element promoter [60–63]. The absence of an insulator allows the flanking promoter to be activated by eve enhancers in a pattern that matches the pattern of repression of eve-lacZ. We further show that the repression is primarily attributable to transcriptional interference by read-through, with a minor contribution from promoter competition. The imbalance is not prevented by the insertion of multiple poly-adenylation (polyA) signals from the α-tubulin gene [64] and SV40 [65], because it does not effectively stop transcriptional read-through. A commonly used core promoter from the heat shock 70 (hsp70) locus [66] does not produce such processive transcripts, and so does not disrupt eve-lacZ expression. These studies add to our knowledge of the mechanisms underlying disruptions of gene expression seen when insulators are removed, or their functions compromised.

Results

Removal of an insulator causes disruption of eve promoter expression by a flanking activity

For this study, we added nhomie [19] to the 5' end of eve-lacZ, making the pseudo-locus identical to the endogenous eve locus including the 5' end of TER94, except for the replacement of protein coding sequences (Fig 1A). Recombinase-mediated cassette exchange (RMCE) [53] was used for transgene insertion, which could occur in either orientation at each site, and both orientations were analyzed. The control construct faithfully reproduced the full eve expression pattern throughout embryogenesis, independent of the orientation of the insert (Fig 1B and 1C, "wt"). In one orientation of the pseudo-locus (the H5 orientation, homie is close to 5'P, Fig 1A and 1B), removing homie disrupted eve-lacZ expression [12], causing early stripes 1, 4, 5, and 6 to be reduced relative to stripes 2, 3, and 7, along with repression of all later tissue-specific expression (including in the mesoderm, "meso", and anal plate ring, "APR", Fig 1A and 1B). In the other orientation (N5, nhomie is close to 5'P, Fig 1A and 1C), the expression pattern without homie was indistinguishable from that of the wild-type locus, although there may be a tendency toward slightly weakened eve-lacZ expression overall, as well as occasional stripe imbalances (Fig 1C, "Δhomie"). However, when nhomie was removed in this N5 orientation,
Fig 1. Effects on expression due to eve insulator removal and replacement. (A) Map of the eve pseudo-locus construct (inserted in either orientation, H5 or N5). The wild-type eve and TER94 coding regions were replaced with lacZ and EGFP, respectively. Rectangles represent regulatory elements: red, nhomie and homie insulators; yellow, Polycomb response elements (PREs); blue, embryonic stripe enhancers labeled with either numbers for early stripes, or “late” for the 7 later stripes; pink, tissue specific enhancers for: APR, anal plate ring; meso, mesoderm (other known elements are either unlabeled or not included). 5’P and 3’P: 5’ and 3’ P-element ends, part of the attP site previously
Insulator deletion allows read-through-induced transcriptional interference

Insert a P-element promoter into the eve pseudo-locus. In all cases, removing homoiec or nhomiec had a strong effect, the repressed enhancers were located toward the end of the loci flanked by the 5′P-element end (a.k.a. P-element border), which contains a P-element promoter [60–63]. Therefore, we hypothesized that the P-element promoter was driving read-through transcription, and that this was involved in repressing eve-lacZ enhancer activity. To begin to test this, we quantified transcripts in the eve regulatory regions using RT-qPCR. First, we found that transcripts were barely detectable throughout the endogenous eve locus (using yw flies not containing the pseudo-locus, Fig 2A, “yw”). With the wild-type pseudo-locus, slightly higher transcript levels were seen (Fig 2A, “wt” vs. “yw”). However, without homoiec (Fig 2A, “Δhomoiec”), transcript levels were increased. Consistent with our hypothesis, deletion of nhomiec in the H5 orientation showed similar levels of transcripts to wild-type (Fig 2A, “Δhomoiec”). This is potentially attributable to a blocking activity of insulators, since nhomiec in the H5 orientation is not between the enhancers and P-element promoter (see Fig 1A map). In contrast, in the N5 orientation, deletion of nhomiec caused

both early stripe 3 at stage 5 (Fig 1C, “Δhomoiec”, compare stripe 3 intensity to stripe 4 at “stg5”) and the later 7-stripe expression at stage 7 (driven by the enhancer labeled “late” in Fig 1A) was somewhat reduced (Fig 1C, “Δhomoiec”, black dots mark affected stripes at stage 7). In contrast, in the H5 orientation where removal of homoiec had a dramatic effect, removing nhomiec had little effect (Fig 1B, Δhomoiec; here again, there may be slightly weakened eve-lacZ expression overall, as well as occasional stripe imbalances). We tested the same pseudo-locus transgenes inserted at other P-element-attP sites, and they showed the same tendencies, but with even stronger repressive effects when the insulator at the “5′P′ end of the locus was removed (Fig 1D and 1E). Therefore, removing either homoiec or nhomiec affects different sets of enhancers, and the effects strongly depend on the orientation of the transgenic eve locus, implicating a specific flanking activity in causing the disruptions.

Insulators are known to block both E-P interactions and the spreading of repressive chromatin from a nucleation site, such as a PRE [1,7,12]. Is the flanking activity that is responsible for eve pseudo-locus disruption either of these known insulator-blocked activities? As a first step to test this, several other insulators were used to replace homoiec in the pseudo-locus in the H5 orientation, and we asked whether they could prevent repression of eve-lacZ. All transgenes with either the Fab7, Fab8, or gypsy insulators expressed eve-lacZ similarly to wild-type (Fig 1F), showing that they all can protect the eve locus. Thus, this protective activity is a common feature of insulators.

The flanking disruptive activity is attributable to a transposable element promoter

What is causing repression of eve-lacZ in the absence of an intervening insulator? In all cases where removing homoiec or nhomiec had a strong effect, the repressed enhancers were located toward the end of the locus flanked by the 5′P′-element end (a.k.a. P-element border), which contains a P-element promoter [60–63]. Therefore, we hypothesized that the P-element promoter was driving read-through transcription, and that this was involved in repressing eve-lacZ enhancer activity. To begin to test this, we quantified transcripts in the eve regulatory regions using RT-qPCR. First, we found that transcripts were barely detectable throughout the endogenous eve locus (using yw flies not containing the pseudo-locus, Fig 2A, “yw”). With the wild-type pseudo-locus, slightly higher transcript levels were seen (Fig 2A, “wt” vs. “yw”). However, without homoiec (Fig 2A, “Δhomoiec”), transcript levels were increased. Consistent with our hypothesis, deletion of nhomiec in the H5 orientation showed similar levels of transcripts to wild-type (Fig 2A, “Δhomoiec”). This is potentially attributable to a blocking activity of insulators, since nhomiec in the H5 orientation is not between the eve enhancers and P-element promoter (see Fig 1A map). In contrast, in the N5 orientation, deletion of nhomiec caused
increased transcript levels, while the transcript levels remained low in wild-type (Fig 2C, "Δnhomie", compare to "wt"). The ratio of Δhomie or Δnhomie transcripts to wild-type showed a maximum near the 5'P-element end, and tended to decrease throughout the locus (Fig 2B and 2D). Similar effects were seen at another transgene landing site (S1 Fig). These data suggest that the absence of an insulator causes transcription through the eve enhancers, which is strongest through those enhancers that are most disrupted. This, in turn, may suggest that transcription through eve enhancers represses eve-lacZ expression.

What is activating the P-element promoter? The TER94 portion of the pseudo-locus contains ubiquitously active enhancers [12]. In a previous study, we showed that TER94-GFP is activated by the eve enhancers when homie is removed [12]. It is possible that the P-element promoter is activated by TER94 enhancers, and/or by enhancers flanking the landing site. Therefore, we analyzed embryos using in situ hybridization to see the expression pattern of transcripts reading through eve enhancers. The eve regulatory regions were surveyed using in situ RNA probes that detect transcripts coming from the 5'P end. In the wild-type pseudo-locus in the H5 orientation, there was no detectable expression pattern in most of the enhancer regions (Fig 3A, "wt", probes 2+7, 4+6, 1+5, GFP, black arrows above the map). The one exception is very faint expression at the late stripe enhancer, in the pattern of stripes 1, 2, 3, and 7 (Fig 3A, "wt", "late" probe). In contrast, when homie was deleted, early stripe 1, 4, 5, and 6 expression was clearly observed throughout the eve locus (Fig 3A, "Δhomie", probes late, 2+7, 4+6, 1+5, GFP), along with faint stripes 2, 3, and 7. Importantly, this expression pattern correlates with the pattern of repression of eve-lacZ (Fig 3A, lacZ probe, "Δhomie" vs. "wt").
In order to test whether this non-coding RNA expression is unidirectional, consistent with its coming from the P-element promoter, we used probes that recognize the opposite strand. With these probes, no detectable expression pattern was observed in any of the enhancer regions (Fig 3A, Δhomie, all probes tested).

In the N5 orientation, weak expression in the pattern of both early stripes and the 7 late stripes was observed downstream of the P-promoter, even in the presence of nhomie (Fig 3B, “wt”, probe (late)R). When nhomie was deleted, considerably stronger expression of early stripes 2, 3, 7, as well as somewhat stronger expression of stripe 1 and the 7 late stripes, was observed (Fig 3B, “Δnhomie”, probe (late)R). Weak stripe 1 expression has been shown to be driven by sequences upstream of the eve start site [58], in addition to the stronger expression driven by the stripe 1 enhancer located near the 3’ end of the locus [55]. Therefore, all of these
aspects of eve expression are driven by enhancers upstream of the eve promoter. This expression pattern can be seen throughout the locus, suggesting that the transcripts detected are continuous, long non-coding RNAs (S2 Fig, Δnhomie, probes (late)R, (2+7)R, (4+6)R, (1+5)R). We did not observe any expression pattern with probes that recognize the opposite strand (S2 Fig, Δhomie, probes 2+7, 4+6, 1+5), although there are faint stripes with “late” probe (S2 Fig, Δnhomie), similar to the ones seen in the H5 orientation (described above).

In order to determine whether the transcripts we detected were continuous, and not, for example, short eRNAs initiated within each enhancer, we further analyzed transcripts of the lines in the H5 orientation (used in Fig 1B, “wt” and “Δhomie”) using RT-qPCR. We produced cDNA using specific primers to late or EL enhancer regions, which anneal to transcripts coming from the direction of the P-element promoter (S3 Fig, green arrows above the map). If the transcripts were continuous, using these cDNAs as template, we would detect qPCR amplification products at downstream enhancer regions. Indeed, we observed high qPCR signals at downstream enhancer sequences in the Δhomie line. The cDNA primed from the “late” enhancer is continuous to the 3+7 enhancer region, and the cDNA from the “EL” region is continuous to the 4+6 and 1+5 enhancer regions, but not to a region beyond the P-element promoter (“wD5”, S3A Fig), consistent with the expected initiation of transcripts at the P-promoter. In summary, the expression patterns show that the P-element promoter is driven by eve enhancers, which is permitted when the intervening insulator is removed, with expression being stronger for those enhancers that are closer to it. This then causes read-through that represses eve-lacZ expression. Thus, the relevant insulator mechanism preventing repression of the eve promoter may be primarily the enhancer blocking function.

In order to confirm that the 5’ P-element end is the source of the transcriptional read-through, we used a MiMIC line [67], which does not carry a flanking P-element promoter. Without nhomie, the eve-lacZ pattern was similar to that of the wild-type pseudo-locus shown in Fig 1B, in either orientation (S4A Fig, Δnhomie), although we detected some stripe imbalances when both homie and nhomie were deleted (S4A Fig, Δnhomie, Δhomie). However, these imbalances were more variable and less severe than those seen in Fig 1B and 1D, and were similar to those seen frequently when eve rescue constructs lacking the insulators were inserted in random locations throughout the genome [48,55]. Therefore, P-element promoter activity driven by the eve enhancers is the main cause of the repression of eve-lacZ expression in the absence of an intervening insulator.

Transcriptional read-through is essential for the disruptive effect of the flankng promoter

The above experiments implicate the flanking P-element promoter in causing eve-lacZ repression when the intervening insulator is removed. What are the mechanisms behind this? It is possible that the read-through itself disrupts eve enhancer activity, as read-through from both P-element [45] and UAS-driven promoters has been seen to affect gene expression [44]. In the case of read-through coming from downstream of eve-lacZ (for example, in Fig 1B and 1D), continuous transcription would produce long anti-sense RNA, which might also cause RNAi-mediated degradation of eve-lacZ RNA. Another possibility is that promoter competition between the eve and P-element promoters reduces eve-lacZ expression. In order to test whether promoter competition is sufficient for repression, we introduced another set of transgenes into the MiMIC attP site (used in S4 Fig) that showed little effect from the surrounding chromatin environment. For the following experiments, the TER94 portion of the pseudo-locus was removed to reduce the number of promoters involved. The construct without the TER94 promoter expressed eve-lacZ similarly to the one with the TER94 promoter, in both
orientations of the transgene in the chromosome (S4B Fig compared to Fig 1B and 1C, "wt"). The P-element promoter was then added to the construct (Fig 4A, "P pro" on the map). In the "wild-type" construct, the P-element promoter transcribes toward the eve pseudo-locus, and homie is intact (Fig 4A, "Pwt"). In the second construct (Fig 4B, "PΔ"), homie was replaced by phage λ DNA. In the third construct, the P-element promoter was turned around relative to the "PΔ" construct, so that it transcribes away from the eve pseudo-locus, and homie is not present (Fig 4C, "PinvΔ"). In Pwt, there is no expression pattern detected in enhancer regions (Fig 4A, Pwt, probes 2+7, 4+6, Ppro, 24B, similar to that shown in Fig 3A), except for the same faint expression described above in the "late" region, which is independent of the P-promoter (Fig 4A, Pwt, "late" probe, similar to that shown in Fig 3A and S2 Fig; this expression, which is stronger in stripes 1, 2, 3, and 7 and weaker in stripes 4, 5, and 6, is more clearly seen in this insertion site than in the others). When homie is deleted (PΔ), stripes 1, 4, 5, and 6 were observed in the enhancer regions (Fig 4B, PΔ, probes "late", 2+7, 4+6), as well as just downstream of the P-element promoter ("Ppro" probe). In PinvΔ, there is again expression in stripes 1, 4, 5, and 6 downstream of the P-promoter, which is now pointing away from the eve promoter (Fig 4C, PinvΔ, probes Ppro and 24B). As expected, no expression pattern was observed in the 2+7 or 4+6 region (Fig 4C, PinvΔ, probes 2+7, 4+6), while the late region showed the same P-promoter independent, faint expression in stripes 1, 2, 3, and 7. Probes recognizing the opposite strand did not show any expression pattern (S5 Fig, probes (late)R, (2+7)R, (4+6) R). Again, cDNA produced from specific primers showed that there are continuous transcripts from enhancer to enhancer (S3B Fig), consistent with the expression patterns being attributable to long non-coding RNAs initiated at the P-promoter. Quantification by RT-qPCR
showed the expected trends, where transcript levels are elevated in PΔ lines relative to levels in lines that do not have the P-promoter pointing toward the eve promoter (S6 Fig, PΔ vs. wt,yw, and PinvΔ).

If repression of eve-lacZ is caused by transcriptional read-through, then lacZ RNA would be reduced in PΔ, but not in PinvΔ. If promoter competition is the cause, then lacZ RNA would be reduced in both PinvΔ and PΔ. In fact, lacZ RNA is reduced in PΔ, but not in PinvΔ. Therefore, eve-lacZ repression requires transcriptional read-through, while promoter competition alone is insufficient. This is the case even though when read-through is occurring, the enhancers driving the read-through expression are themselves subject to the read-through. We also cannot rule out a contribution to eve-lacZ repression from read-through of the eve promoter (in addition to the enhancers), which could disrupt either initiation itself, or E-P communication. It is also possible that promoter competition plays a role, even though it is not sufficient by itself to cause significant repression (see Discussion).

In the case where read-through transcription is coming from downstream of eve-lacZ, we cannot rule out a contribution to repression by an RNAi-based mechanism. However, in the N5 orientation shown in Fig 1C and 1E, the P-promoter is upstream of the eve promoter, so that it does not produce anti-sense RNA in the eve-lacZ coding region. Yet, we observed repression of lacZ in stripe 3 and in the 7 late stripes (Fig 1C and 1E). Although in this situation, the repression of eve-lacZ cannot be caused by RNAi, quantifying a reduction in lacZ expression by RT-qPCR is not straightforward, since the lacZ region is included in transcripts initiated by both the P-promoter and the eve promoter. That is, detected lacZ RNA (S7 Fig) is produced by both eve-lacZ expression and transcription from the P-element promoter that is repressing eve-lacZ. Nonetheless, RT-qPCR showed that lacZ RNA is reduced both in Δhome vs wild-type (S7A Fig, same lines used in Fig 1B), and in Δnhomie compared to wild-type, (S7B Fig, same lines used in Fig 1C). Therefore, while a contribution from RNAi cannot be ruled out when the P-promoter is downstream of the eve locus, transcriptional interference caused by read-through from the flanking P-promoter does, indeed, occur in the absence of an intervening insulator.

Do insulators block transcriptional read-through?

An intervening insulator blocks communication between the eve enhancers and the P-element promoter. Can it block transcriptional read-through itself? To test this, a heterologous rhomboid enhancer, NEE [68], was added upstream of the P-element promoter (Fig 5), where it should activate the P-element promoter regardless of the presence or absence of homie. In the “NEE-Pwt” control, the P-element promoter is expressed in the NEE pattern of two ventrolateral “racing stripes” running anterior to posterior (Fig 5A, “NEE-Pwt”, probe ”Ppro”). In addition, early eve stripes 1, 4, 5, and 6 are seen between the two NEE stripes on the ventral side of the embryo (Fig 5A, “NEE-Pwt”, probe “Ppro”). However, the eve stripe pattern is not seen outside of this ventral region. This may be due to the action of NEE-bound activators in the ventral region that help to generate the racing stripes, acting in combination with activators bound to the eve enhancers that generate the eve stripes, to overcome enhancer blocking by homie. This same composite expression pattern was seen with a probe of the eve enhancer region (Fig 5A, probe “4+6”), suggesting that read-through from the P-element promoter is not significantly blocked by homie, regardless of which enhancers drive it. Wherever P-element transcripts were seen, eve-lacZ was repressed (Fig 5A, probe "lacZ"). When homie was removed, stripes in the ventral region became stronger, and the same stripes were also visible in the lateral and dorsal region, consistent with the removal of homie’s enhancer blocking activity (Fig 5B vs. Fig 5A). Again, eve-lacZ was repressed wherever these transcripts were
present (Fig 5B). When the P-element promoter was turned around (with homie still not present), NEE-driven transcript expression was somewhat reduced, but the pattern of expression was the same as with the original orientation (Fig 5C, probe “Ppro”). However, consistent with our previous conclusion that repression is due primarily to read-through, and not to promoter competition, there was no detectable repression of eve-lacZ. In fact, eve-lacZ was also enhanced in the NEE pattern (Fig 5C, probe “lacZ”), showing that the “potentially competing” promoters are sharing NEE enhancer activity in the absence of homie. Interestingly, when there is read-through of the eve promoter, lacZ is not expressed in the NEE pattern, suggesting that read-through of the eve promoter region is sufficient for repression, since the NEE itself is not experiencing read-through (Fig 5B). In summary, driving read-through from a heterologous enhancer (the rhomboid NEE) causes disruption of eve expression, indicating that read-through is sufficient for repression. When homie is present, read-through transcription is driven primarily by the NEE, and eve-lacZ is repressed only where there is read-through. This shows that homie blocks only E-P interactions, not read-through transcription itself, at least of those transcripts initiated by the P-element promoter (which are highly processive, see below).

Different promoters have distinct enhancer capturing capabilities, and different degrees of transcriptional processivity

To examine the effects of promoter specificity on the actions of flanking promoters in this system, we turned to the hsp70 core promoter [66], which has been used frequently as a “general” regulated promoter in transgenic reporters [69]. To this end, we used it to drive a GFP reporter
gene without a polyA signal, activated by the NEE (NEE-hspGFP). With home in its normal place in the pseudo-locus, GFP transcripts are expressed in a similar pattern to the transcripts detected in the comparable construct with the P-element promoter, NEE-Pwt (Fig 6A, GFP probe; compare the pattern to Fig 5A, probe “Ppro”). When home is removed, the eve-like stripes in the ventral region are a bit stronger, relative to the NEE-driven “racing stripes”, than with home (Fig 6B, compare to Fig 6A, both with GFP probe), consistent with the loss of enhancer blocking activity. However, NEE-driven expression is almost undetectable in the eve regulatory region, with only very faint eve stripes seen on the ventral side (Fig 6B, probe 1+5). Reflecting this weak “read-through” expression pattern, eve-lacZ is repressed only in the ventral-most part of stripe 5 (Fig 6B, lacZ probe). When this NEE-hspGFP cassette is turned around to read away from eve-lacZ, NEE-driven expression of GFP transcripts, as well as strong ventral eve stripes with weak dorsal/lateral stripes, are seen (Fig 6C, probes GFP and 24B). Consistent with read-through being required for repression, eve-lacZ is expressed similarly to wild-type (Fig 6C, lacZ probe), except that some NEE-driven expression is also seen. It is likely that home blocks this E-P communication in the “wt” construct (Fig 6A, lacZ probe). These data suggest that the hsp70 core promoter may not respond as well to the eve enhancers as does the P-element promoter, at least at distances of several kb. They also suggest that the hsp70 core promoter initiates transcription that has low processivity, relative to that initiated by the P-element promoter, when driven by the same enhancers.
In order to eliminate the possibility that the hsp70 promoter’s response to eve enhancers is weak due to competition with the NEE, we removed the NEE, as well as the GFP coding region. Thus, this construct is equivalent to PΔ, but with the hsp70 core promoter replacing the P-element promoter (Fig 7A map). To compare activities of the two promoters directly, we used a probe to the λ sequence used to replace homie. The “λ DNA” probe showed expression driven by the P-promoter in eve stripes 1, 4, 5, and 6 (Fig 7A, PΔ). However, simultaneously stained embryos showed much fainter stripes driven by the hsp70 core promoter (Fig 7A, hspΔ, probe "λ DNA"). Consistent with this difference in transcription levels, eve-lacZ expression was not noticeably repressed in hspΔ, while it was repressed in PΔ. RT-qPCR analysis showed that RNA expression at the enhancer regions from the hsp70 promoter are 10–30% of those from the P-element promoter (Fig 7B, hspΔ/PΔ). These data suggest that the hsp70 core promoter is not activated as well by the eve enhancers as is the P-promoter (however, see Discussion).

In order to test more directly whether transcription initiated at the P-promoter has higher processivity than that initiated at hsp70 core, both NEE-PGFPΔ and NEE-hspGFPΔ (both of which have the NEE and the GFP coding sequence without a polyA site) were stained with probes located at different distances downstream. The promoter-proximal probe showed comparable levels of transcripts in both (Fig 7C, NEE-PGFPΔ and NEE-hspGFPΔ, probe "GFP"). In contrast, by the time transcription reached the stripes 1+5 enhancer region, expression was much lower in NEE-hspGFPΔ than in NEE-PGFPΔ (Fig 7C, probe "1+5"). RT-qPCR analysis showed a similarly high level of transcripts at promoter proximal GFP and PRE regions in NEE-hspGFPΔ and NEE-PGFPΔ (Fig 7D, GFP and PRE primer sets). However, consistent with the levels seen in Fig 7C, when the transcripts reach the RP2 neuronal enhancer and beyond, the transcription levels are reduced for both, but much more so for those initiated at the hsp70 promoter (Fig 7D, RP2, 1, 4+6, and EL primer sets; note that primer efficiencies can vary, so that comparisons between primer sets are not as quantitatively valid as are the ratios with a given primer set, which are graphed on the right). These data indicate that the processivity of transcription initiated at the P-element promoter is considerably higher than that initiated at the hsp70 core promoter, when driven by the same enhancers.

An intervening poly-adenylation signal only partially blocks transcriptional read-through

In order to test whether the transcriptional read-through effect is due more to read-through of the eve promoter or the enhancers (and/or disrupting E-P interactions), the commonly used α-tubulin polyA signal [64] was inserted between the 4+6 and mesodermal enhancers in the PΔ construct (which carries the P-element promoter without homie, used in Figs 4B and 7A). If read-through is inactivating only the eve promoter, stopping transcription before it reaches the promoter would rescue all eve-lacZ expression. If read-through is inactivating only enhancers, stripes 1 and 5 would still be repressed. Unexpectedly, the transcription pattern beyond the α-tubulin polyA signal did not diminish much at the 4+6 enhancer region, indicating that the polyA signal did not effectively stop read-through (S8A and S8B Fig, probe 4+6). However, the introduction of the polyA signal did cause a slight increase in eve-lacZ expression, most noticeably in stripes 4 and 6 relative to 5 (compare intensity of stripes 4 and 6 to stripe 5 with lacZ probe in S8A Fig vs. S8B Fig), suggesting that the polyA signal reduces read-through from the P-element promoter a bit, which results in partial rescue of stripes driven by enhancers downstream of the polyA site. This, in turn, suggests that read-through of enhancers reduces expression. In order to try to get a clearer result, we introduced a pair of tandem polyA signals from SV40 [65], in addition to the α-tubulin polyA signal, in the PΔ construct (Fig 8A and 8B). Again, we did not observe a clear decrease in expression beyond the polyA signals (Fig 8A and 8B;
Fig 7. Processivity of read-through transcription from flanking promoters correlates with degree of repression of the eve promoter. Transgenes are at the same MiMIC site, and in the same orientation, as in Fig 4. All loci have homie replaced with phage λ DNA (refer to map above each one). (A) eve-lacZ and flanking promoter–driven RNA expression in stage 5 embryos. Positions and orientations of the RNA probes used are shown as black and red arrows in the map. Images are labeled (above) with black lettering corresponding to black arrows, or red lettering corresponding to red arrows. Top row: transgene with P-element promoter (PΔ, also used in Fig 4B). Bottom row: transgene with hsp70 core promoter (hspΔ). Note that neither promoter has either the NEE or a GFP coding region. (B) Quantification of total RNA (normalized to RP49 RNA) from the indicated enhancer regions (probed regions are shown as red bars in the map) in the PΔ and hspΔ lines. Averages with standard deviations of 4 biological samples each from hspΔ and PΔ are graphed on the left, and the ratios of average signals (with standard deviations) are graphed on the right. (C) Same as in A, except that the both promoters have the NEE and a GFP coding region (but no polyA signal). (D) Same as in B, except that the promoters are as described in C.
probe 4+6 vs. 1+5). And again, lacZ expression was only weakly rescued in stripes 4 and 6 relative to stripe 5 (Fig 8, probe lacZ, compare intensity of stripes 4 and 6 to stripe 5 in A vs. B). There may also be an increase in expression of the other stripes relative to that of stripe 5, but the effect remains subtle. Quantitation of transcript levels by RT-qPCR showed that introduction of the polyA signals caused lower levels from the P-promoter overall (S9A Fig). The ratio of transcript levels in PpA2Δ to those in PΔ was 82% and 71% at the λDNA and the stripe 1 enhancer, respectively (both upstream of the polyA signals). Downstream of the polyA signals, this ratio was decreased to 58% and 56% at the stripe 4+6 and 3+7 enhancers, respectively, suggesting again that the polyA signals may be causing termination of a small fraction of P-promoter initiated transcripts. Finally, we tested the NEE-PGFPΔ construct with and without the same polyA signals to see if heterologous enhancer-driven transcription from the P-element promoter is affected in the same way, with similar results (S8C and S8D Fig). As before, transcript levels appeared to be slightly reduced at the 4+6 enhancer relative to the 1+5 enhancer region, and lacZ expression in stripes 4 and 6 was rescued just noticeably relative to expression in stripe 5, again suggesting that enhancer read-through may have a repressive effect on expression.

**Discussion**

**A myriad of insulator functions in gene regulation**

It is widely appreciated that removing insulators can cause the influences of both enhancers and silencers to spread inappropriately along the chromosome, changing gene expression in both flanking regions [1]. What have not been clearly described are the important secondary
effects that changes in transcription can produce, one of the most direct of which is disruption of enhancer and promoter function by transcriptional read-through coming from outside the TAD. Here, we describe such an effect at the eve pseudo-locus, which we have analyzed in detail to show that promoter competition is not sufficient for the disruption of eve-lacZ expression that ensues when an insulator is removed. There are two main lines of evidence for this. First, turning the flanking promoter around causes loss of most or all of the repression. Second, read-through driven by an added enhancer is sufficient to disrupt expression driven by the other enhancers.

We further show that insulators do not block read-through per se, consistent with data previously reported [44]. Instead, insulators prevent enhancers within one locus from activating a flanking promoter, which, without the insulator, would disrupt enhancer action on the appropriate promoter. Such indirect effects may be common when insulator activity is compromised, as we have also found that the P-element promoter produces transcripts that read through not only an insulator, but also polyA addition signals from both α-tubulin and SV40, suggesting that significant read-through may be a common phenomenon, even though the relevant polyA site(s) is intact.

A recent study showed that TADs in Drosophila usually correspond to a single locus [40]. This suggests that enhancer-promoter interactions may be a major driving force underlying TAD-level architecture. We showed that removing either homie or nhomie allows a promoter outside the locus to interact with eve enhancers, and by reading toward the eve locus, capture their activity and repress the eve promoter. Similarly, a recent study showed that removing the Fub insulator in the BX-C caused collapse of the corresponding TAD boundary [31]. Our data suggest that different genes will react differently to inactivation of insulators, since each gene is in a different chromatin context. Thus, understanding the effects of removing insulators on individual genes may be as important as identifying the general effects of insulators.

**Transcriptional read-through in gene regulation**

In this study, we showed that transcriptional read-through effectively represses eve-lacZ expression, and that homie does not block read-through. Is this an unusual case caused by a P-element promoter? Studies of the BX-C showed that long non-coding RNAs (ncRNAs) can read through insulators and affect gene regulation [47]. For example, the iab-8 ncRNA extends for 92 kb in the posterior segments of the embryo where abd-A is expressed at a relatively low level, and is required for repression of abd-A in the central nervous system. Two repression mechanisms were suggested, a trans effect due to a microRNA produced from the iab-8 ncRNA, and a second effect acting in cis. The cis effect is most likely transcriptional interference caused by transcription through abd-A [47]. The iab-8 ncRNA extends through several abd-A regulatory modules containing early- and late-acting enhancers that are separated by insulators [70]. Consistent with our study, these insulators do not stop transcriptional read-through. However, the enhancers there also do not activate the iab-8 ncRNA promoter, because the intervening insulators do block enhancer-promoter interactions. Taken together, these studies suggest that gene repression by transcriptional read-through is a likely outcome when insulator function is disrupted.

The eve enhancers can activate a flanking P-element promoter in the absence of an intervening insulator. In particular, when homie was present, a flanking P-element promoter was not detectably activated by the eve enhancers. On the other hand, we did observe some weak stripe expression when nhomie was the intervening insulator. This expression was, as expected, much stronger when nhomie was removed (Fig 3B, “wt” and “Δnhomie”). These data show that nhomie’s enhancer blocking activity is somewhat weaker than that of homie. More generally,
our results suggest that while blocking of cis effects along chromosomes by insulators may not be absolute, inappropriate interactions become weak enough when they are present so that genes can be considered separate functional genetic units, in most cases.

The transcripts we detected at various enhancers throughout the locus are continuous from the flanking P-element promoter, and are not short non-coding RNAs (e.g., eRNAs). One line of evidence for this is that the RNAs are unidirectional, since they were not detected by in situ hybridization using probes against the opposite strand (Fig 3A, S2 and S5 Figs). In addition, RT-qPCR analysis of cDNA produced from specific primers showed that the RNAs are continuous from one enhancer to the next (S3 Fig). This confirms that it is transcription initiated from the flanking promoter in response to eve enhancers that is responsible for disrupting eve promoter activity when an intervening insulator is removed.

What are the mechanisms of transcriptional interference by read-through? One possibility is inactivation of enhancers, since the transcriptional machinery must at least transiently displace DNA binding proteins as it passes. How strongly such displacement affects promoter activity is expected to depend on the dynamics of the E-P interactions in any given case. We attempted to see if this is the case for eve enhancers by adding polyA signals to terminate read-through transcription between enhancers. We were surprised to find that the read-through transcription was not effectively stopped even by a tandem array of 3 polyA-addition signals, one from \( \alpha \)-tubulin and 2 from SV40. We did see evidence of weak attenuation, accompanied by correspondingly weak rescue of a downstream enhancer’s activity on the eve promoter (Fig 8 and S8 Fig, compare stripes 4+6 to stripe 5 within individual embryos). Because the effect is small, we can only suggest that read-through of enhancers may be sufficient for transcriptional interference. It is possible that most of the effect we see here is due to read-through of the eve promoter (see below). Consistent with the P-element promoter initiating highly processive transcription, wild-type P-elements have two polyA signals.

In light of the fact that many genes have enhancers in their introns, it may seem implausible that enhancers would be inactivated by read-through. However, such an effect would only be expected to reduce the rate of transcription, since a reduced rate of transcription would result in a reduced amount of repression, and eventually a steady-state balance would be reached. Since most genes are not expressed at very high levels, such an effect is entirely plausible.

Read-through driven by a heterologous enhancer inserted upstream of the P-promoter (Fig 5, NEE enhancer), so that the enhancer itself is not being read through, seems sufficient to repress eve promoter activity. Although we cannot completely rule out that the repression we observe is due to enhancer read-through, it is more likely the result of disruption of both enhancers and the eve-lacZ promoter. Thus, our data are consistent with the idea that enhancer-promoter interactions are disrupted by read-through of either interacting element.

In the case where the flanking promoter that is responsible for read-through (and for the resulting transcriptional interference) is downstream of the eve locus, and reads through the coding region as well as the eve promoter, another possible mechanism for the observed repression is RNAi (Figs 3A and 4B). Although we cannot rule this out as a contributing mechanism when the read-through is coming from downstream of eve-lacZ (e.g., Fig 1B and 1D), when read-through is coming from upstream, no anti-sense RNA is produced. In such cases (e.g., Fig 1C and 1E), we observed significant repression of lacZ in the pattern of the non-coding RNA produced from the flanking promoter, just as we did when the flanking promoter was downstream (S7 Fig). This is the case even though the RNA transcribed from the lacZ coding region is the sum of transcripts initiating at both the eve promoter and the flanking promoter that is responsible for the repression. This suggests that the transcriptional interference observed in this study is quite “efficient”, producing a reduction in eve promoter-initiated
transcripts that is greater than the number of read-through transcripts causing the repression. Thus, our data show that read-through can cause repression by transcriptional interference without the involvement of anti-sense RNA.

It was reported that a P-element inserted into the BX-C caused segment A1 to A2 transformation, and this effect was ascribed to a P-promoter that produced a long ncRNA [45]. Thus, a P-element promoter in other contexts can generate read-through that disrupts gene expression. Such effects are not limited to P-element promoters. For example, it was shown that ncRNAs initiated in the bxd region of the BX-C repress Ubx expression in cis by transcriptional interference [73]. More complex effects of read through are also known to occur. For example, one of the promoters contained within the scs insulator generates read-through transcription that can inactivate a PRE [46]. These results, along with our observation that removal of insulator function allows flanking promoters to capture enhancers that they would not otherwise have access to, suggest that preventing read-through caused by inappropriate enhancer capture may be a common function of many insulators throughout the genome.

There are 3 ncRNAs in the eve locus, according to Flybase [74]. One is initiated just 5' of TER94 (lncRNA:CR45324); however, this initiation site is not present in any transgenes used in Fig 4 or later. Another (asRNA:CR43948) begins just 5' of the eve coding region. This initiation site is not present in any of our constructs, since we replaced the eve coding region with that of lacZ. Furthermore, according to Flybase, neither of these RNAs is expressed at any of the stages used in this study. The initiation site of the 3rd ncRNA (lnc-RNA:CR46455) is present in our transgenes. It is near the 5' end of the eve locus, within the late 7-stripe element, and is transcribed toward the eve coding region. This transcript should be recognized by our “(late)” R probe, but we did not detect any pattern with this probe (Fig 3A, Δhomie). RT-qPCR showed very low expression in this region in our control yw fly strain (Fig 2A and S6 Fig). Therefore, it is extremely unlikely that this ncRNA is involved in any of our results. Interestingly, in situ hybridization with “late” probe detected faint 1, 2, 3, 7 stripe expression in our transgenic lines (Fig 3A, wt; Fig 4, Pwt and PinvΔ; S2 Fig, Δnhomie), but not in embryos without a transgene (S2 Fig, yw). The “late” probe detects transcripts coming from downstream of the “late” enhancer. However, the pattern is not the one expected for transcripts coming from the downstream P-element promoter in Figs 3A and 4 (which would be in stripes 1, 4, 5, 6, driven by enhancers in the 3’ region). Instead, this weak expression is stronger in stripes 1, 2, 3, and 7 (and so is likely driven by enhancers 5’ of the eve start site). Therefore, it is likely that there is another ncRNA start site downstream of the “late” stripe enhancer, but 5’ of the eve transcription initiation site. Nonetheless, this weak expression does not correlate with any of the results in this study, and so we conclude that it is unlikely to be consequential for either eve or eve-lacZ expression.

Promoter specificity and processivity

When a core hsp70 promoter is used in place of the P-element promoter, read-through transcripts are weakened, as is repression. This suggests that the P-element promoter is relatively efficient at responding to eve enhancers (Fig 7A). In an earlier study, we reported that the eve upstream promoter region can facilitate homie-homie long-range interaction activity. Replacing the eve promoter with the hsp70 core promoter reduced this activity [7]. This is consistent with the idea that the efficiency (or, more specifically, the stability) of enhancer-promoter interactions can affect the stability of long-range insulator interactions [75]. This illustrates the dynamic interplay between chromosome architecture and gene regulation, and the complex mechanisms by which insulators affect gene expression.
Importantly, little reduction in intensity is seen between transcripts detected at different distances downstream of the P-element promoter, suggesting that read-through is processive (Figs 3A and 4). In contrast, when the hsp70 promoter is used, while there are easily detectable transcripts near the start site, they are almost undetectable a few kb downstream (Fig 6). This is true when expression is driven by either the eve enhancers or the NEE (Fig 7). For NEE-driven expression (Fig 7D), analysis by RT-qPCR showed a level of expression from the hsp70 promoter comparable to that from the P-promoter at 0.7 and 1.7 kb from the start site (at the positions of the GFP and PRE primer sets). However, the level of transcripts from the hsp70 promoter dropped relative to those from the P-promoter between the position of the 3’ PRE and the RP2 neuronal enhancer (2.3 kb from the start site). We consider two possibilities for this apparent termination of transcripts from the hsp70 promoter. First, the eve locus is known to be a Polycomb domain, which is characterized by the histone methylation mark H3K27me3 [76,77]. This repressive chromatin environment might affect processivity of transcription, causing the less processive transcription from hsp to terminate more frequently than that from the P-promoter. A second possibility is that transcription from hsp is responding to a termination signal where ncRNA CR45324 ends, which is between the RP2 enhancer and the 3’ PRE. (This ncRNA is initiated within the 1st exon of TER94.) In summary, these data suggest that the P-promoter drives more processive transcription in combination with a variety of enhancers than does the hsp70 core promoter. This processivity allows RNAPII to read through eve enhancers located many kb away, when there is an intervening insulator, and even through and far beyond multiple polyA signals. It will be interesting to investigate how different promoters, activated by the same enhancers, cause differences in RNAPII processivity.

As discussed above, when polyA-addition signals were introduced downstream of the flanking P-promoter, RNAPII did not efficiently terminate transcription. Two models for how transcription termination occurs have been proposed [78]. A recent study in C. elegans showed that the promoter can influence which mode is used [79]. Further study will be required to determine whether the two promoters we have compared here use either of these modes of termination, as well as to understand how some promoters generate transcription that fails to terminate downstream of polyA signals.

**Promoter competition vs. transcriptional interference by read-through**

In most cases we examined, there is a close correlation between the amount of transcription driven by the competing P-element promoter and the degree of repression of eve-lacZ. This is consistent with either promoter competition or transcriptional interference as a mechanism of repression. However, when the P-promoter is turned around so that read-through is eliminated, repression is strongly reduced, showing that read-through is necessary for the repression (Fig 4). Furthermore, read-through driven by a heterologous enhancer is sufficient for repression, implicating transcriptional interference caused by read-through as the primary mechanism of repression (Fig 5). Thus, our data are consistent with there being little promoter competition at work, which in turn suggests that enhancer-promoter interactions are highly dynamic while not approaching saturation. This scenario allows both promoters to be activated in an eve stripe pattern without significant competition.

There is an important caveat to this view, however. When the P-element promoter is turned around, its level of transcription appears to be reduced. If this is indeed the case, it suggests another interesting possibility, one that involves significant promoter competition. If we suppose that eve E-P interactions are more stable than those that occur between eve enhancers and the heterologous P-promoter, we might expect that eve enhancers would preferentially interact with the eve promoter when a competing P-promoter is brought into the same TAD.
(either by insertion of the P-promoter or by removing an intervening insulator). However, if transcription from the P-promoter reads toward the eve locus, and if that transcription interrupts stable eve E-P interactions, freeing the enhancers to interact with the P-promoter, it could facilitate enhancer capture by the P-promoter, allowing it to better compete for the enhancers. This in turn would boost P-promoter activity at the expense of eve promoter activity, repressing eve. This mechanism might also explain why the hsp70 core promoter is not activated as well by eve enhancers as is the P-promoter, since transcription from it can’t effectively release the eve E-P contacts, due to its low processivity. Additional work will be required to understand the dynamics of these interactions, and to tease out how much of the repression that we see is due to transcriptional interference caused by read-through of enhancers, how much is due to transcriptional interference caused by read-through of the eve promoter, and whether promoter competition plays a significant role. These repression mechanisms are not mutually exclusive, and all can be a consequence of insulator removal.

Methods

Plasmids and transgene production

The original eve pseudo-locus constructs have been described previously [12]. A modified version which includes the complete nhome insulator [19] was used in this study. The DNA fragment from –6.5 kb (instead of the –6.4 kb end point used in the original construct) to +166 bp relative to the eve TSS was fused to the lacZ coding region. The 3’ end of the lacZ coding region was fused to DNA from +1.3 to +11.4 kb, which includes the eve polyA signal, and extends into the 3rd exon of TER94. The EGFP coding region, followed by the polyA signal of α-tubulin, was added at the 3’ end. The entire construct was placed between two oppositely oriented attB sequences [53]. Replacement of homie (from +9.3 to +9.8 kb) in these constructs by λ DNA or by heterologous insulators was described previously [12]. The original 5’ end point of the pseudo-locus at –6.4 kb was used as our nhome deletion (described herein as Δnhomie). The heterologous insulator assays of Fig 1F (including wt and Δhomie in that figure) were done in this Δhomie context. Exact end points are shown in S10 Fig. These transgenic vectors were inserted using φC31 RMCE [53] as described previously, into chromosomal attP sites that had previously been introduced into the Drosophila genome by P-element transgenesis [12]. The direction of each insertion was determined by PCR. Orientations of inserts are listed in the text as either the "H5 orientation", in which the 3’ ("homie") end of the locus is close to the 5’ P-element end, or the "N5 orientation", in which the 5’ ("nhomie") end of the locus is close to the 5’ P-element end. The attP sites used are those at cytological locations 95E5, for Figs 1B, 1C, 1F, 2, 3A, 3B and S3A Fig, and 23C4 for Fig 1D and 1E and S1 and S2 Figs.

To test the effects of transcriptional read-through, the following new transgenic vectors were made. Starting with the modified pseudo-locus described above (which includes nhome), TER94-EGFP was removed, and the 3’ end was shortened to +9.8 kb, which is the 3’ end of homie. The homie sequence (from +9.3 to +9.8 kb) was then replaced by λ DNA. Modified forms of these with either “wt” or “Δ” in the name were used in the figures after Fig 4 and S5 Fig, except S7 Fig. Either the P-element promoter alone (P) [60–63], the P-element promoter with the rhomboid neuroectodermal enhancer (NEE-P) [68], or the heat shock 70 core promoter [66] driving EGFP without a polyA signal (NEE-hspGFP) was then inserted at the 3’ end of each of these, in either orientation, as diagrammed in Figs 4–8. The following additional modifications were also made: the P-element promoter with the GFP coding region (without a polyA signal) driven by the NEE was inserted into the construct without homie to give NEE-PGFPA (used in Fig 7C and S8C Fig); an α-tubulin polyA signal [64] was inserted at +6621 nt in PΔ, between the stripes 4+6 and mesodermal enhancers, to yield PpAΔ (used in
S8B Fig); an α-tubulin polyA signal [64] plus two SV40 polyA [65] signals were inserted at +6621 nt in both \( P \Delta \) and NEE-PGFP to yield \( PpA2 \Delta \) (used in Fig 8) and NEE-PGFP-pA2Δ (used in S8D Fig), respectively. Each of these vectors was inserted into the MiMIC target site Mi(MIC)Drgx [MI04684] (cytological location 24B1) [67,74]. Orientations of the inserts are as noted in the figure legends. Relevant sequences are given in S10 Fig.

**RT-PCR**

Total RNA was purified from 2–4 h embryos (or from 2.5–4 h embryos for S7B Fig), using an RNA purification kit (Roche Applied Science). cDNA was synthesized using the Transcriptor first strand cDNA synthesis kit with random primer, following the manufacturer’s protocol (Roche Applied Science) for Figs 2 and 6 and S1, S6, S7A and S9 Figs. For S3 Fig, specific primers for “late” or “EL” along with “RP49”-specific primer were used to make cDNA. Each biological sample was analyzed in triplicate by real-time PCR (Life Technologies, StepOnePlus) using SYBR Green Master Mix with ROX dye (Roche Applied Science). PCR data were analyzed with StepOne software, using the standard curve method. \( RP49 \) was used as a reference gene, while \( eve \) was also used as a reference gene for \( lacZ \) expression in S7B Fig. Each experimental value was divided by the \( RP49 \) value (and \( eve \) in S7B Fig) from the same sample. Primers used for RT-qPCR are given in S10 Fig.

**In situ hybridization**

Embryos were collected and subjected to in situ hybridization as described previously [12]. Enhancer regions were cloned in vector pSP72 (Promega), then digested with appropriate enzymes to make labeled run-off transcripts using T7 or SP6 RNA transcriptase, with the DIG RNA labeling kit (Roche Applied Science). The two probes produced by T7 or SP6 recognize the same enhancer region, but different strands. The genomic regions used for probes are listed in S10 Fig. In order to reduce staining variability when lines were to be compared, in situ hybridization was performed in parallel at the same time. Representative embryos from the stained populations are shown.

**Supporting information**

**S1 Fig. Non-coding RNA expression is increased by \( eve \) insulator removal.** (A, B) transgenes in the H5 orientation (see Fig 1A) at site 23C4. (C, D) transgenes in the N5 orientation (see Fig 1A) at 23C4. (A, C) RT-qPCR quantification of total RNA (normalized to \( RP49 \) RNA) from the indicated enhancer regions (probe locations shown as red bars below the map), in the \( wt \) and \( \Delta homie \) transgenic lines (used in Fig 1D) in A, or in \( wt \) and \( \Delta nhomie \) (used in Fig 1E) in C. Averages with standard deviations of 3 biological samples each are graphed. (B, D) The ratios of average signals (with standard deviations) from \( wt \) and \( \Delta homie \) in B, and \( wt \) and \( \Delta nhomie \) in D are graphed. Note the general trend toward a decrease in average signal moving away from the location of the 5’ P-element end (the right side in B and the left side in D).

**S2 Fig. Patterns of non-coding RNA expression caused by \( nhomie \) removal.** Map: Transgene construct, showing location of the 5’ P-element end, where read-through transcripts are initiated. Images: RNA in situ hybridization to embryos at stage 5 carrying \( \Delta nhomie \) at 23C4 (used in Fig 1E). Probes are shown as either red arrows recognizing transcripts transcribed from left to right in the map, or black arrows recognizing transcripts transcribed from right to left. Labels use either black lettering corresponding to black arrows, or red lettering with R
corresponding to red arrows. (DOCX)

S3 Fig. Non-coding transcripts are continuous across enhancers. cDNA primed in either the "late" or "EL" (green block arrows) enhancer regions were analyzed by qPCR using primers recognizing other enhancer regions (red bars below the map) (normalized to RP49, analyzed in parallel for each sample, using RP49-specific primers). (A) cDNA from wt and Δhomie transgenes at 74A2 (used in Fig 1B). Left graph: cDNA from "late" primer analyzed with "3+7" primer set. Right graph: cDNA from "EL" primer analyzed with "4+6", "1", and "wD5" primer sets. The "wD5" primer set was used to recognize cDNA representing the region upstream of the P-element promoter (a negative control). (B) Same as A, except that cDNA is from Pwt and PA transgenes (used in Fig 4A and 4B) at 24B1, and the "24B" primer set was used to recognize cDNA representing the region upstream of the P-element promoter (negative control). (DOCX)

S4 Fig. Insulator removal does not cause significant changes in expression in the absence of a flanking P-element promoter. (A) Top: Map of the eve pseudo-locus. miR and miL are Minos inverted repeats. Bottom: Expression of eve-lacZ from the pseudo-locus inserted into a MiMIC site at cytological location 24B1 detected by in situ hybridization. Both orientations of insertion are shown (left and right panels) at embryonic stages 5, 7, 11, 13, (as indicated), with either nhomie alone (Δhomie), or both nhomie and homie (Δhomie, Δhomie), deleted. (B) Top: Map of the eve pseudo-locus modified by removal of TER94-GFP. Bottom: Same as in A, using a line carrying this modified pseudo-locus. (DOCX)

S5 Fig. There is no apparent expression from upstream in the modified eve pseudo-locus at the 24B1 MiMIC site. Same as Fig 4, except probes recognizing the opposite strand are used. Positions of probes are shown as red arrows, and images are labeled with red lettering with R. (DOCX)

S6 Fig. Modified eve pseudo-loci show read-through transcription when a P-element promoter is added to the original constructs at the 24B1 MiMIC site. Map: The wt transgene with locations of PCR products used for transcript detection shown as red bars under the map. (A) Loci used in Fig 4A (wt), Fig 4B (PA), Fig 4C (PinvΔ), and yw (without transgene; signals come only from endogenous eve) were subjected to RT-qPCR quantification of total RNA (normalized to RP49 RNA) from the indicated enhancer regions. Averages (with standard deviations) of 4 biological samples each are graphed. Note that transcript levels are strongly increased throughout the locus in PA, but not in wt or PinvΔ. (B) The ratios of average signals (with standard deviations) from PA and wt are graphed. Note the general trend toward a decrease in the relative PA signal moving away from the location of the P-element promoter. (C) Similar to A, except that the set of wt, PA, and PinvΔ transgenes are inserted in the opposite orientation at the same MiMIC site, and averages (with standard deviations) of 5 biological samples each are graphed. (D) The ratios of average signals (with standard deviations) from PA and wt in C are graphed. (DOCX)

S7 Fig. Quantification of expression downstream of the eve promoter shows a significant reduction caused by removal of nhomie, even though this expression is a combination of that driven by the eve promoter and the upstream P-element promoter. RT-qPCR quantification of total RNA from the lacZ coding region in embryos, in either wt and Δhomie (in A),
or wt and Δ
homie (in B), normalized to control RNA from either RP49 or endogenous eve, as indicated. (A) Line used in Fig 1B (H5 orientation). The reduction in expression is significant at the P < 0.01 level (one-tailed t-test assuming unequal variances). (B) Line used in Fig 1C (N5 orientation). The reduction in expression relative to RP49 is not significant at the P < 0.05 level, but relative to eve it is significant at the P < 0.05 level (one-tailed t-test assuming unequal variances). Normalizing to eve expression may better control for variations in the developmental stages represented in the embryo collections that were the source of the RNA.

S8 Fig. Insertion of polyA-addition signals does not stop P-element promoter-initiated transcription. eve-lacZ and flanking P-element promoter-driven RNA expression in stage 5 embryos from eve pseudo-loci inserted at the 24B1 MiMIC site. Orientation of inserts are the same as in Fig 8. All loci have homie replaced with phage λ DNA (refer to maps). Positions and orientations of the RNA probes used are shown as black and red arrows in the map. Insertion site of polyA signals is shown in the maps of B and D. Affected lacZ stripes are labeled as 4, 5, and 6 (compare the intensity of stripes 4 and 6 to stripe 5 in the same embryos). (A, B) Same as in Fig 8A and 8B (ncRNA is driven by the P-element promoter), except that only the α-tubulin polyA signal is present here in B. (C, D) Same as in Fig 8A and 8B, except that here, the NEE-PGFP cassette used in Fig 7C is driving the ncRNA.

S9 Fig. There may be a slight reduction of P-element promoter-driven ncRNA downstream of polyA-addition signals. Quantification of total RNA (normalized to RP49 RNA) from the indicated enhancer regions (shown as red bars) in the PΔ and PpA2Δ lines used in Fig 8. (A) Averages (with standard deviations) of 4 biological samples each are graphed. (B) Ratios of signals (average and standard deviation) from PΔ and PpA2Δ are graphed. (C) Quantification of lacZ RNA (normalized to RP49 RNA). The increase in lacZ RNA in PpA2Δ relative to that in PΔ is significant at the P < 0.01 level (one-tailed t-test assuming unequal variances).

S10 Fig. Relevant end points and sequences used in this study. (A) End-point coordinates are given according to Flybase [74] (FB2021_02 release, R6.39). Numbers in parentheses are nt positions relative to the eve transcription start site. (B) List of sequences and primers used in this study.

S11 Fig. Data used for RT-qPCR graphs.

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