Polarity of transcriptional enhancement revealed by an insulator element

Wei Wei and Mark D. Brennan*

Department of Biochemistry and Molecular Biology, University of Louisville Medical School, Louisville, KY 40202

Communicated by Anthony P. Mahowald, University of Chicago, Chicago, IL, November 6, 2000 (received for review July 11, 2000)

Transcriptional enhancers for genes transcribed by RNA polymerase II may be localized upstream or downstream of the stimulated promoter in their normal chromosomal context. They stimulate transcription in an orientation-independent manner when assayed on circular plasmids. We describe a transient transformation system to evaluate the orientation preference of transcriptional enhancers in *Drosophila*. To accomplish this, the *gypsy* insulator element was used to block bidirectional action of an enhancer on circular plasmids. In this system, as in the chromosome, blocking of enhancer activity requires wild-type levels of the su(Hw) protein. We evaluated the orientation preference for the relatively large (4.4 kb) *Adh* larval enhancer from *Drosophila melanogaster*, used in conjunction with a luciferase reporter gene under the control of a minimal *Adh* promoter. An orientation preference was revealed by insertion of a single copy of the insulator between the enhancer and the promoter. This orientation effect was greatly amplified when the promoter was weakened by removing binding sites for critical transcription factors, consistent with a mechanism of insulator action in which the insulator intercepts signals from the enhancer by competing with the promoter. The orientation preference, as much as 100-fold, is a property of the enhancer itself because it is displayed by gene constructions introduced into the chromosome regardless of the presence of the insulator in a distal location. These findings are most easily reconciled with a facilitated tracking mechanism for enhancer function in a native chromosomal environment.

Transcriptional enhancers are DNA sequences that can act over long distances to control the spatial and temporal patterns of gene expression required for the proper development of multicellular organisms (1, 2). They activate transcription by facilitating the assembly of limiting protein components at the target promoter (2, 3). In their native chromosomal context, transcriptional enhancers for genes transcribed by RNA polymerase II may be located upstream or downstream of the stimulated promoter. Through the use of plasmids bearing reporter genes, many studies have shown that a given enhancer can stimulate transcription when placed either "upstream" or "downstream" of a promoter on a circular plasmid.

Several lines of evidence support the possibility that such enhancers function by a looping mechanism, in which proteins bound to the enhancer interact with the promoter by looping out of the intervening DNA. The observation that two closely spaced promoters can be activated to an equal extent by a remotely located enhancer is consistent with this model (4). Furthermore, RNA polymerase II promoters can be stimulated by enhancers located on DNA molecules physically linked to, but not contiguous with, the segment of DNA carrying the promoter (5, 6). The phenomenon of transvection in *Drosophila*, in which an enhancer on one chromosome activates a promoter on the paired, homologous chromosome in trans apparently represents an analogous situation (7).

Although these examples clearly establish that enhancers can act via a looping mechanism, they do not address the question of whether or not enhancers normally act by a simple looping mechanism in a native chromosomal environment. In fact, there is a growing body of evidence suggesting that, in a chromosomal context, at least some enhancers may work by a tracking or scanning mechanism that may involve localized looping (see ref. 8 for review). According to this model, either proteins initially bound to the enhancer, or, more likely, enhancer-bound proteins track along the chromosome until a promoter is encountered. Assuming that there are physical constraints on this process within the chromosome, this model predicts that an enhancer would preferentially activate a promoter located on the same DNA molecule (in cis) even if a potential target promoter is located on a paired homologous chromosome (in trans). Indeed, transvection in *Drosophila* displays just such a cis preference for a functional promoter (9–13). Recent experiments indicating that enhancer-proximal genes within a gene cluster are preferentially activated by a distant enhancer, unless their promoters are masked by repressive chromatin structures (14–16), are also consistent with a tracking mechanism. Finally, genetic screens for genes influencing the ability of distant enhancers to activate target promoters provide evidence for sophisticated biochemical mechanisms needed to mediate enhancer–promoter interactions *in vivo* (reviewed in ref. 17). Implicit in facilitated tracking models is the assumption that the DNA sequences between the site where proteins initially bind the enhancer and the target promoter must participate in delivering stimulatory signals to the promoter.

As such, enhancers might be expected to display an orientation preference if assayed within a region of DNA sufficiently large to mimic the natural chromosomal context. However, most experiments using transient transfection methods to test for orientation preferences for enhancers have used relatively small, core enhancer elements that may not be representative of native chromosomal enhancers. Moreover, as such experiments typically involve circular plasmid molecules, any orientation preference would be obscured by the ability of the enhancer to function either "upstream" or "downstream" of the promoter. Recent results reported for the murine *GATA-1* gene hematopoietic enhancer may be a case in point (18). This enhancer shows only a modest orientation preference in a plasmid context but a more dramatic orientation dependence when inserted into the chromosome.

Here, we describe a transient transformation method to evaluate the orientation preference of transcriptional enhancers. For this we used the relatively large (4.4 kb) *Adh* larval enhancer (ALE) from *Drosophila melanogaster* (19). The ALE showed a distinct orientation preference when a single copy of the *gypsy* insulator, which blocks enhancer promoter interactions (20, 21), was inserted between it and the promoter. Blocking by a single copy of the insulator was greatly increased by weakening the promoter, thus supporting a mechanism of insulator action in

Abbreviations: ADH, alcohol dehydrogenase; ALE, alcohol dehydrogenase larval enhancer; su(Hw), suppressor of *Hairy-wing*.

*To whom reprint requests should be addressed. E-mail: mdbren01@louisville.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.011529598. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.011529598

which the insulator competes with the promoter by intercepting signals from the enhancer (22). Insertion of ALE-containing genes into the chromosome confirmed that the polarity is an inherent property of the ALE. These results are consistent with a tracking mechanism for RNA polymerase II enhancers in a native context.

Materials and Methods

Plasmid Construction. Details of plasmid construction will be provided on request. In general, for experimental constructs, the firefly luciferase gene carried in the pGL3-Basic vector (Promega) was used as a reporter. This was fused to the minimal promoter of the *Drosophila affinidisjuncta Adh* gene (defined by an *Nde*I site at −203 and an *Eco*RI site at +18) or to a crippled form of this promoter (MutAB) carrying two clustered point mutations that abolish critical protein binding sites needed for full levels of transcription *in vitro* and *in vivo* (23, 24). The 4.4-kb ALE from the *D. melanogaster Adh* gene (19) was removed from psAF2 (25) by digestion with *Xba*I and inserted into the multiple cloning site of the pBluescript vector (26). Both orientations of the insert were recovered for use in further constructions. The ALE was then removed by digestion with various enzymes for insertion into reporter plasmids. The 410-bp *gypsy* insulator carried in the multiple cloning site of pREP-1 (kindly provided by P. Geyer) was similarly inserted into appropriate reporter constructions. Including polylinker sequences, the *gypsy* insulator-containing fragments inserted into reporter constructs ranged in size from 415 to 455 bp. For germ-line transformation, the reporter genes controlled by the crippled *Adh* promoter and carrying either orientation of ALE, with or without the *gypsy* insulator, were inserted into the P-element vector, pCaSpeR, carrying a mini-*white* gene (kindly provided by C. Thummel; see ref. 27). Plasmids used for injection were purified by CsCl gradient centrifugation and quantified by fluorimetry with Hoechst 33258 dye using a Hoefer, DNA Quant fluorometer.

***Drosophila* Stocks.** For transient transformation, *D. melanogaster Adh*-null stocks were used because an alcohol dehydrogenase (ADH)-encoding plasmid served as an internal control for the efficiency of the transient transformation procedure. The *Adh*-null strain, Adh^{fn6}, *cn;* ry^{506}, is homozygous for the wild-type *su(Hw)* gene. The recessive, visible eye color markers, *cn* and ry^{506}, were included to allow facile monitoring of genetic homogeneity of the stock by visual inspection. To assess the role of the *Su(Hw)* protein in insulator function, the stock, y^1, w^{1118}, ct^6, f^1; Adh^{fn6}, *cn;* $su(Hw)^v/su(Hw)^f$, was used. This was constructed by standard methods using *Binsc*, *CyO*, and *TM6* as balancer chromosomes (28). The $su(Hw)^v/su(Hw)^f$ compound heterozygotes display greatly reduced levels of *Su(Hw)* but are fertile (21, 29). The *gypsy*-induced ct^6 and f^1 alleles in this stock are suppressed, thus allowing visual verification of the $su(Hw)^v/su(Hw)^f$ genotype. Stocks carrying the $su(Hw)^v/su(Hw)^f$ mutations, as well as the y^1, w^{1118}, ct^6, f^1 chromosome, were provided by P. Geyer.

Analysis of Gene Expression. Transient transformation was performed by injection of supercoiled plasmid DNAs into the ventral midline of preblastoderm *Drosophila* embryos so as to optimize for expression in the larval fat body, as described by McKenzie and coworkers (23). Equimolar mixtures (40 nM each) of two plasmids, one being the variant construct under study encoding firefly luciferase and the other being an internal control, were injected. The control plasmid was p11BXB2 (30), which encodes ADH.

After developing to the third instar (7 days at 25°C), groups of five larvae were pooled and homogenized in 200 μl of Promega cell culture lysis buffer. Homogenates were clarified by centrifugation at 10,000 × *g* for 2 min at 4°C. ADH activity in 100 μl of extract was measured as described by McKenzie *et al.* (23), and luciferase activity in 5 μl of extract was measured with reagents and methods suggested by Promega using a Lumat (Berthold) luminometer. The luciferase assay is linear over at least 4 orders of magnitude without dilution of the samples. Where necessary, luciferase activity was determined after a 10-fold dilution of the sample in cell culture lysis buffer. Typically, between 10^3 and 10^8 relative light units were obtained in a 10-s measurement with the luminometer. Five independent groups of five larvae were analyzed for each construction. Where specified in the text, levels of expression were compared statistically by a two-tailed Student's *t* test (31).

P-element transformation was performed by standard methods using the w^{1118} strain as the host (32–34). Injected flies were crossed to w^{1118} flies, and progeny with pigmented eyes (w^+) were individually crossed back to w^{1118} flies. Homozygous lines were obtained by using *CyO* and *TM3* as balancer chromosomes (28). Only homozygous viable lines having single copies of the transposons in autosomal locations were tested for expression.

Five to eight different transformed lines were analyzed for each gene. For each line, three samples consisting of five third instar larvae were homogenized in cell lysis buffer, and luciferase activity was determined as described above. Protein concentrations of homogenates were determined by using DC Protein Assay kit (Bio-Rad). Luciferase-specific activities were then calculated as relative light units per microgram of protein. Values for different genes were compared by nested analysis of variance, using the SPSS statistical package (SPSS, Chicago), with the factors "gene type," "transformed line within gene type," and "observations within transformed line" (31). The conservative, post hoc Tukey test was used to perform multiple comparisons between the different genes (31).

Results

The *gypsy* Insulator Blocks an Enhancer in Transient Transformation. To test for enhancer activity, plasmid mixtures were injected into the ventral midline of preblastoderm embryos so as to ensure expression in the larval fat body (23). Previous studies have shown that the minimal *Adh* promoter is active in this tissue as assayed by both transient and germ-line transformation (23, 35). Germ-line transformation experiments also indicate that the ALE is most active in this tissue (36).

Initially, we wanted to determine if the ALE stimulated transcription in this transient transformation system. Thus, plasmids carrying the 4.4-kb ALE or a 4.8-kb fragment of bacteriophage λ DNA were microinjected, along with an internal control plasmid expressing ADH, into *Adh* null embryos. The construct with the *Adh* promoter, but lacking the enhancer, produced approximately 1,000-fold more luciferase than the pGL3-Basic vector without the promoter (not shown). However, when the ALE was placed upstream of the promoter, the expression increased about 850-fold above this basal level (Fig. 1, gene 1, shaded bar). These results confirm that the ALE stimulates expression in this transient transformation assay.

To determine whether the *gypsy* insulator can block an enhancer in a transient transformation system, we tested the ability of the *gypsy* insulator (20, 37) to block stimulation by the ALE. When one copy of the insulator was placed downstream of the enhancer, expression dropped 20-fold but remained about 40-fold higher than the basal level (*cf.* Fig. 1, genes 1 and 2, shaded bars). The decrease in transcription upon insertion of the insulator is not due to spacing because a control plasmid carrying a 420-bp fragment of bacteriophage λ DNA downstream of the enhancer had levels of expression identical to the plasmid with the enhancer only (data not shown). In contrast, when the *gypsy* insulator was inserted upstream of the enhancer, there was no decrease in expression (gene 3, shaded bar). However, flanking the enhancer with two copies of the insulator lowered expression

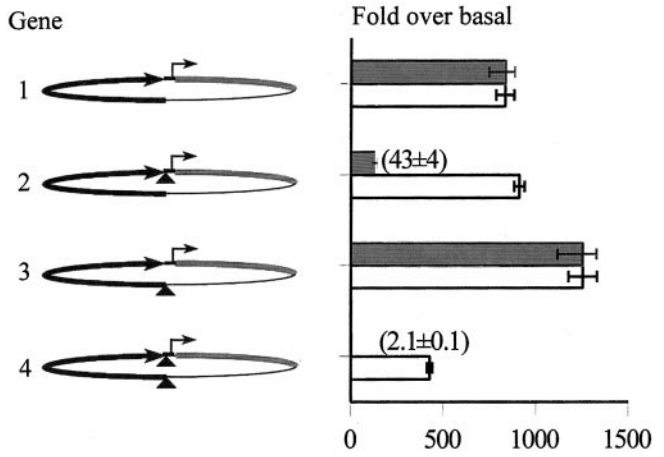

Fig. 1. The *gypsy* insulator prevents enhancer stimulation in an Su(Hw)-dependent manner. (*Left*) The genes shown are drawn to scale. All plasmids carry the firefly luciferase reporter gene (gray box) under the control of the minimal promoter (−203 to +18) of the *D. affinidisjuncta Adh* gene (thick line and small arrow). The 4.4-kb ALE is represented by a large curved arrow. Triangles indicate the *gypsy* insulator. Transient transformation was performed by injecting plasmid mixtures, containing equimolar amounts of the experimental plasmid and an ADH-encoding control plasmid, into the ventral midline of embryos (see *Materials and Methods*). (*Right*) The bars represent relative expression of genes (ratios of luciferase activity to ADH activity) normalized to that of a gene carrying a similarly sized fragment of bacteriophage λ DNA instead of the ALE. The shaded bars show results obtained with the standard *Adh*-null strain, which carries a wild-type *su(Hw)* gene. The open bars show results for the *Adh*-null, $su(Hw)^f/su(Hw)^v$ strain, which contains low levels of Su(Hw) protein. Bars represent means ± SE for five independent samples for each gene normalized relative to the value obtained for the control gene in the same genetic background. In addition, numerical values are given for genes having low levels of expression that are not readily evaluated graphically.

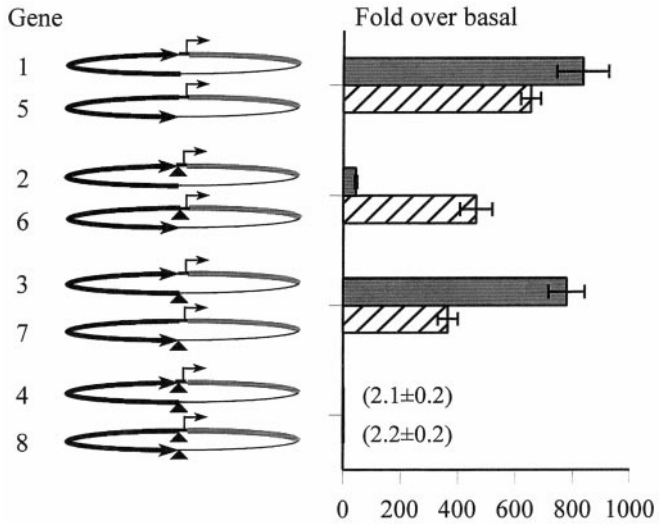

Fig. 2. The ALE displays an orientation preference in transient transformation. Genes (drawn to scale) are displayed as in Fig. 1. The different orientations of ALE are represented by the large curved arrows pointing in opposite directions. The *Adh*-null, $su(Hw)^+$ strain was used. Gray bars show results for normal orientation of ALE, and striped bars show the results for the reversed orientation of ALE. Values are normalized relative that for the control same gene carrying the λ DNA insert in place of the ALE.

nearly to basal levels (gene 4, shaded bar). Thus, the *gypsy* insulator blocks enhancer stimulation in this transient transformation system and does not require insertion into the chromosome to display its enhancer-blocking activity.

The suppressor of *Hairy-wing* protein, Su(Hw), is required for the enhancer-blocking activity of the *gypsy* insulator in a chromosomal context (21, 38–41). The work of others indicates that Su(Hw), a protein with nearly ubiquitous tissue distribution, is produced in the larval fat body (42). Therefore, we predicted that Su(Hw) is involved in the enhancer blocking seen here.

To test this, the above constructions were injected into embryos from a *Drosophila* stock that produces reduced levels of Su(Hw) activity. In this case, a single copy of the insulator located either downstream or upstream of the enhancer did not significantly reduce stimulation by the enhancer (Fig. 1, genes 2 and 3, open bars). Moreover, with two copies of the insulator flanking the enhancer, the degree of blocking was reduced by at least 200-fold in the *su(Hw)* mutant background (gene 4, open bar).

The ALE Displays an Orientation Preference in Transient Transformation. The ALE behaves as a classical enhancer in that it can stimulate transcription from a heterologous promoter and does so regardless of orientation (19). However, previously published experiments have not quantitatively addressed the level of stimulation mediated by the two alternative orientations of the ALE. If a facilitated tracking mechanism were in place, we would expect that ALE would function better in the normal than in the reversed orientation, provided we can prevent it from acting bidirectionally on a circular plasmid.

To address this, the ALE was inserted in the reversed orientation upstream of the promoter. As can be seen in Fig. 2, the reversed orientation of ALE stimulates transcription in a circular plasmid as well as the normal orientation, with no significant difference between the two constructions (gene 1 vs. gene 5, $P > 0.1$). This result is not surprising because even a polar enhancer would be expected to work either upstream or downstream of the promoter on a circular plasmid. In this sense, it is possible that the reversed orientation of ALE placed upstream of the promoter could function by acting "downstream" of the promoter in a plasmid context.

To test this possibility, the *gypsy* insulator was used to block enhancer activity. Remarkably, when a single copy of the insulator was located immediately upstream of the promoter, the expression dropped 20-fold for the normal orientation of ALE (Fig. 2, gene 2) but only modestly for the reversed orientation (Fig. 2, gene 6). The difference between these two genes is highly significant (gene 2 vs. gene 5, $P < 0.0005$). Conversely, when one copy of the insulator was inserted upstream of ALE, a 2-fold decrease in expression was observed for the reversed orientation (gene 7) but not for the normal orientation (gene 3) of the ALE (gene 3 vs. gene 7, $P < 0.00001$). These results clearly show that one orientation of ALE works better than the other depending on the position of the insulator and suggest an orientation preference of the ALE enhancer. Not surprisingly, two copies of the insulator flanking ALE resulted in near basal expression regardless of ALE orientation (genes 4 and 8), as expected for enhancer blocking.

Weakening the Promoter Magnifies the Effects of the Insulator and of Enhancer Orientation. The above results are consistent with a facilitated tracking mechanism in that the DNA sequences between the site(s) where proteins initially bind the enhancer and the target promoter participate in and, therefore, influence the delivery of stimulatory signals to the promoter. These results harmonize with the transcriptional decoy model of *gypsy* insulator function, which predicts that a single copy of the insulator interposed between an enhancer and a target promoter will result in enhancer blocking (22). Still, because the ALE orientation preference is not absolute, blocking by one copy of the insulator is not complete in the above cases. A critical, although heretofore untested, prediction of the transcriptional decoy

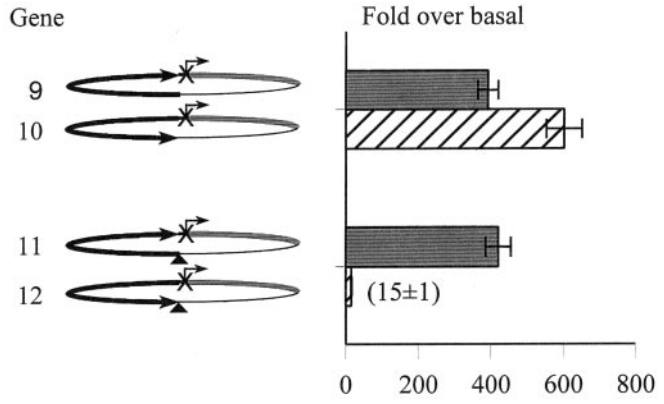

Fig. 3. Weakening the promoter magnifies the orientation preference of the ALE. Genes, displayed as in previous figures, carry the crippled (MutAB) promoter as indicated by the ''X'' (24). The values are normalized relative to that of the gene containing the same modified promoter and a similarly sized fragment of bacteriophage λ DNA instead of the ALE.

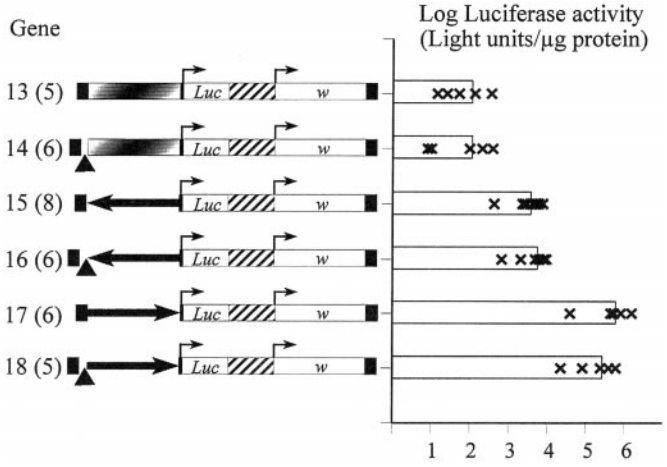

Fig. 4. The ALE displays an orientation preference in a chromosomal context. Genes are drawn to scale. After the gene number, the number of individual transformed lines is given in parentheses. Each construct contains a firefly luciferase gene (*Luc*) and mini-*white* gene (*w*). The small arrows represent the promoters for each gene. Note that the promoter driving luciferase expression in each case is the crippled *Adh* promoter. The black small boxes flanking each construct represent P-element sequences, and the central, slashed boxes represent plasmid vector sequences. As in previous figures, the *gypsy* insulator is shown as a small triangle. For genes 13 and 14, the shaded boxes represent a 4.8-kb fragment of λ DNA. For genes 15 through 18, the large arrows pointing to the right represent the normal orientation of ALE, while the ones pointing left represent the reversed orientation of ALE. The graph shows the $\log_{10}$ of luciferase specific activity (light units per μg protein). Each data point shows the mean of three independent samples from a given transformed line. Bars are the grand means of the luciferase specific activities for all the transformed lines for a given gene.

model is that the insulator should compete more effectively, and block more completely, if the promoter is weakened.

To address this, a crippled form of the *Adh* larval promoter (MutAB) was used in place of the wild-type promoter. This crippled promoter was obtained by introducing clustered point mutations that abolish two conserved sequences, GATCGC and a GATA factor-binding site, needed for full levels of transcription *in vitro* and *in vivo* (23, 24). Absolute levels of expression for this promoter were nearly a 1,000-fold lower than for the wild-type promoter (data not shown). With the weakened form of the promoter, both orientations of ALE were able to increase expression by about 400- to 600-fold (Fig. 3, genes 9 and 10). Also, as was seen with the wild-type promoter, insertion of one copy of the insulator upstream of the normal orientation of ALE had no effect on transcription (Fig. 3, gene 11). In contrast, when one copy of the insulator was inserted upstream of the reversed orientation of ALE, stimulation decreased from about 600-fold to only 15-fold above basal levels (Fig. 3, gene 12). This orientation effect is highly significant (gene 11 vs. gene 12, $P < 0.000002$) and much more dramatic than that observed for the wild-type promoter, for which placing the insulator upstream of the reversed orientation of ALE decreased expression by only 2-fold rather than the approximately 40-fold seen with the weak promoter.

The ALE Displays an Orientation Preference in the Chromosome. All of the above results are consistent with the interpretation that ALE works better in one orientation than in the other in the transient transformation system. In a circular plasmid, the orientation preference of ALE can be seen using one copy of the *gypsy* insulator. Presumably, the insulator prevents the enhancer from working bidirectionally on a circular plasmid. Still, it is a formal possibility that this orientation preference could be due to a property of the insulator (such as particular interactions between the *gypsy* insulator and the two ends of the ALE) rather than to a property of the enhancer itself. If this orientation preference is a property of ALE, then it should still be seen when genes (with or without the insulator) are assayed as linear constructs inserted into the chromosome.

Therefore, it was important to determine whether this orientation preference reflected the activity of the enhancer in a chromosomal context. To evaluate this, reporter genes were stably inserted into chromosomes by P-element transformation. Because the crippled form of the promoter displayed the greatest dependence on ALE orientation, luciferase reporter genes controlled by the crippled *Adh* promoter were used for this experiment. Three types of genes were made. Upstream of the promoter, these carried a 4.8-kb fragment of bacteriophage λ DNA, ALE in the normal orientation, or ALE in the reversed orientation. To test for a role of the insulator in the apparent polarity of the ALE, three similar genes were constructed that carried, in addition, a far upstream copy of the *gypsy* insulator (Fig. 4).

Fig. 4 clearly indicates that the ALE stimulates transcription regardless of its orientation but that there is a profound difference in the degree of stimulation supported by the two orientations. As is typical of germ-line transformation in *Drosophila*, analysis of variance indicated that all experimental genes showed significant variation due to chromosomal position effects. The presence of the *gypsy* insulator in a far upstream location made no difference for any of the three gene types. Analysis of variance grouped gene 13 with gene 14, gene 15 with gene 16, and gene 17 with gene 18 as homogenous subgroups. On average, the reverse orientation of ALE stimulated transcription by a significant, but relative moderate, 40-fold (genes 13 and 14 vs. genes 15 and 16, $P < 0.01$). However, the normal orientation of the ALE resulted in an average 4,100-fold stimulation regardless of the presence of the insulator. Even omitting the one stock having exceptionally high levels of expression (carrying gene 17), the normal orientation of ALE supports an average of 3,200-fold stimulation. The post hoc analysis confirmed a highly significant difference in the levels of expression supported by the two orientations of the ALE (genes 15 and 16 vs. genes 17 and 18, $P < 0.001$).

Discussion

The use of a transient assay incorporating the *gypsy* insulator allowed us to detect an orientation preference for ALE when inserted into plasmids. This suggests a general strategy for evaluating orientation preference of enhancers and the function of

GENETICS

insulators. A *Drosophila*-based system allows one to combine the power of *Drosophila* genetics with the speed and convenience of a transient assay, as demonstrated by our experiments using the *su(Hw)*^{v}/*su(Hw)*^{f} stock. This offers clear advantages over heterologous systems for which the relevant interacting proteins may not be present (43, 44). The luciferase assay is sensitive enough that it may be used with single embryos or larvae, and the approach could be modified by incorporation of *in situ* hybridization or immunohistochemical methods to evaluate cell lineage-specific effects.

A simple looping mechanism for ALE action cannot be reconciled with the results we obtain when the enhancer is assayed on plasmids. Rather, the observed polarity implies a tracking mechanism, analogous to that proposed for the *Xenopus laevis* rRNA enhancer (45). The blocking seen with a single copy of the *gypsy* insulator supports the notion that enhancers can stimulate expression in circular plasmids either by working "upstream" of the promoter or, perhaps with somewhat lower efficiency, by working "downstream" of the promoter through a mechanism that involves participation of the DNA sequences lying between the enhancer and the promoter. A simple looping mechanism would predict that random collisions between proteins bound to the enhancer and the insulator would not be sensitive to enhancer orientation. Contrast this with the fact that one copy of the insulator inserted upstream of the ALE in a plasmid carrying a weakened promoter displays either negligible or nearly complete blocking depending upon ALE orientation.

Several lines of evidence indicated that the polarity is not due simply to a spacing effect such that sequences near the (native) promoter-proximal end of ALE must be located near the promoter for full levels of transcriptional stimulation. The ALE works equally well in either orientation on plasmids in the absence of the insulator. It is only when the insulator is placed near the promoter-proximal end of the enhancer that a reduction in transcriptional stimulation is seen. However, the germ-line transformation experiments verify that the polarity is a property inherent to ALE and does not result from idiosyncratic interactions of the ALE with the *gypsy* insulator. Furthermore, the orientation preference remains when 5 kb of bacteriophage λ DNA is inserted between enhancer and the promoter (unpublished observations). Nor does it appear, following similar reasoning, that the (native) promoter-distal portion of the ALE contains a spacing-sensitive silencer element. Not only do both orientations work equally well in a plasmid context in the absence of the insulator, but the reversed orientation of the ALE significantly stimulates transcription in a chromosomal context.

Although it is clear that RNA polymerase II enhancers, including the ALE, can and do function bidirectionally in some cases, surprisingly few enhancers have been evaluated quantitatively with reference to orientation when inserted into the chromosome. For example, it is known that a single enhancer can activate divergently transcribed promoters in a chromosomal context in *Drosophila* (46–49). However, all of the enhancers used in these experiments are small (less than 1 kb), and with the exception of the *yolk-protein* gene enhancer, which normally activates divergently transcribed genes, all were assayed outside of their normal chromosomal context (46). Moreover, direct quantitative comparisons of the levels of activation for the divergently transcribed genes, in these cases, have not been reported.

It seems unlikely that the ALE is exceptional regarding its orientation preference, but the relatively large size of this enhancer probably plays a role in its polarity. The cis-stimulatory sequences in the ALE are spread throughout the 4.4-kb enhancer, possibly reflecting the presence of two families of dispersed repeated sequences (36). In addition to these repeats, ALE contains a total of 13 dispersed copies of the consensus binding sequence (T/AGATAA) for the GATA transcription factor (ABF or Serpent; refs. 24 and 50). In this respect, the ALE may resemble the 1.3-kb, murine *GATA-1* gene hematopoietic enhancer, which contains multiple GATA factor binding sites and shows a strong orientation preference (of undetermined magnitude) when inserted into the chromosome (18). Presumably, if sufficiently large chromosomal regions were evaluated for other enhancers, orientation preferences would be revealed in many cases. Polarity in transcriptional enhancement for RNA polymerase II genes has been hinted at by other studies. Notably, others have found roles of extended regions of DNA sequence in the communication between distant regulatory elements and a target promoter (11, 17, 51). We propose the phrase, "enhancing regions," to distinguish DNA stimulatory sequences displaying polarity from core promoter elements that display orientation independence.

The transcriptional decoy model of *gypsy* insulator function (22) fits nicely with a facilitated tracking mechanism for enhancer action and makes two basic predictions that are consistent with our results. First, a single insulator interposed between an enhancer and a promoter should display blocking, without needing to interact with other insulator elements to define a chromatin domain. Strictly speaking, this can never be tested by inserting genes into a chromosome replete with endogenous insulator elements. It does, however, agree with what we observe for plasmids. Second, weakening the promoter should increase blocking by the insulator, which we also observe. Significantly, even reversing the orientation of the polar enhancing region and weakening the promoter do not allow transcriptional silencing by a distally located *gypsy* insulator in the chromosome. This confirms the prevailing notion that the mechanisms of silencing and enhancer blocking are fundamentally different (17, 22, 52). Indeed, it may be impossible to test whether any insulator functions as a transcriptional decoy when placed in a distal location in the chromosome. Logically, there would always be some promoter or insulator lying distal to the transposon insertion site, making the presence of the experimental insulator irrelevant.

There are at least three models that can account for the observed polarity. First, there may be a cryptic insulator element within the distal portion of the ALE itself. Second, there might be a discrete polarity element where chromatin remodeling complexes (53–55) interact with the enhancing region and processively direct limiting transcription factors to the promoter. The third model, which we favor, is that many sequences, likely with graded affinities for limiting transcription factors or with weak insulator activity, work in concert to facilitate movement of transcription factors preferentially, although not exclusively, in one direction. Such an arrangement for native enhancing regions would explain why the sequences flanking various *Adh* genes invariably play cis-stimulatory roles in the context of the native gene but sometimes lower expression (possibly by inappropriate competition) when substituted for the homologous sequences of a gene from a closely related species (56–58).

It is likely that polarity of enhancing regions will prove to be a rather general finding. A facilitated tracking model is consistent with a mechanical role of actin-related proteins in chromatin remodeling complexes (59, 60) and the proposed role of adherins in facilitating long-range enhancer–promoter interactions (17). For example, recent evidence suggests that the locus control region of the murine β-globin locus, once thought to be a binary switch necessary for establishment of a broad domain of open chromatin structure, may more appropriately be thought of as a key component of a large enhancing region (14, 15, 61–64). Indeed, a facilitated tracking mechanism has important implications for the regulation of genes within a gene cluster, such as the homeotic genes in mammals and *Drosophila* (11, 16, 65), that may be controlled by a single large enhancing region.

We thank P. Geyer for providing *Drosophila* stocks. This work was supported by University of Louisville and National Institutes of Health monies (to M.D.B.). W.W. was supported in part by a Merit Fellowship from the University of Louisville Center for Genetics and Molecular Medicine.

1. Ptashne, M. (1986) *Nature (London)* **322,** 697–701.
2. Tjian, R. & Maniatis, T. (1994) *Cell* **77,** 5–8.
3. Ptashne, M. & Gann, A. (1997) *Nature (London)* **386,** 569–577.
4. Heuchel, R., Matthias, P. & Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 8931–8947.
5. Müller, H.-P., Sogo, J. M. & Schaffner, W. (1989) *Cell* **58,** 767–777.
6. Rothberg, I., Hotaling, E. & Sofer, W. (1991) *Nucleic Acids Res.* **19,** 5713–5717.
7. Wu, C.-T. (1993) *J. Cell Biol.* **120,** 587–590.
8. Blackwood, E. M. & Kadonaga, J. T. (1998) *Science* **281,** 60–63.
9. Martínez-Laborda, A., González-Reyes, A. & Morata, G. (1992) *EMBO J.* **11,** 3645–3652.
10. Casares, F., Bender, W., Merriam, J. & Sanchez-Herrero, E. (1997) *Genetics* **145,** 123–137.
11. Sipos, L., Mihaly, J., Karch, F., Schedl, P., Gausz, J. & Gyurkovics, H. (1998) *Genetics* **149,** 1031–1050.
12. Morris, J. R., Geyer, P. K. & Wu., C.-T. (1999) *Genes Dev.* **13,** 253–258.
13. Morris, J. R., Chen., J., Filandrinos, S. T., Dunn, R. C., Fisk, R., Geyer, P. K. & Wu, C.-T. (1999) *Genetics* **151,** 633–651.
14. Dillon, N., Trimborn, T., Strouboulis, J., Fraser, P. & Grosveld, F. (1997) *Mol. Cell* **1,** 131–139.
15. Epner, E., Reik, A., Cimbora, D., Telling, A., Bender, M. A., Fiering, S., Enver, T., Martin, D. I., Kennedy, M., Keller, G. & Groudine, M. (1998) *Mol. Cell* **2,** 447–455.
16. Kondo, T., Zakany, J. & Duboule, D. (1998) *Mol. Cell* **1,** 289–300.
17. Dorsett, D. (1999) *Curr. Opin. Genet. Dev.* **9,** 505–514.
18. Nishimura, S., Takahashi, S., Kuroha, T., Suwabe, N., Nagasawa, T., Trainor, C. & Yamamoto, M. (2000) *Mol. Cell. Biol.* **20,** 713–723.
19. Corbin, V. & Maniatis, T. (1989) *Genes Dev.* **3,** 2191–2200.
20. Corces, V. G. & Geyer, P. K. (1991) *Trends Genet.* **7,** 86–90.
21. Geyer, P. K. & Corces, V. G. (1992) *Genes Dev.* **6,** 1865–1873.
22. Geyer, P. K. (1997) *Curr. Opin. Genet. Dev.* **7,** 242–248.
23. McKenzie, R. W., Hu, J. & Brennan, M. D. (1994) *Nucleic Acids Res.* **22,** 1257–1264.
24. Hu, J., Qazzaz, H. & Brennan, M. D. (1995) *J. Mol. Biol.* **249,** 259–269.
25. Goldberg, D. A. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5794–5798.
26. Alting-Mees, M. A., Sorge, J. A. & Short, J. M. (1992) *Methods Enzymol.* **216,** 483–495.
27. Pirrotta, V. (1988) in *Vectors: A Survey of Molecular Cloning Vectors and Their Uses*, eds. Rodriguez, R. L. & Denhardt, D. T. (Butterworth–Heinemann, Boston), pp. 437–456.
28. Lindsley, D. L. & Zimm, G. G. (1992) *The Genome of Drosophila melanogaster* (Academic, New York).
29. Harrison, D. A., Gdula, D. A., Coyne, R. S. & Corces, V. G. (1993) *Genes Dev.* **7,** 1966–1978.
30. Brennan, M. D. & Dickinson, W. J. (1988) *Dev. Biol.* **125,** 64–74.
31. Zar, J. H. (1984) *Biostatistical Analysis* (Prentice–Hall, Englewood Cliffs, NJ).
32. Rubin, G. M. & Spradling, A. C. (1982) *Science* **218,** 348–353.
33. Spradling, A. C. & Rubin, G. M. (1982) *Science* **218,** 341–347.
34. Steller, H. & Pirrotta, V. (1986) *EMBO J.* **4,** 167–171.
35. McKenzie, R. W. & Brennan, M. D. (1998) *Insect Biochem. Mol. Biol.* **28,** 869–873.
36. Corbin, V. & Maniatis, T. (1990) *Genetics* **124,** 637–646.
37. Spana, C., Harrison, D. A. & Corces, V. G. (1988) *Genes Dev.* **2,** 1414–1423.
38. Holdridge, C. & Dorsett, D. (1991) *Mol. Cell. Biol.* **11,** 1894–1900.
39. Smith, P. A. & Corces, V. G. (1992) *Mol. Gen. Genet.* **233,** 65–70.
40. Scott, K. C., Taubman, A. D. & Geyer, P. K. (1999) *Genetics* **153,** 787–798.
41. Modolell, J., Bender, W. & Meselson, M. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1678–1682.
42. Smith, P. A. & Corces, V. G. (1995) *Genetics* **139,** 215–228.
43. Dunaway, M., Hwang, J. Y., Xiong, M. & Yuen, H.-L. (1997) *Mol. Cell. Biol.* **17,** 182–189.
44. van der Vlag, J., den Blaauwen, J. L., Sewalt, R. G., van Driel, R. & Otte, A. P. (2000) *J. Biol. Chem.* **275,** 697–704.
45. Krebs, J. E. & Dunaway, M. (1998) *Mol. Cell* **1,** 301–308.
46. Barnett, T. & Wensink, P. C. (1981) in *Developmental Biology Using Purified Genes*, *UCLA-ICN Symposium*, eds. Brown, D. D. & Fox, C. F. (University of California, Berkeley), Vol. 23, pp. 97–106.
47. Scott, K. S. & Geyer, P. K. (1995) *EMBO J.* **14,** 6258–6279.
48. Cai, H. & Levine, M. (1995) *Nature (London)* **376,** 533–536.
49. Ohtsuki, S., Levine, M. & Cai, H. N. (1998) *Genes Dev.* **12,** 547–556.
50. Abel, T., Michelson, A. M. & Maniatis, T. (1993) *Development (Cambridge, U.K.)* **119,** 623–633.
51. Bonifer, C., Jagle, U. & Huber, M. C. (1997) *J. Biol. Chem.* **272,** 26075–26078.
52. Mannervik, M., Nibu, Y., Zhang, H. & Levine, M. (1999) *Science* **284,** 606–609.
53. Kingston, R. E. & Narlikar, G. J. (1999) *Genes Dev.* **13,** 2339–2352.
54. Travers, A. (1999) *Cell* **96,** 311–314.
55. Peterson, C. L. & Logie, C. (2000) *J. Cell. Biochem.* **78,** 179–185.
56. Fang, X. M., Wu, C. Y. & Brennan, M. D. (1991) *J. Mol. Evol.* **32,** 220–226.
57. Fang, X. M. & Brennan, M. D. (1992) *Genetics* **131,** 333–343.
58. Wu, C. Y. & Brennan, M. D. (1993) *Mol. Gen. Genet.* **240,** 58–64.
59. Cairns, B. R., Erdjument-Bromage, H., Tempst, P., Winston, F. & Kornberg, R. D. (1998) *Mol. Cell* **2,** 639–651.
60. Boyer, L. A. & Peterson, C. L. (2000) *BioEssays* **22,** 666–672.
61. Reik, A., Telling, A., Zitnik, G., Cimbora, D., Epner, E. & Groudine, M. (1998) *Mol. Cell. Biol.* **18,** 5992–6000.
62. Tanimoto, K., Liu, Q., Bungert, J. & Engel, J. D. (1999) *Nucleic Acids Res.* **27,** 3130–3137.
63. Li, Q., Harju, S. & Peterson, K. R. (1999) *Trends Genet.* **15,** 403–408.
64. Bender, M. A., Bulger, M., Close, J. & Groudine, M. (2000) *Mol. Cell* **5,** 387–393.
65. Duboule, D. & Morata, G. (1994) *Trends Genet.* **10,** 358–364.