Core promoter elements can regulate transcription on a separate chromosome in trans

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Transvection can cause the expression of a gene to be sensitive to the proximity of a homolog. It can account for many cases of intragenic complementation at the Drosophila yellow gene, where one mode of transvection involves the action of enhancers in trans on a promoter present on a separate chromosome. Our goal was to identify cis-acting elements that regulate the trans action of enhancers. Using gene replacement, we altered two core promoter elements at yellow and tested the resulting alleles for their ability to support transvection. We found that the TATA box and initiator element can regulate transvection.

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Homologous sequences can influence each other in profound ways, resulting in changes in gene structure and expression (for review, see Henikoff and Comai 1998). A good example of homolog interaction is transvection, which was defined in Drosophila [Lewis 1954] and is now known to occur elsewhere (for review, see Henikoff and Comai 1998). In Drosophila, somatic homolog pairing brings homologous sequences into proximity. The expression of some genes is sensitive to somatic pairing and these genes are said to exhibit transvection effects. One example of such a gene is yellow (Geyer et al. 1990). The yellow gene is required for cuticle pigmentation and is controlled by several independent tissue-specific enhancers. Mutations in yellow result in decreased pigmentation. Interestingly, some mutant yellow alleles that reduce pigmentation in the same cuticular structures are able to complement each other in pairwise combinations to give a nearly wild-type phenotype. This form of intragenic complementation can be explained by transvection.

One mode of transvection at yellow entails the action of enhancers in trans on the promoter of a paired homolog (Geyer et al. 1990; Morris et al. 1998). Figure 1A illustrates this mechanism with the y2 and y186 alleles (Geyer et al. 1990). The y2 allele results from the insertion of a gypsy retrotransposable element between the promoter and the two enhancers directing expression in the wings and body. When gypsy is bound by the Suppressor of Hairy wing [Su(Hw)] protein, it establishes a chromatin insulator (for review, see Dorsett 1996; Gdula et al. 1996; Geyer 1997) that prevents the wing and body enhancers from communicating with the promoter, and therefore, y2 flies have mutant wing and body pigmentation. The y186 allele is a null that is associated with a 780-bp deletion removing the promoter. Although homozygous y2 and y186 flies show mutant wing and body pigmentation, y2/y186 flies show almost wild-type pigmentation. This intragenic complementation can be explained by the action of the wing and body enhancers of y186 in trans on the y2 promoter (Geyer et al. 1990).

Although the wing and body enhancers of some yellow alleles are able to act in trans on the y2 promoter, the enhancers of other yellow alleles cannot. For example, the y2 allele, which is caused by an A to C mutation in the translation initiation codon, has intact wing and body enhancers, but fails to complement y2 (Fig. 1B; Geyer et al. 1990). This observation suggests that the action of enhancers in trans on a paired promoter is not always possible, that it occurs only under certain circumstances. In this way, transvection is a regulated process at yellow.

We are interested in the molecular basis for the regulation of transvection. Previous studies at yellow delineated a large region encompassing the promoter as a key player in that its presence appeared to inhibit transvection (Geyer et al. 1990; Morris et al. 1999). For example, y186, which lacks this region, can participate in transvection, while y1, which carries this region, cannot. The importance of the promoter region is also highlighted by findings at the Ultrabithorax (Ubx) (Martínez-Laborda et al. 1992; Casares et al. 1997) and Abdominal-B (Hendrickson and Sakonju 1995; Sipos et al. 1998) genes. Our

Figure 1. A model to explain complementation patterns at yellow (Geyer et al. 1990). (A) The y186 allele complements y2 to give flies with nearly wild-type pigmentation. The model suggests that the wing and body enhancers of y186 act in trans on the promoter of the y2 allele. (B) The y2 allele does not complement y1. The intact promoter region of y2 may preclude the action of enhancers in trans. (W) Wing enhancer; (B) body enhancer; (Br) bristle enhancer; (T) tarsal claw enhancer; (solid rectangle) exon; (open rectangle) gypsy long-terminal repeat (LTR); (large arrow above exon) transcription from yellow promoter; (small arrow above LTR) transcription of gypsy; (●) su(Hw)-binding region. There are 17 bp of P-element sequence at the breakpoints of y186 (Geyer et al. 1990).
goal has been to identify the elements that regulate transvection at yellow. Here, we present our studies that were prompted by the presence of the promoter within the delineated region. It has been proposed that cis-preference of enhancers for their own promoter precludes their action in trans (Geyer et al. 1990; Martínez-Laborda et al. 1992; Hendrickson and Sakonju 1995; Casares et al. 1997; Sipos et al. 1998; Morris et al. 1999). Our strategy involved the introduction of mutations into putative core promoter elements and assessment of the resulting yellow alleles for their ability to support transvection.

Results and Discussion

We focused first on the putative TATA box of yellow, extending from −32 to −24 relative to the transcription start site (Chia et al. 1986; Geyer et al. 1986). Our method of mutagenesis was targeted gene replacement at the endogenous locus. This approach does not compromise the ability of yellow alleles to pair and is required because ectopically located yellow transgenes have not been observed to pair and promote transvection with the endogenous yellow gene (Geyer et al. 1990).

Targeted gene replacement in Drosophila involves the excision of a marked P element followed by gap repair from a template (Fig. 2, Keeler et al. 1996). To generate a marked P element in yellow, we began with an allele, yh12, that is associated with an unmarked P element at +4 relative to the transcription initiation site (Geyer et al. 1990). We then used targeted transposition (e.g., see Heslip and Hodgetts 1994) to substitute the internal sequences of the yh12 P element with sequences containing the white marker gene (Fig. 2A). The resulting yellow allele, called yh12ws, became our target for the introduction of sequence changes from a plasmid template (Fig. 2B). Using this strategy, we replaced 6 bp of the wild-type TATA box with the 6-bp recognition site for the Smal restriction enzyme (Table 1). Introduction of the Smal site allowed us to use restriction analysis of PCR-amplified fragments in the identification of gene converts. Changes were subsequently confirmed by sequencing, and the integrity of the yellow locus was ascertained by Southern analysis. The new yellow allele with a mutant TATA box is called yata (Table 1).

Homozygous yata/yata and hemizygous yata/Df flies showed a reduction in the pigmentation of all adult cuticular structures (Table 2). For example, the wings showed intermediate pigmentation, the body showed low-level pigmentation, and the bristles showed fully mutant pigmentation. These observations demonstrate that yata compromises transcription, and therefore, that the putative TATA box is functional.

We placed yata in trans to y2 and discovered that yata can support transvection. The wings and body of y2/yata flies were darker than the wings and body of flies homozygous or hemizygous for either y2 or yata (Table 2). This finding has two implications. First, yata identifies 6 bp that, when mutant, allow transvection to proceed, and represents the most precise definition, to date, of an element that acts in cis to determine whether transvection can occur. Second, the 6 bp implicate a promoter element in the regulation of transvection.

Although the model for transvection shown in Figure 1 predicts that complementation between y2 and yata results from the yata enhancers activating transcription from the y2 promoter, it is formally possible that complementation arises from pairing-mediated activation of the yata promoter. For instance, pairing-mediated activation of transcription has been reported for Ubx
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Table 1. Targeted gene replacement at yellow

| Allele  | TATA | Inr | ATG | Number |
|---------|------|-----|-----|--------|
| y\textit{\textsuperscript{ata}} | m   | +   | +   | 300    |
| y\textit{\textsuperscript{inr}} | m   | +   | +   | 600    |
| y\textit{\textsuperscript{ata-inr}} | m   | m   | m   | 600    |
| y\textit{\textsuperscript{ata-1}} | m   | m   | m   | 550    |
| y\textit{\textsuperscript{inr-1}} | m   | m   | m   | 600    |
| y\textit{\textsuperscript{ata-inr-1}} | m   | m   | m   | 900    |

A. Alleles generated by targeted gene replacement

Motif

Allele | TATA | Inr | ATG | embryos | males | convertants
|-------|------|-----|-----|---------|-------|----------------|
| y\textit{\textsuperscript{ata}} | m   | +   | +   | 300    | 51    | 2
| y\textit{\textsuperscript{inr}} | +   | m   | +   | 600    | 96    | 5
| y\textit{\textsuperscript{ata-inr}} | m   | m   | m   | 600    | 104   | 5
| y\textit{\textsuperscript{ata-1}} | m   | m   | m   | 550    | 112   | 5
| y\textit{\textsuperscript{inr-1}} | +   | m   | m   | 600    | 92    | 8
| y\textit{\textsuperscript{ata-inr-1}} | m   | m   | m   | 900    | 102   | 10

B. Consensus and mutant sequences

| TATA box | Inr | ATG |
|----------|-----|-----|
| C       | T   |     |
| A       | G   | T   |
| G       | T   | T   |
| A       | GG  |     |
| T       |     |     |
| A       | ATCGTCG | ATG |
| T       |     |     |
| A       | ACCGTCG | ATG |
| T       |     |     |
| A       | ACATCGAT | GTGCACTG |
| T       |     |     |

| Smal | Clal | Alw441 |
|------|------|--------|
| G    | T    |        |
| T    | G    |        |
| T    | T    |        |

The TATA box, Inr, and ATG translation initiation codon were mutated, (+) wild-type; (m) mutant.

The data regarding the TATA box suggest that core promoter elements can participate in the regulation of transvection at yellow. To pursue this idea, we determined the role of another potential yellow core promoter element, the putative initiator [Inr] that encompasses the transcription start site [Arkhipova 1995; Emami et al. 1997]. We replaced 6 bp of the putative Inr with the 6 bp recognition site for the Clal restriction enzyme and called the new allele y\textit{\textsuperscript{inr}} [Table 1]. In contrast to the mutation in the TATA box, mutation of the Inr caused only a slight reduction in yellow expression as judged by pigmentation levels [Table 2]. Homozygous y\textit{\textsuperscript{ata}} / y\textit{\textsuperscript{inr}} flies showed wild-type wing, body, and bristle pigmentation, while hemizygous y\textit{\textsuperscript{ata}} / y\textit{\textsuperscript{inr}} flies showed only a slight reduction in body pigmentation as compared to pigmentation levels seen in y\textit{\textsuperscript{inr}} / y\textit{\textsuperscript{inr}} flies.

To determine the effect of the Inr mutation on transvection, we made a companion allele, as was done for y\textit{\textsuperscript{ata}}. This allele, y\textit{\textsuperscript{inr-1}}, carried an A → C change in the translation initiation codon in addition to the changes in the Inr and produced a fully mutant pigmentation phenotype. We placed y\textit{\textsuperscript{inr-1}} in trans to the four tester alleles and found that it supports complementation (Table 2). The level of complementation was similar to that seen with y\textit{\textsuperscript{ata-1}}.

Finally, we made two additional alleles, y\textit{\textsuperscript{ata-inr}} and y\textit{\textsuperscript{ata-inr-1}}, in which both the TATA box and Inr had been mutated and where y\textit{\textsuperscript{ata-inr-1}} also carried a mutated translation initiation codon (Table 1). The doubly mutant y\textit{\textsuperscript{ata-inr}} / y\textit{\textsuperscript{ata-inr}} flies showed a reduction in wing, body, and bristle pigmentation that was more severe than that seen in flies homozygous for either y\textit{\textsuperscript{ata}} or y\textit{\textsuperscript{inr}} [Table 2]. These phenotypes are consistent with studies showing functional synergy between the TATA box and Inr [Emami et al. 1997]. We placed y\textit{\textsuperscript{ata-inr}} in trans to all four tester alleles and observed complementation in each case. The level of complementation was greater than levels seen with either y\textit{\textsuperscript{ata-1}} or y\textit{\textsuperscript{inr-1}} [Table 2].

In short, our data show that mutations in the TATA box and Inr induce transvection and that they do so by activating gene expression from a promoter in trans. How might promoter mutations in one allele induce expression from the promoter of a paired allele? One attractive explanation proposes that y\textit{\textsuperscript{ata}} and y\textit{\textsuperscript{inr}} influence transvection as a direct consequence of their effects on the transcriptional process. As noted earlier, transvection may be regulated by cis preference of enhancers for
If so, y\textsuperscript{tata} and y\textsuperscript{inr} may induce transvection because disruption of transcription leads to the release of enhancers from cis preference. In this way, our observations parallel studies addressing the mechanism by which enhancers select among multiple cis-linked promoters (Blackwood and Kadonaga 1998) and indicate that the parameters governing promoter choice in cis may also apply in trans. Specifically, the commitment of an enhancer for a particular promoter may reflect promoter strength, accessibility (e.g., see Geyer et al. 1990; Martinez-Laborda et al. 1992, Cai and Levine 1995; Hendrickson and Sakonju 1995; Scott and Geyer 1995; Casares et al. 1997; Dilllon et al. 1997; Krebs and Dunaway 1998; Sharpe et al. 1998; Sipos et al. 1998; Tilghman et al. 1998, Morris et al.1999) and/or identity as determined by sequence or associated factors (e.g., see Hirschman et al. 1988; Li and Noll 1994; Hansen and Tjian 1995; Merli et al. 1996; Ohtsuki et al. 1998; Sharpe et al. 1998). Likewise, at yellow, mutations in core promoter elements may release cis-acting enhancers to act on a promoter in trans because they compromise promoter strength through reduction in transcriptional efficiency, decrease promoter accessibility through alterations in chromatin structure or DNA topology, or change promoter identity through disruption of the TATA box or Inr. The consequence of such events could be that the intact promoter in trans becomes more competitive for the input of the enhancers in cis to the promoter mutations. The weakening of a promoter such that it cannot retain transcription factors could also lead to an increased concentration of transcription factors at a paired and functional second promoter, again, causing enhancers to be drawn to the second promoter.

If promoter choice reflects relative promoter appeal, how do yellow enhancers act when paired promoters are identical? The inability of y\textsuperscript{1} to complement the four tester alleles is informative. As the promoters of the tester alleles are intact and, at this level, identical to the promoter of y\textsuperscript{1}, lack of complementation indicates that cis preference dominates. It may be that mechanisms of enhancer–promoter interaction [Blackwood and Kadonaga 1998] favor interactions between cis-linked, over unlinked, elements. We note, however, one caveat. The lesions of the tester alleles, while outside the promoter region, may compromise the promoters of the tester alleles such that they are unable to compete effectively with the promoter of y\textsuperscript{1}. That is, cis preference of y\textsuperscript{1} enhancers may be accentuated by a reduced potential of the promoter in trans.

How, then, might yellow enhancers act when the paired alleles are identical throughout, such as in the case of paired wild-type genes? It is plausible that cis action will prevail. Again, arguments of linkage may favor cis action. Trans action might also be inhibited because a wild-type promoter that is fully engaged with its cis enhancers might not be able to receive input in trans [see also Sipos et al. 1998], although this does not appear to be the case at Ubx [Martinez-Laborda et al. 1992]. It is equally plausible that when faced with identical promoters, enhancers will be free to act in cis or trans, as has been demonstrated with catenated plasmids in a Xenopus oocyte system [Krebs and Dunaway 1998]. While our data do not address the situation where genes are identical, they do suggest that enhancers that are able to act in trans may still be able to act in cis, consistent with the behavior of regulatory elements at the bithorax gene complex (Lewis 1955; and most recently, Goldbrough and Kornberg 1996; Casares et al. 1997; Sipos et al. 1998).

In the case of yellow, the pigmentation seen in flies that bear a tester allele in trans to y\textsuperscript{tata} or y\textsuperscript{inr} is darker than that of flies bearing the tester allele in cis or trans, as has been demonstrated with catenated plasmids in a Xenopus oocyte system [Krebs and Dunaway 1998]. While our data do not address the situation where genes are identical, they do suggest that enhancers that are able to act in trans may still be able to act in cis, consistent with the behavior of regulatory elements at the bithorax gene complex (Lewis 1955; and most recently, Goldbrough and Kornberg 1996; Casares et al. 1997; Sipos et al. 1998).

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### Table 2. Complementation data

|     | Df Homozygous | 2 | 62a | 82f29 | 2374 |
|-----|---------------|---|-----|-------|------|
| Df  | 1, 1          | 1, 1| 1, 1| 1, 1  | 1, 1 |
| Homozygous | — — | 1, 1–2| 1, 3| 1, 1a | 1, 1–2 |
| tata | 2–3, 1        | 3, 2| 4, 3| 4, 4  | 3, 1–2| 4, 3 |
| inr  | 5, 4          | 5, 5| 5, 5| 5, 5  | 5, 5 |
| tata–inr | 2, 1         | 2, 1| 4, 3–4| 4, 4  | 3, 1–2 | 4, 3 |
| tata–1 | 1, 1         | 1, 1| 3, 2–3| 2, 4  | 2, 1–2 | 3, 2–3 |
| inr–1 | 1, 1          | 1, 1| 3, 2–3| 2, 4  | 2, 1–2 | 3, 2–3 |
| tata–inr–1 | 1, 1       | 1, 1| 4, 3–4| 3, 4  | 3, 1–2 | 4, 3 |
| 188  | 1, 1          | 1, 1| 4, 4| 4, 4  | 3, 3 |

Pigmentation scores for wings and body (wings, body) when alleles are in trans to a deficiency (Df), homozygous, or in trans to one of the alleles listed at the top. Scores in boldface type indicate complementation. The y\textsuperscript{1} allele behaved like Df in complementation tests involving each promoter mutation, as did y\textsuperscript{tata} in trans to y\textsuperscript{tata–1}, y\textsuperscript{inr–1}, and y\textsuperscript{tata–inr–1}. Flies bearing y\textsuperscript{tata} in trans to y\textsuperscript{tata}, y\textsuperscript{inr}, or y\textsuperscript{tata–inr} showed slightly darker pigmentation compared to flies bearing Df in trans to these three promoter mutations, suggesting that these altered promoters can receive enhancer input in trans.

aThe scores given were all obtained in the laboratory of C.-t.W. We note that the parameters governing promoter choice in cis may also apply in trans.

The Pigmentation scores for wings and body (wings, body) when alleles are in trans to a deficiency (Df), homozygous, or in trans to one of the alleles listed at the top. Scores in boldface type indicate complementation. The y\textsuperscript{1} allele behaved like Df in complementation tests involving each promoter mutation, as did y\textsuperscript{tata} in trans to y\textsuperscript{tata–1}, y\textsuperscript{inr–1}, and y\textsuperscript{tata–inr–1}. Flies bearing y\textsuperscript{tata} in trans to y\textsuperscript{tata}, y\textsuperscript{inr}, or y\textsuperscript{tata–inr} showed slightly darker pigmentation compared to flies bearing Df in trans to these three promoter mutations, suggesting that these altered promoters can receive enhancer input in trans. The scores given were all obtained in the laboratory of P.K.G. The difference may be due to culture condition.
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promoter elements. Nevertheless, we are considering the possibility that promoter elements also influence transvection via routes that are independent of the transcriptional process. For instance, transvection might normally be inhibited by the packaging of homologous chromosomes in a way that favors intramolecular over intermolecular interactions [Martínez-Laborda et al. 1992, Wu and Howe 1995, Sipos et al. 1998]. If so, small sequence changes, such as y\textsuperscript{tata} and y\textsuperscript{inr}, might alter relative homolog accessibilities or, if sequences within a wild-type promoter are important for homolog sequestration, y\textsuperscript{tata} and y\textsuperscript{inr} might act by disrupting the insular state. Alternatively, the changes in y\textsuperscript{tata} and y\textsuperscript{inr} may influence a promoter in trans by virtue of their effect on pairing. For example, if sequences in, or factors associated with, the promoter region are essential for homolog pairing [Jorgensen 1990, Cook 1997, McKee 1998], y\textsuperscript{tata} and y\textsuperscript{inr} may lead to local unpairing of the promoter in trans, resulting in its greater accessibility and ultimate activation. Intriguingly, this structural interpretation can explain why y\textsuperscript{tata} and y\textsuperscript{inr} support transvection equally; the magnitude of their lesions is the same. Also consistent with this view are studies suggesting that yellow alleles bearing deletions or insertions in the promoter region induce transvection by causing pairing-mediated changes in gene topology [Morris et al. 1998, 1999, Sipos et al. 1998]. In fact, in genotypes involving y\textsuperscript{p}, such changes may be responsible for a second mode of transvection in which the y\textsuperscript{p} wing and body enhancers bypass the gypsy insulator to activate the y\textsuperscript{p} promoter [Morris et al. 1998]. If y\textsuperscript{tata} and y\textsuperscript{inr} can cause this mode of transvection, then insulator bypass may contribute to the complementation observed when these alleles are paired with y\textsuperscript{p}.

Finally, we note that none of our promoter mutations achieves the uniformly high level of transvection that is seen with y\textsuperscript{198} (Table 2). It may be that stronger degrees of transvection require maximal disruption of transcription and/or severe structural disruption of the promoter, both of which characterize y\textsuperscript{198}. For example, yellow may carry promoter elements, in addition to the TATA box and Inr, whose presence inhibits transvection. Alternatively, there may be as-yet-unidentified elements present in our mutant promoter alleles, but absent from y\textsuperscript{198}, that are independent of promoter function but which down-regulate transvection. In fact, there is evidence for the ability of special elements, in particular insulators, to confer cis preference on enhancer–promoter interactions in plasmid-based assays [Krebs and Dunaway 1998] and for the existence of nonpromoter elements regulating transvection at Abdominal-B [Hendrickson and Saksouju 1995, Hopmann et al. 1995, Sipos et al. 1998].

In summary, our data demonstrate that the TATA and Inr promoter elements play key roles in the regulation of transvection at yellow. These results are particularly exciting in light of suggestions that the promoter may participate in other pairing-mediated processes [Jorgensen 1990, Lichten and Goldman 1995; Cook 1997, McKee 1998]. Specifically, promoter elements have been implicated in meiotic recombination in yeast [Lichten and Goldman 1995] and in meiotic pairing and segregation of the X and Y chromosomes in Drosophila [McKee 1998]. The importance of promoter elements in these meiotic events, as well as in transvection-modulated gene expression in nonmeiotic tissues, argues strongly that transcriptional regulatory elements and pairing-mediated processes, while distinct, are intimately linked.

Materials and methods

Drosophila stocks

Df represents the Df(1)\textsuperscript{ac} w\textsuperscript{118} chromosome, which lacks the entire yellow gene [Morris et al. 1998]. The w\textsuperscript{118} marker is a null allele of the white eye color gene, and the C\textsubscript{y} wing and S\textsubscript{b} bristle mutations are dominant markers on the Cy\textsubscript{O} second chromosome balancer and TMS third chromosome balancer, respectively [Lindsley and Zimm 1992]. For simplicity, Figure 2 notes the dominant markers but does not give the names of the balancer chromosomes. The attached X chromosome we used was FMA43 [Lindsley and Zimm 1992]. P[y\textsuperscript{w}] is a transposon carrying a wild-type w\textsuperscript{+} gene, and P[y\textsuperscript{w} Δ3·3988], abbreviated Δ3·3, is a transposon encoding transposase (Keeler et al. 1996 and references within).

Culture condition

Flies were cultured at 25 ± 1°C on standard Drosophila cornmeal, yeast, sugar, and agar medium with p-hydroxybenzoic acid methyl ester added as a mold inhibitor. Three females were mated with three or more males in vials and brooded daily. Temperature and crowding were carefully controlled as both affect pigmentation.

Scoring of pigmentation

Pigmentation was scored in 1- to 3-day-old flies on a scale of 1–5, where 1 represents the null or nearly null state, and 5 represents the wild-type or nearly wild-type state. The null phenotype is defined by the pigmentation seen in flies that are homozygous or hemizygous for y\textsuperscript{1} or Df(y\textsuperscript{1}y\textsuperscript{1+}), and the wild-type phenotype is defined by the pigmentation seen in our wild-type Canton S strain. The wing pigmentation score refers to pigmentation of the wing blade but not the wing bristles, and body pigmentation refers to pigmentation of the abdominal stripes. At least two independent crosses were set up for each genotype. Complementation between two alleles was judged to occur when wing or body pigmentation was at least one point darker on the pigmentation scale than the pigmentation of analogous tissues in females that were homozygous for either allele and Df.

Template construction

The templates used for gene replacement were full-length yellow genes, carrying appropriate sequence changes, cloned into a Bluescript plasmid [Stratagene]. The following constructs are named according to the yellow allele that they were used to generate. pUC8\textsuperscript{ySB} is a pUC8 plasmid containing the 5‘–3.1-kb Sal–BamHI yellow fragment, pUC8\textsuperscript{ySBtata} is a Bluescript plasmid containing the 3‘–4.7-kb Bam–BglII yellow fragment, and pUC8\textsuperscript{ySBinr} is a pUC8 plasmid containing the entire yellow gene in a 7.8-kb Sal–BglII fragment. For the tata construct, primers 3 and 5 (see below) were used with pUC8\textsuperscript{ySB} to PCR amplify a product that was digested with KpnI and EagI and then cloned into the KpnI and EagI sites of pUC8\textsuperscript{ySB} to make pUC8ySBtata. The Sal–BamHI yellow fragment of pUC8\textsuperscript{ySBtata} was then cloned into the Sal and BamHI sites of pBS\textsuperscript{ySB} to generate a full-length yellow gene. For the remaining constructs, the following strategy was followed. Primers were used to PCR amplify regions of pUC8\textsuperscript{ySB}, and the products were digested with EagI and BamHI and cloned into those sites of either pUC8\textsuperscript{ySBtata} or pUC8\textsuperscript{ySB}. The resulting plasmids were digested with Sal and BamHI and the yellow sequence cloned into those sites of pBS\textsuperscript{ySB}. For the tata–1 construct, primers 3 and 6 were used, and the digested PCR product was cloned into pUC8\textsuperscript{ySBtata}. For the inr and tata–inr constructs, primers 7 and 8 were used, and the digested PCR products were cloned into pUC8\textsuperscript{ySB} and pUC8\textsuperscript{ySBtata}, respectively. For the inr–1 and tata–inr constructs, primers 7 and 6 were used, and the digested PCR products were cloned into pUC8\textsuperscript{ySBtata} and pUC8\textsuperscript{ySB}, respectively.
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Primer sequences

The primer sequences used in this study are indicated below. Mutant nucleotides are noted in boldface type and the position of the 5' base is noted in parentheses after the primer number: 1 (-448) 5'-GAGCTTCTCGGCTTTCAAATTTAC 2 (71) 5'-GTTCCTTCTGTACGCTTCCGTGTCAG 3 (547) 5'-GAGCTGCGGCTGGAAGAAAATGGAACACAG 4 (426) 5'-ATTTAAGCCCTTACGACATCAATCGCAC 5 (-7) 5'-CTTCTATATGGCGGCTGTTGGGGTCGAAAGTTTCTCGGAGACGGCAAGACAGACG 6 (+206) 5'-CTGCAAGATTGCACCCCTTTTCTCGTTGGAACAGTCACGTATTAATGCAATATCGCCCACTACATGATTACCCCGCAGTCTGACCTAGCTCTAAGCTGACAATCAC 7 (-30) 5'-ATACAGGGAGGCGCGACATATTATGGCCACCTGGCGGTTGAAGGTTTCTCCGAATACGTGCAAATCTGCTACCTGACTA 8 (-16) 5'-TCTGATGCCCTAAGTACCTGTTATGCTCAC

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