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Enhancer Choice *in Cis* and *in Trans* in *Drosophila melanogaster*: Role of the Promoter

James R. Morris,[1] Dmitri A. Petrov,[2] Anne M. Lee and Chao-ting Wu

Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT

Eukaryotic enhancers act over very long distances, yet still show remarkable specificity for their own promoter. To better understand mechanisms underlying this enhancer-promoter specificity, we used transvection to analyze enhancer choice between two promoters, one located *in cis* to the enhancer and the other *in trans* to the enhancer, at the *yellow* gene of *Drosophila melanogaster*. Previously, we demonstrated that enhancers at *yellow* prefer to act on the *cis*-linked promoter, but that mutation of core promoter elements in the *cis*-linked promoter releases enhancers to act *in trans*. Here, we address the mechanism by which these elements affect enhancer choice. We consider and explicitly test three models that are based on promoter competency, promoter pairing, and promoter identity. Through targeted gene replacement of the endogenous *yellow* gene, we show that competency of the *cis*-linked promoter is a key parameter in the *cis-trans* choice of an enhancer. In fact, complete replacement of the *yellow* promoter with both TATA-containing and TATA-less heterologous promoters maintains enhancer action *in cis*.

EUKARYOTIC enhancers are able to act over long distances, sometimes interacting with promoters hundreds of kilobase pairs away. At the same time, enhancers can show a high degree of specificity, finding and interacting with their own promoter but not with other nontarget promoters. One mechanism for how this specificity is achieved suggests that intrinsic properties of enhancers and promoters limit an enhancer to a particular promoter (Li and Noll 1994; Hansen and Tjian 1995; Kapoun and Kaufman 1995; Merli *et al.* 1996; Ohtsuki *et al.* 1998; Sharpe *et al.* 1998; Butler and Kadonaga 2001; Cai *et al.* 2001; Conte *et al.* 2002). Here, we explore this mechanism using the phenomenon of transvection.

Transvection is a process by which a gene can affect the expression of its homologous gene on a separate chromosome in a pairing-dependent manner. It has been observed in Drosophila, where homologous chromosomes are aligned and paired in somatic cells. In addition, transvection and related processes have been observed in fungi, plants, and mammals, where extensive somatic homolog pairing has not been demonstrated, suggesting that even transient pairing interactions might have long-term consequences on gene expression (reviewed in Pirrotta 1999; Wu and Morris 1999; Burgess 2002; Duncan 2002; Kennison and Southworth 2002; most recently Bean *et al.* 2004).

[1]*Corresponding author:* Center for Genomics Research, Room 210, Harvard University, 7 Divinity Ave., Cambridge, MA 02138. E-mail: jmorris@cgr.harvard.edu

[2]*Present address:* Department of Biological Sciences, Stanford University, Stanford, CA 94305.

Our studies explore transvection at the *yellow* gene of Drosophila. The *yellow* gene is required for dark pigmentation of the cuticle, including the wings, body, and bristles, and is expressed under the control of tissue-specific enhancers located in the 5′ upstream region and intron of the gene (Geyer and Corces 1987; Martin *et al.* 1989; Wittkopp *et al.* 2002). Mutations at *yellow* reduce pigmentation, sometimes to a fully mutant yellow color. Interestingly, some combinations of mutant *yellow* alleles that reduce pigmentation in the same tissues show intragenic complementation, such that flies heterozygous for two mutant alleles show nearly wild-type pigmentation (Geyer *et al.* 1990; Morris *et al.* 1999a). This intragenic complementation depends on the somatic pairing of *yellow* genes (Geyer *et al.* 1990; Chen *et al.* 2002; Savitsky *et al.* 2003; S. Ou, J. R. Morris and C.-t. Wu, unpublished results). One mechanism of transvection at *yellow* involves the action of the wing and body enhancers of one gene *in trans* on the promoter of its homolog on a separate chromosome (Geyer *et al.* 1990; Morris *et al.* 1998; Chen *et al.* 2002). As these enhancers usually prefer to act on their own promoter *in cis*, that is, show *cis* preference (Geyer *et al.* 1990; Morris *et al.* 1999a,b), transvection at *yellow* provides a system where enhancer choice is changed from a state of *cis* preference to one that also includes *trans* action.

The goal of our studies is to clarify how an enhancer chooses between a *cis*-linked promoter and one located *in trans*. It has been shown that enhancer choice can be modulated by the state of the *cis*-linked promoter (Geyer *et al.* 1990; Martínez-Laborda *et al.* 1992; Hendrickson and Sakonju 1995; Casares *et al.* 1997; Sipos *et al.* 1998; Morris *et al.* 1999a,b). Previously, we found

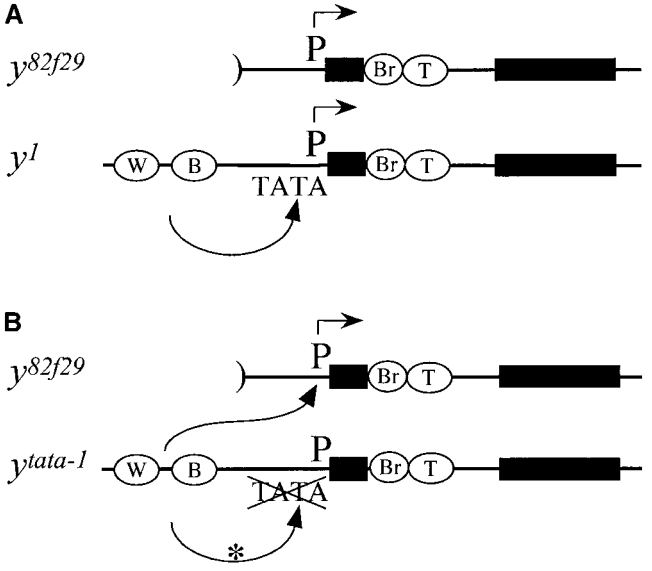

FIGURE 1.—Enhancer choice *in cis* and *in trans*. (A) *Cis* preference of enhancers. The wing and body enhancers of y^{1}, with an intact promoter (GEYER *et al.* 1990), are restricted to *cis* action and do not activate the promoter of y^{82f29}, which is a deletion of the wing and body enhancers (MORRIS *et al.* 1998). (B) Enhancer action *in trans*. In contrast, the wing and body enhancers of $y^{tata\text{-}1}$, which has a 6-bp mutation in the TATA box (MORRIS *et al.* 1999b), are able to act *in trans* on the promoter of y^{82f29} as well as *in cis* on their own promoter. Both y^{1} and $y^{tata\text{-}1}$ have a mutated translation initiation codon to eliminate the contribution of pigmentation from enhancer action *in cis*. An asterisk indicates that evidence for *cis* action of the $y^{tata\text{-}1}$ enhancers is inferred from analysis of other genotypes (MORRIS *et al.* 1999b). The *yellow* gene is drawn approximately to scale and spans 7.8 kbp. W, wing enhancer; B, body enhancer; Br, bristle enhancer; T, tarsal claw enhancer; P, promoter; solid rectangle, exon.

that mutations introduced by targeted gene replacement (GLOOR *et al.* 1991; KEELER *et al.* 1996) of the *yellow* TATA box or initiator (Inr), two core promoter elements (reviewed in SMALE and KADONAGA 2003), released the upstream wing and body enhancers to act *in trans* (MORRIS *et al.* 1999b). This model can be illustrated by contrasting the abilities of two alleles, y^{1} and $y^{tata\text{-}1}$, to contribute enhancer activity *in trans* to y^{82f29}, which lacks both the wing and the body enhancers (MORRIS *et al.* 1998). As shown in Figure 1, the wing and body enhancers of y^{1}, which carries an intact promoter, are restricted to *cis* action while those of $y^{tata\text{-}1}$, in which the TATA box has been altered, can act *in trans* as well as *in cis* (MORRIS *et al.* 1999b).

These observations suggest three broad classes of models for enhancer choice *in cis* and *in trans*. One proposes that enhancer choice is sensitive to some aspect of competency involving the *cis*-linked promoter, such as transcription rate, the binding of specific transcription factors to the promoter, or a particular chromatin state. This model is in line with studies of enhancers and promoters in *cis*-linked configurations (SHARPE *et al.* 1998; CAI *et al.* 2001; CONTE *et al.* 2002). Another model proposes that enhancer choice is influenced by the effect of promoter mutations on the state of pairing between two *yellow* alleles. That is, sequence heterology resulting from mutations in the *cis*-linked promoter or flanking regions may lead to promoter unpairing, and this unpairing may then render the intact promoter *in trans* to an enhancer more attractive, perhaps through conformational changes or by making it more accessible to transcription factors (SIPOS *et al.* 1998; MORRIS *et al.* 1999a). A third model suggests that mutations in core promoter elements, by altering key promoter sequences, exert their effects on enhancer action by changing the identity or signature of the promoter. Such changes might cause an enhancer to seek a native promoter on a separate chromosome rather than interact with a *cis*-linked promoter that is of the wrong identity. Studies of *cis*-linked enhancers and promoters have revealed that enhancers can discriminate among different promoters on the basis of the presence or absence of core promoter motifs (OHTSUKI *et al.* 1998; BUTLER and KADONAGA 2001; CONTE *et al.* 2002).

Here, we use targeted gene replacement at *yellow* to further examine these three models. In particular, we ask whether a mutation of a *yellow* core promoter element, the TATA box, releases enhancers to act *in trans* due to its effects on promoter competency, pairing, or identity. Surprisingly, we find that while a 6-bp mutation of a core promoter element releases enhancers, complete replacement of 193 bp of the *yellow* promoter with any of three heterologous promoters fails to release enhancers to *trans* action. We argue that the roles of promoter pairing and identity in the *cis-trans* decision are likely to be at most secondary to that of promoter competency. In addition, we consider our data in light of the possibility that competency of a promoter may reflect features of the promoter in addition to transcription strength.

MATERIALS AND METHODS

Scoring of pigmentation: Pigmentation was scored as previously described (MORRIS *et al.* 1998). We scored 1- to 3-day-old females using a five-point scale, where 1 represents the null or nearly null state and 5 represents the wild-type or nearly wild-type state. For consistency, flies were scored by comparison to flies whose wing and body pigmentation was previously reported, including y^{1}/y^{1} (1, 1), y^{2}/y^{2} (1, 1–2), $y^{tata\text{-}1}/y^{82f29}$ (2, 1–2), y^{tata}/y^{tata} (3, 2), $y^{1\#8}/y^{82f29}$ (3, 3), $y^{1\#8}/y^{2}$ (4, 4), and y^{+}/y^{+} (5, 5). However, because pigmentation represents a continuum, there is some variation of pigmentation for flies scored at the same value. Complementation between two alleles required that wing or body pigmentation was one point darker on the pigmentation scale than that of corresponding tissues in females homozygous for either allele. Flies were cultured at 25° ± 1° as previously described (MORRIS *et al.* 1998). Crosses were done with three to four females mated to three or more males in vials that were brooded daily. Both temperature and crowding were carefully controlled.

Plasmid construction: The templates used in the targeted

gene replacement experiments were full-length 7.8-kbp *yellow* genes (GenBank accession nos. X06481 and X04427) with the designated sequence changes (Figure 2) cloned into a modified Bluescript plasmid (Stratagene, La Jolla, CA) in which the *Kpn*I site is replaced by an *Xba*I site (pBSX). pUC8ySB is a pUC8 plasmid containing the wild-type 3.1-kbp *Sal*I-*Bam*HI *yellow* fragment; pUC8y1SB contains the A-to-C mutation of y^{1}; pUC8ySBtata contains the 6-bp sequence change in the TATA box present in y^{tata}; pUC8ySBtata-1 contains the 6-bp TATA mutation and the A-to-C mutation of y^{1}; pBSXyBG is pBSX containing the 4.7-kbp *Bam*HI-*Bgl*II *yellow* fragment (Morris *et al.* 1999b). All sequence changes were first incorporated into pUC8ySB or one of its derivatives noted above, which was then digested with *Sal*I and *Bam*HI and cloned into pBSXyBG to generate full-length templates in pBSX. All changes were confirmed by sequencing.

The four insertional promoter templates, tT, tT-1, TT, and TT-1, were made by introducing a double-stranded linker (see below) at the *Eag*I site of pUC8ySB, pUC8y1SB, pUC8ySBtata, or pUC8ySBtata-1, respectively.

The eight promoter scan (ps) templates were made using a PCR strategy with one of the two primers carrying the desired sequence changes. For ps1 and ps1-1, primers 1 and m13rev (primer sequences shown below) were used to generate PCR products with pUC8ySB or pUC8y1SB as a template, respectively. The resulting PCR products were digested with *Eag*I and *Bam*HI and cloned into pUC8ySB. For ps2 and ps2-1, primers 2 and m13rev were used in a similar strategy. For ps3 and ps3-1, primers 3 and 4 were used with pUC8ySB as a template, and the resulting PCR products were digested with *Kpn*I and *Eag*I and cloned into pUC8ySB or pUC8y1SB, respectively. For ps4 and ps4-1, primers 3 and 5 were used in a similar strategy.

The six heterologous promoter templates were made by first cloning the *Kpn*I-*Bam*HI *yellow* fragment from pUC8ySBps4 into pBS to make pBSyKBps4. Primer pairs 6/7, 8/9, and 10/11 were then used to PCR amplify a 193-bp fragment from the *even skipped* (*eve*), *heat-shock protein 70* (*hsp70*), and *white* (*w*) promoter regions of wild-type Canton-S genomic DNA, respectively. These were TA cloned (Invitrogen, San Diego), confirmed by sequence analysis, cut with *Cla*I and *Afl*II, and cloned into pBSyKBps4. The resulting plasmid was digested with *Kpn*I and *Bam*HI and cloned into pUC8ySB.

Constructs for *P*-element-mediated transformation were made by digesting the three pBSX plasmids containing the heterologous promoters with *Hin*dIII and *Not*I, religating the resulting 2.9-kbp pBSX fragment and the 5.5-kbp 3′ *yellow* fragment, digesting the resulting plasmid with *Xba*I, and cloning this fragment into the *Xba*I site of pCaSper3.

Targeted gene replacement and *P*-element-mediated germline transformation: Targeted gene replacement was carried out as previously described using y^{h12w+} as a target allele on a chromosome that also carried w^{1118} and plasmid sources of templates and transposase at concentrations of 1.0 and 0.25 mg/ml, respectively (Keeler *et al.* 1996; Morris *et al.* 1999b). On average, 500 embryos were injected per construct, with a larval survival rate of 40% and a conversion rate of 1% (of total embryos injected). Candidate lines were screened by single-fly PCR using primers 12 and 13, which give an 874-bp PCR product. Those lines giving wild-type-sized products were confirmed by digesting the PCR product with restriction enzymes diagnostic of the desired changes and sequence analysis. Southern analysis of genomic DNA digested separately with *Hin*dIII/*Bam*HI and *Pst*I and hybridized to a full-length *yellow* probe was performed to confirm the integrity of the *yellow* gene (data not shown). *P*-element-mediated transformation was done as previously described (Rubin and Spradling 1982; Morris *et al.* 1998) using 0.5 mg/ml construct, 0.1 mg/ml helper plasmid, and the $Df(1)y^{-}\ ac^{-}\ w^{1118}$ stock as the host.

Northern analysis: Flies were allowed to lay eggs for 24 hr in bottles at 25° and pupae were collected 8 days later. Total RNA was collected [Eppendorf (Madison, WI) Eukaryotic Perfect RNA] and poly(A)$^{+}$ RNA was isolated [Promega (Madison, WI) PolyATtract] and electrophoresed (5 μg/lane) on a 1% agarose/formaldehyde gel followed by hybridization with a ^{32}P-labeled *Eco*RI-*Bgl*II *yellow* fragment from the second exon and *rp49* as a loading control. Quantitation was based on band intensity relative to the average of the band intensities for the four data points from two independent y^{+} lines generated by targeted gene replacement. The average level of steady-state mRNA in our wild-type Canton-S line was 91% (over three trials) of the level in our y^{+} control lines (data not shown). Preliminary experiments using Canton-S pupae collected daily from days 6–10 indicated that day 8 showed maximal steady-state *yellow* mRNA expression (data not shown). On the basis of these results, day 8 pupae were used in subsequent experiments. However, because time courses were not done for each of the alleles, it is possible that maximum expression is shifted earlier or later compared to that of Canton-S pupae.

Primers and linkers: Primer and linker sequences used in this study are indicated below in 5′ to 3′ orientation with mutated nucleotides in boldface type.

1. AAAACGCGGCCG**GGTACC**TATGGCCACCAGTCGTT ACCGCGCCACGGTCCACAGAAG;
2. AAAACGCGGCCGACATAT**AGATCT**CACCAGTCGTTA CCGCGCCACGGTCCACAGAAG;
3. GCAGTCGCCGATAAAGATGAACACAG;
4. ATATGTCGGCCGCGTTTTATATGAAGGTTTTTTTCT CC**ACTAGT**GAAGACAGGCCAATGAAAATGAAAACG;
5. ATATGTCGGCCGCGTTTTATATGAAGGTTTTTTTCT CCGAAGACGAAGACAGG**ATCGAT**AAAATGAAAACGA AGGCG;
6. ATCGATGACGGCGGCCATTTGCCTGCAGAGCGCAG CGGTATAAA;
7. CTTAAGGGCTCTCCAGGTTGTAGGTTCGGTATCCGT GAATGTTT;
8. ATCGATCGCCTCGAATGTTCGCGAAAAGAG;
9. CTTAAGCTGGTTACTTTTAATTGATTCACT;
10. ATCGATCGCTGCGTCCGCTATCTCTTTCGCCACC;
11. CTTAAGTCACCACCCCAATCACTCAAAAAACAAA;
12. GAGCCTCCTGGCCTTACAATTTAC;
13. ATTTAACTTCCACTTACCATCACGCC;

Linker: 5′-GGCCAAAAAAACCTTCATATAAAACGC-3′
3′-TTTTTTTGGAAGTATATTTTGCGCCGG-5′.

RESULTS

Testing the role of transcription in the control of enhancer choice: Previously, we demonstrated that a 6-bp mutation of the TATA box (y^{tata}), the initiator (y^{inr}), or the TATA box and initiator ($y^{tata\text{-}inr}$) is sufficient to support transvection by releasing the wing and body enhancers to act *in trans* (Morris *et al.* 1999b; Table 1; Figure 2). Because these mutations were made in core promoter elements, the data suggested a model in which disruption of promoter competency, in particular its ability to support transcription, releases enhancers to act *in trans*. Here, we tested this model by determining the steady-state level of *yellow* expression through Northern analyses. We compared the level of *yellow* mRNA in flies carrying y^{tata}, y^{inr}, or $y^{tata\text{-}inr}$ to that in control flies carrying a wild-type y^{+} gene. Our y^{+} control flies were generated by the protocol used to generate y^{tata}, y^{inr}, and

TABLE 1

Complementation data

	Df	Homozygous	*82f29*	*2*	*62a*	*2374*
Df	1, 1	—	1, 1	1, 1	1, 4	1, 1
Homozygous	—	—	1, 1	1, 1–2	1, 3	1, 1–2
tata	2-3, 1	3, 2	*3*, 1–*2*	*4*, *3*	*4*, 4	*4*, *3*
inr	5, 4	5, 5	5, 5	5, 5	5, 5	5, 5
tata-inr	2, 1	2, 1	*3*, 1–*2*	*4*, *3*–*4*	*4*, 4	*4*, *3*
tata-1	1, 1	1, 1	*2*, 1–*2*	*3*, 2–*3*	*2*, 4	*3*, 2–*3*
inr-1	1, 1	1, 1	*2*, 1–*2*	*3*, 2–*3*	*2*, 4	*3*, 2–*3*
tata-inr-1	1, 1	1, 1	*3*, 1–*2*	*4*, *3*–*4*	*3*, 4	*4*, *3*
tT, TT	5, 5	5, 5	5, 5	5, 5	5, 5	5, 5
tT-1, TT-1	1, 1	1, 1	1, 1	1, 1–2	1, 4	1, 1–2
ps1,2,3,4	5, 5	5, 5	5, 5	5, 5	5, 5	5, 5
ps1,2,3,4-1	1, 1	1, 1	1, 1	1, 1–2	1, 4	1, 1–2
eve	5, 5	5, 5	5, 5	5, 5	5, 5	5, 5
hsp70	5, 5	5, 5[a]	5, 5	5, 5	5, 5	5, 5
w	4, 4	4, 4	4, 4	4, 4	4, 4	4, 4
eve, hsp70, w-1	1, 1	1, 1	1, 1	1, 1–2	1, 4	1, 1–2

Pigmentation scores for wings and body when alleles are *in trans* to a deficiency (*Df*) of *yellow*, homozygous, or *in trans* to one of the *yellow* alleles listed in the column headings. A score of 1 is null or nearly null; 5 is wild type or nearly wild type. Scores in italic type indicate complementation. Alleles showing similar phenotypes are grouped together.

[a] These flies have a dusky appearance.

$y^{tata\text{-}inr}$, that is, through targeted gene replacement (Gloor *et al.* 1991; Keeler *et al.* 1996) of the mutant *yellow* allele in our standard starting line, y^{h12w+} w^{1118} (see materials and methods; the w^{1118} null mutation of the *white* gene is present in all our convertant lines, but is not mentioned further). We found that steady-state levels of *yellow* mRNA are reduced in y^{tata}, y^{inr}, and $y^{tata\text{-}inr}$ to 1, 3, and 1%, respectively, of the level found in control y^{+} flies (Figure 3). These data are consistent with a model in which promoters that have been transcriptionally compromised release their enhancers to act *in trans*.

This interpretation further predicts that restoration of transcription to a transcriptionally compromised allele will recapture the upstream enhancers and prevent transvection. We tested this prediction by inserting a wild-type TATA box downstream of the mutated TATA box in y^{tata} (Figure 2) and then testing the resulting allele for transcription and the ability of its enhancers to act *in trans*. Note that we chose to restore transcription to y^{tata} instead of y^{inr} because y^{tata} is the more transcriptionally compromised of the two (Figure 3) and therefore provides a stronger test. Specifically, we used targeted gene replacement to insert the 27-bp region extending from position −44 to −18 of the wild-type *yellow* gene into the y^{tata} allele at position −17/−18 (nucleotides numbered relative to the transcription start site of +1). The resulting allele carried a wild-type promoter 3′ of position −44. We called the new allele y^{tT}, where *t* and *T* represent the mutated and wild-type TATA boxes, respectively, and their order represents their 5′ to 3′ order *in vivo*. Homozygous y^{tT}/y^{tT} and hemizygous y^{tT}/Df (where *Df* represents the y^{-} ac^{-} w^{1118} chromosome that is deficient for all *yellow* sequences; Morris *et al.* 1998) flies showed fully wild-type pigmentation in all cuticular structures, including the wings, body, and bristles, similar to our y^{+} control flies (Table 1). However, by Northern analysis, steady-state *yellow* mRNA levels were only ~11% of the level seen in y^{+} control flies (Figure 3).

Next we determined the ability of the wing and body enhancers of y^{tT} to act *in trans*. To this end, we first generated a companion protein-null derivative of y^{tT} such that translation of the transcripts made in the presence of the promoter alterations would not obscure our tests of the ability of the upstream enhancers to act on a promoter *in trans*. As was done for all subsequent companion alleles mentioned below, this protein-null derivative was generated by targeted gene replacement and bore, in addition to the changes in the promoter region, an A-to-C change in the ATG translation initiation codon. This change is identical to that found in the y^{1} protein-null allele (Geyer *et al.* 1990) and therefore, as expected, the companion allele for y^{tT}, called $y^{tT\text{-}1}$, gives a fully mutant phenotype (Table 1). We then determined whether $y^{tT\text{-}1}$ could release its wing and body enhancers to act *in trans* by asking whether it complements y^{82f29} (Figure 1) and three other alleles, y^{2}, y^{62a}, and y^{2374}, which, like y^{82f29}, lack strong wing and/or body enhancer activity (Morris *et al.* 1999b). We found that $y^{tT\text{-}1}$ does not complement each of these tester alleles. This finding suggests that restoration of transcriptional competency, as revealed by increased pigmentation and

FIGURE 2.—Partial sequences of wild-type and mutant *yellow* alleles generated by targeted gene replacement. Underlined bases, heat-shock element, TATA box, and Inr; bases in boldface type, mutant sequences; asterisk, transcription initiation site; //, break in sequence. For *yellow*, // indicates TTACCGCGCCACGGTCCACAGAAGAGGATTAAAAAAATATCACACAGCCGAAGGCTAGAG AAGAACCCCCTTAGCTGAACATATATAAACAAATATATTTTTTTTTTATTGCCAACACACT. For *eve*, // indicates CGAGCTGTGA CCGCCGCACAGTCAACAACTAACTGCCTTCGTTAATATCCTCTGAATAAGCCAACTTTGAATCACAAGACGCATACCAAACAT TCACGGATACCGAACCTACAACCTGGAG. For *hsp70*, // indicates CAATTCAAACAAGCAAAGTGAACACGTCGCTAAGCGAAA GCTAAGCAAATAAACAAGCGCAGCTGAACAAGCTAAACAATCTGCAGTAAAGTGCAAGTTAAAGTGAATCAATTAAGAGTAA. For *w*, // indicates CACTTTGTCAGCGGTTTCGTGACGAAGCTCCAAGCGGTTTACGCCATCAATTAAACACAAAGTGCTGTGC CAAAACTCCTCTCGCTTCTTATTTTTGTTTGTTTTTTGAGTGATTGGGGTG.

transcript levels in y^{tT} compared to those of y^{tata}, is sufficient to recapture the upstream enhancers and prevent transvection. Interestingly, *yellow* transcript levels in y^{tT} flies reach only to the 11% level compared to the y^{+} control (Figure 3), indicating that transcription need not be restored to wild-type levels for the upstream enhancers to be restricted to *cis* action.

These data also argue against a model in which local promoter unpairing as a result of structural heterozygosity between two alleles in the promoter region is sufficient to release enhancers to *trans* action. Specifically, the 27-bp insertion present in y^{tT} would be predicted to unpair the promoter region when paired with a tester allele, yet it does not support transvection. To test this interpretation, we made a second insertional *yellow* allele, called y^{TT}, that differs from y^{tT} only in that it carries a wild-type TATA box in place of the mutated TATA box (Figure 2). The y^{TT} allele produced wild-type pigmentation in homozygous and hemizygous flies, and its protein-null companion allele, $y^{TT\text{-}1}$, failed to complement each of the four tester alleles (Table 1). These findings provide additional support for the interpretation that local promoter unpairing as a result of sequence heterology between two alleles is not sufficient to release enhancers to act *in trans*.

Not all sequence changes in the promoter release enhancer to *trans* action: These results indicate a strong correlation between promoter elements involved in transcription and those involved in enhancer choice. We next decided to test the strength of this correlation by introducing mutations in noncore promoter sequences. Will all elements that affect transcription also affect enhancer choice and vice versa, or are there elements dedicated to one process but not the other? Using targeted gene replacement, we made four ps mutations, y^{ps1}, y^{ps2}, y^{ps3}, and y^{ps4} (Figure 2), each of which carried a 6-bp substitution in the promoter region, and four corresponding protein-null companion alleles, $y^{ps1\text{-}1}$, $y^{ps2\text{-}1}$, $y^{ps3\text{-}1}$, and $y^{ps4\text{-}1}$. Our design of these alleles was guided by the presence within the *yellow* promoter of an 80-bp

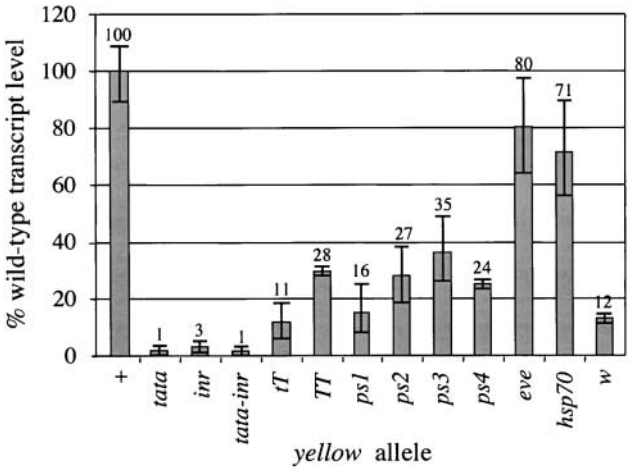

FIGURE 3.—Quantitation of transcript levels following Northern analysis of *yellow* alleles. Transcript levels from the y^+ control lines were averaged and set at 100%, and transcript levels for other alleles are expressed as a percentage of this average. Quantitation was based on band intensity. Height of bars (indicated by number over bar) represents the mean of two or more experiments. Error bars indicate one standard error on either side of the mean.

region, extending from position −43 to +37, that shows 91% sequence identity with the homologous promoter region of the *yellow* gene of *Drosophila subobscura*, a species that diverged from *D. melanogaster* ~30 million years ago (MUNTÉ *et al.* 1997). This sequence identity suggests that there might be important functional elements in this region, a prediction supported by sequence comparisons with other Drosophila species (MUNTÉ *et al.* 2000, 2001; WITTKOPP *et al.* 2002). With this in mind, we placed two mutations (y^{ps1} and y^{ps2}) within and two (y^{ps3} and y^{ps4}) outside this region of conservation.

In spite of the different placements relative to the conserved region, however, all four mutations have similar pigmentation and transcriptional phenotypes. Homozygous or hemizygous y^{ps} flies show wild-type pigmentation (Table 1) and steady-state *yellow* mRNA levels in these mutants are between 16% (y^{ps1}) and 35% (y^{ps3}) of levels seen in our y^+ control flies (Figure 3). The finding that mutations in noncore promoter elements nevertheless have a strong negative effect on steady-state mRNA levels is consistent with mutational analyses of noncore elements in other systems (for example, DUDLEY *et al.* 1999; WRAY *et al.* 2003).

None of the companion alleles supported transvection when placed *in trans* to the four tester alleles (Table 1). These data suggest that not any 6-bp mutation in the promoter region allows transvection, even though all that we tested reduce transcript levels. In light of our observation that mutations of the *yellow* TATA box and Inr do release enhancers (MORRIS *et al.* 1999b), these data demonstrate the importance of core promoter elements in enhancer choice. Furthermore, as mutations in core elements reduce transcript levels more significantly than mutations in noncore sequences (Figure 3), one interpretation is that only mutations that most severely compromise transcription release enhancers to *trans* action.

The data also suggest a second way to look at the role of promoter competency in enhancer choice. Specifically, it may be that the primary feature of the promoter that guides enhancer choice is its integrity as defined, for example, by the array of core elements rather than by an absolute rate of transcription. According to this interpretation, the *ps* alleles maintain *cis* preference of enhancers because their core promoter elements are intact. Furthermore, this model is consistent with the ability of y^{tT} and y^{TT} to recapture enhancers; in spite of their low steady-state transcript levels, they nevertheless have an intact configuration of core promoter elements.

Enhancer action *in cis* can be maintained by heterologous promoters: The data thus far support a key role for promoter competency in the *cis-trans* choice of an enhancer. Here we consider the third model in which promoter identity guides enhancer choice. In particular, we asked whether the 6-bp alteration in the TATA box changes the *yellow* promoter from a TATA-containing to a TATA-less promoter, thereby making it a poor target for the enhancers. Specifically, will the upstream enhancers of a *yellow* allele with a functional but heterologous promoter in place of the native promoter choose the heterologous promoter *in cis* rather than the native promoter *in trans*?

To answer this question, we used targeted gene replacement to substitute 193 bp of the *yellow* promoter from position −63 to +130, including the TATA box and Inr but not the translation initiation codon, with 193 bp of sequence from the promoter regions of the Drosophila *eve*, *hsp70*, and *w* genes (Figure 2). The resulting alleles were called y^{eve}, y^{hsp70}, and y^{w}, and their protein-null companion alleles were called $y^{eve\text{-}1}$, $y^{hsp70\text{-}1}$, and $y^{w\text{-}1}$. The *eve* promoter most closely resembles that of *yellow* in that both have a TATA box and Inr (MACDONALD *et al.* 1986). The *hsp70* promoter, like that of *yellow* and *eve*, has a TATA box and Inr but, in addition, contains multiple heat-shock elements (PERISIC *et al.* 1989), one of which is included in the 193-bp sequence inserted into *yellow*. The *white* promoter is TATA-less, but does have an Inr and downstream promoter element sequences (KADONAGA 2002).

All three heterologous promoters gave dark pigmentation in homozygous or hemizygous flies (Table 1), indicating that they can direct *yellow* transcription. Flies bearing the y^{eve} allele were indistinguishable from wild-type flies, while flies bearing the y^{hsp70} allele were at least as dark, if not darker, than wild-type flies. In addition, y^{hsp70} flies had a dusky appearance with dark pigmentation even in abdominal interbands, which are the normally lightly pigmented regions between the dark abdominal bands. The y^{w} flies were less dark than wild-

type flies. Consistent with these observations, steady-state levels of *yellow* transcripts reached 80 and 71% of wild-type levels in y^{eve} and y^{hsp70} flies, respectively, but only 12% in y^{w} flies (Figure 3).

To verify that transcription from these heterologous promoters is enhancer driven and not constitutive, we made constructs that carried promoter replacements identical to those present in y^{eve}, y^{hsp70}, and y^{w}, but that were deleted for the wing and body enhancers, and determined the pigmentation levels that they direct when integrated into the genome by *P*-element-mediated germ-line transformation. If transcription of y^{eve}, y^{hsp70}, and y^{w} is enhancer dependent, then flies bearing these enhancerless transgenes should show mutant pigmentation. If expression of y^{eve}, y^{hsp70}, and y^{w} is constitutive, then the transgenic flies should instead show dark pigmentation. Flies carrying the *eve* and *white* enhancerless transgenes showed fully mutant wing and body pigmentation in all five and three, respectively, independent transgenic lines, indicating that the wing and body pigmentation seen in y^{eve} and y^{w} flies is in fact the result of enhancer-dependent transcription. The eight independent lines bearing the *hsp70* enhancerless transgene differed from each other, showing a range of wing and body pigmentation from fully mutant to nearly wild type. This observation may reflect position effects or might suggest that the dark pigmentation seen in y^{hsp70} flies is partially constitutive, but that to achieve consistently full pigmentation, input from an enhancer is required. Significantly, all of the transgenic lines carried an intact bristle enhancer and showed wild-type bristle pigmentation, indicating that the transgenes are capable of *yellow* expression in their ectopic locations.

We then tested the ability of y^{eve}, y^{hsp70}, and y^{w} to release the wing and body enhancers to act *in trans* by placing each of their corresponding companion alleles *in trans* to the four tester alleles and assaying complementation. None of the companion alleles complemented the tester alleles (Table 1), suggesting that *yellow* enhancers prefer to act *in cis*, even on a foreign promoter, rather than act *in trans* on a wild-type *yellow* promoter. As these heterologous promoters maintain *cis* preference of the *yellow* enhancers in spite of their different sequences and identities relative to the native *yellow* promoter, our data indicate that promoter pairing and identity are unlikely to play primary roles in enhancer choice at *yellow*.

DISCUSSION

At the outset of our studies, we considered three mechanisms that may govern how an enhancer chooses between a *cis*-linked promoter and one located *in trans*: by some aspect of promoter competency, by pairing-mediated changes in gene topology, and by promoter identity. Although our data do not rule out any model, they argue that the latter two mechanisms do not, on their own and in their simplest form, dictate *cis-trans* decisions of the wing and body enhancers of *yellow*. In contrast, our data draw attention to the transcriptional process and the integrity of the promoter.

Significantly, consideration of the alleles from the point of view of transcription shows that those supporting transvection reduce transcript levels below ~3% compared to levels in our y^{+} control flies, while those that do not support transvection maintain transcript levels above ~11% (Figure 3). The difference in transcript levels between the two groups of alleles is significant (Mann-Whitney *U*-test, $P = 0.005$) and suggests that the trancriptional competency of a promoter may need to be compromised below a certain threshold, as assayed by steady-state transcript levels, before the *cis*-linked enhancers can be released to act *in trans*.

What this hypothetical transcription-based threshold represents in biological terms is not clear. It may reflect a particular rate of transcription, the binding of a specific transcription factor, the adoption of a particular chromatin state, or another aspect of promoters that can affect the attractiveness of a promoter to an enhancer (Li and Noll 1994; Hansen and Tjian 1995; Kapoun and Kaufman 1995; Merli *et al.* 1996; Ohtsuki *et al.* 1998; Sharpe *et al.* 1998; Butler and Kadonaga 2001; Cai *et al.* 2001; Conte *et al.* 2002). Importantly, as transvection requires many events before transcription, such as homology sensing and pairing, the *cis-trans* choice of a *yellow* enhancer may occur much earlier than transcription, possibly making a threshold defined by transcript levels a poor reporter of an earlier key step. Choice may also be a dynamic process, varying from cell to cell or tissue to tissue (Golic and Golic 1996; Gubb *et al.* 1997; reviewed in Wu and Morris 1999; Kennison and Southworth 2002). Indeed, we have not yet determined whether *yellow* enhancers can alternate from moment to moment between the *cis* and *trans* promoters, perhaps interacting simultaneously with both. Finally, as has been observed at the bithorax complex (Goldsborough and Kornberg 1996; Casares *et al.* 1997; Sipos *et al.* 1998), it is formally possible that *yellow* enhancers are released to *trans* action at a threshold higher than we have observed, even when the *cis* promoter is entirely wild type and fully functional. If true for any of the noncomplementing genotypes we have tested, such interactions are nonproductive, ineffective, or too infrequent to affect pigmentation.

Interestingly, all nine alleles that maintain *cis* preference of enhancers also have an intact configuration of known core promoter elements. The insertional alleles have an intact configuration 3′ of position −44, the promoter scan alleles do not affect core elements, and the heterologous promoter alleles have foreign but otherwise intact promoter sequences. By contrast, the three alleles that do support transvection (y^{tata}, y^{inr}, and $y^{tata\text{-}inr}$) all have mutations in core promoter elements (Morris *et al.* 1999b). This observation highlights the possibility

that enhancer choice between two promoters may be influenced by the integrity of the promoter as determined by the configuration of core elements, the factors bound to them, and/or the chromatin state that they generate. That is, the nine alleles that maintain *cis* preference of their enhancers might do so because they each have an intact array of core promoter elements in spite of their differences in sequence, identity, and transcriptional profiles. This feature may be a molecular mark of a fully competent promoter in terms of transcription or, at the extreme, may reflect a promoter function independent of the transcriptional process.

A comparison of y^{inr} with y^{w} illustrates this point. The y^{inr} allele has a 6-bp mutation in the initiator (Morris *et al.* 1999b), while y^{w} has a *white* promoter substituted for the *yellow* promoter (Figure 2). Steady-state levels of *yellow* mRNA are 3% for y^{inr} and 12% for y^{w} as compared to that of our y^{+} controls and, as predicted by the threshold model, y^{inr} supports transvection while y^{w} does not. Interestingly, however, y^{w} flies show lighter wing and body pigmentation as compared to y^{inr} flies (Table 1) in spite of their higher transcript levels (Figure 3). It may be that *white* sequences in the 5′-untranslated region lead to the production of unstable transcripts, so that while transcript levels are relatively high, protein expression is not. If so, the transcription-based threshold would still apply. However, it may also be that *yellow* expression in tissues other than wing and body accounts for the discrepancy between phenotype and transcript levels of y^{w}. If so, the transcription-based threshold would not apply in this case. Instead, it may be that the enhancers of y^{inr} are released to act *in trans* because y^{inr} carries a mutated core promoter element and therefore they perceive their promoter as inadequate, while the enhancers of y^{w} are not released because they perceive the geography of their promoter as intact.

The ability of y^{w} as well as y^{eve} and y^{hsp70} to maintain *cis* preference also indicates that promoter pairing and identity are unlikely to be key determinants of *cis-trans* choice. Interestingly, at *Ultrabithorax* (*Ubx*), regulatory regions can act both *in cis* with a heterologous *P*-element promoter and *in trans* on their own promoter (Casares *et al.* 1997). This observation could reflect differences between *yellow* and *Ubx* in terms of the involvement of transposable elements, participating enhancers and promoters, or mechanisms of transvection.

In sum, our data suggest that the *cis-trans* choice of the *yellow* wing and body enhancers rests to a significant degree on the competency of the *cis*-linked promoter. Furthermore, our data call attention to two aspects of promoter competency with respect to the *cis-trans* choice: transcriptional competency and intactness of the promoter. A model of enhancer choice based on promoter function and integrity provides an explanation of why enhancers are able to detect small changes in key promoter elements but are apparently insensitive to complete replacement of the promoter.

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LITERATURE CITED

Bean, C. J., C. E. Schaner and W. G. Kelly, 2004 Meiotic pairing and imprinted X chromatin assembly in *Caenorhabditis elegans*. Nat. Genet. **36:** 100–105.

Burgess, S. M., 2002 Homologous chromosome associations and nuclear order in meiotic and mitotically dividing cells of budding yeast. Adv. Genet. **46:** 49–90.

Butler, J. E., and J. T. Kadonaga, 2001 Enhancer-promoter specificity mediated by DPE or TATA core promoter motifs. Genes Dev. **15:** 2515–2519.

Cai, H. N., Z. Zhang, J. R. Adams and P. Shen, 2001 Genomic context modulates insulator activity through promoter competition. Development **128:** 4339–4347.

Casares, F., W. Bender, J. Merriam and E. Sánchez-Herrero, 1997 Interactions of *Drosophila Ultrabithorax* regulatory regions with native and foreign promoters. Genetics **145:** 123–137.

Chen, J. L., K. L. Huisinga, M. M. Viering, S. A. Ou, C.-t. Wu *et al.*, 2002 Enhancer action in *trans* is permitted throughout the *Drosophila* genome. Proc. Natl. Acad. Sci. USA **99:** 3723–3728.

Conte, C., B. Dastugue and C. Vaury, 2002 Promoter competition as a mechanism of transcriptional interference mediated by retrotransposons. EMBO J. **14:** 3908–3916.

Dudley, A. M., L. J. Gansheroff and F. Winston, 1999 Specific components of the SAGA complex are required for Gcn4- and Gcr1-mediated activation of the his4-912Δ promoter in *Saccharomyces cerevisiae*. Genetics **151:** 1365–1378.

Duncan, I. W., 2002 Transvection effects in *Drosophila*. Annu. Rev. Genet. **36:** 521–556.

Geyer, P. K., and V. G. Corces, 1987 Separate regulatory elements are responsible for the complex pattern of tissue-specific and developmental transcription of the *yellow* locus in *Drosophila melanogaster*. Genes Dev. **1:** 996–1004.

Geyer, P. K., M. M. Green and V. G. Corces, 1990 Tissue-specific transcriptional enhancers may act in *trans* on the gene located in the homologous chromosome: the molecular basis of transvection in *Drosophila*. EMBO J. **9:** 2247–2256.

Gloor, G. B., N. A. Nassif, D. M. Johnson-Shlitz, C. R. Preston and W. R. Engels, 1991 Targeted gene replacement in *Drosophila* via P element-induced gap repair. Science **253:** 1110–1117.

Goldsborough, A. S., and T. B. Kornberg, 1996 Reduction of transcription by homologue asynapsis in *Drosophila* imaginal discs. Nature **381:** 807–810.

Golic, M. M., and K. G. Golic, 1996 A quantitative measure of the mitotic pairing of alleles in *Drosophila melanogaster* and the influence of structural heterozygosity. Genetics **143:** 385–400.

Gubb, D., J. Roote, J. Trenear, D. Coulson and M. Ashburner, 1997 Topological constraints on transvection between *white* genes within the transposing element *TE35B* in *Drosophila melanogaster*. Genetics **146:** 919–937.

Hansen, S. K., and R. Tjian, 1995 TAFs and TFIIA mediate differential utilization of the tandem Adh promoters. Cell **82:** 565–575.

Hendrickson, J. E., and S. Sakonju, 1995 *Cis* and *trans* interactions between *iab* regulatory regions and *abdominal-A* and *abdominal-B* in *Drosophila melanogaster*. Genetics **139:** 835–848.

Kadonaga, J. T., 2002 The DPE, a core promoter element for transcription by RNA polymerase II. Exp. Mol. Med. **34:** 259–264.

Kapoun, A. W., and T. C. Kaufman, 1995 A functional analysis of 5′, intronic and promoter regions of the homeotic gene *proboscipedia* in *Drosophila melanogaster*. Development **121:** 2127–2141.

Keeler, K. J., T. Dray, J. E. Penney and G. B. Gloor, 1996 Gene targeting of a plasmid-borne sequence to a double-strand DNA break in *Drosophila melanogaster*. Mol. Cell. Biol. **16:** 522–528.

Kennison, J. A., and J. W. Southworth, 2002 Transvection in *Drosophila*. Adv. Genet. **46:** 399–420.

Li, X., and M. Noll, 1994 Compatibility between enhancers and promoters determines the transcriptional specificity of *gooseberry* and *gooseberry neuro* in the *Drosophila* embryo. EMBO J. **13:** 400–406.

MacDonald, P. M., P. Ingham and G. Struhl, 1986 Isolation, structure, and expression of *even-skipped*: a second pair-rule gene of *Drosophila* containing a homeobox. Cell **47:** 721–734.

Martin, M., Y. B. Meng and W. Chia, 1989 Regulatory elements involved in the tissue-specific expression of the *yellow* gene of *Drosophila*. Mol. Gen. Genet. **218:** 118–126.

Martínez-Laborda, A., A. González-Reyes and G. Morata, 1992 *Trans* regulation in the *Ultrabithorax* gene of *Drosophila*: alterations in the promoter enhance transvection. EMBO J. **11:** 3645–3652.

Merli, C., D. E. Bergstrom, J. A. Cygan and R. K. Blackman, 1996 Promoter specificity mediates the independent regulation of neighboring genes. Genes Dev. **10:** 1260–1270.

Morris, J. R., J.-L. Chen, P. K. Geyer and C.-t. Wu, 1998 Two modes of transvection: enhancer action in *trans* and bypass of a chromatin insulator in *cis*. Proc. Natl. Acad. Sci. USA **95:** 10740–10745.

Morris, J. R., J.-l. Chen, S. T. Filandrinos, R. C. Dunn, R. Fisk *et al.*, 1999a An analysis of transvection at the *yellow* locus of *Drosophila melanogaster*. Genetics **151:** 633–651.

Morris, J. R., P. K. Geyer and C.-t. Wu, 1999b Core promoter elements can regulate transcription on a separate chromosome in *trans*. Genes Dev. **13:** 253–258.

Munté, A., 2000 Nucleotide variation at the *yellow* gene region is not reduced in *Drosophila subobscura*: a study in relation to chromosomal polymorphism. Mol. Biol. Evol. **17:** 1942–1955.

Munté, A., 2001 Changes in the recombinational environment affect divergence in the *yellow* gene of *Drosophila*. Mol. Biol. Evol. **18:** 1045–1056.

Munté, A., M. Aguadé and C. Segarra, 1997 Divergence of the *yellow* gene between *Drosophila melanogaster* and *D. subobscura*: recombination rate, codon bias and synonymous substitutions. Genetics **147:** 165–175.

Ohtsuki, S., M. Levine and H. N. Cai, 1998 Different core promoters possess distinct regulatory activities in the *Drosophila* embryo. Genes Dev. **12:** 547–556.

Perisic, O., H. Xiao and J. T. Lis, 1989 Stable binding of Drosophila heat shock factor to head-to-head and tail-to-tail repeats of a conserved 5 bp recognition unit. Cell **59:** 797–806.

Pirrotta, V., 1999 Transvection and chromosomal *trans*-interaction effects. Biochim. Biophys. Acta **1424:** M1–M8.

Rubin, G. M., and A. C. Spradling, 1982 Genetic transformation of *Drosophila* with transposable element vectors. Science **218:** 348–353.

Savitsky, M., T. Kahn, E. Pomerantseva and P. Georgiev, 2003 Transvection at the end of the truncated chromosome in *Drosophila melanogaster*. Genetics **163:** 1375–1387.

Sharpe, J., S. Nonchev, A. Gould, J. Whiting and R. Krumlauf, 1998 Selectivity, sharing and competitive interactions in the regulation of *Hoxb* genes. EMBO J. **17:** 1788–1798.

Sipos, L., J. Mihaly, F. Karch, P. Schedl, J. Gausz *et al.*, 1998 Transvection in the *Drosophila Abd-B* domain: extensive upstream sequences are involved in anchoring distant *cis*-regulatory regions to the promoter. Genetics **149:** 1031–1050.

Smale, S. T., and J. T. Kadonaga, 2003 The RNA polymerase II core promoter. Annu. Rev. Biochem. **72:** 449–479.

Wittkopp, P. J., K. Vaccaro and S. B. Carroll, 2002 Evolution of *yellow* gene regulation and pigmentation in *Drosophila*. Curr. Biol. **12:** 1547–1556.

Wray, G. A., M. W. Hahn, E. Abouheif, J. P. Balhoff, M. Pizer *et al.*, 2003 The evolution of transcriptional regulation in eukaryotes. Mol. Biol. Evol. **20:** 1377–1419.

Wu, C.-t., and J. R. Morris, 1999 Transvection and other homology effects. Curr. Opin. Genet. Dev. **9:** 237–246.

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