Transcription of the juvenile hormone esterase gene under the control of both an initiator and AT-rich motif

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The binding of transcription factors to the core promoter of the juvenile hormone esterase gene was functionally characterized using both a cell-free *in vitro* transcription functional assay and a cell transfection assay. A core JHE promoter (−61 to +28 bp relative to transcription start site) supported faithful transcription from the *in vivo* transcription start site. The nuclear extracts from the Sf9 insect cell line that provided transcription from that template also bound to that template as a probe in gel-mobility shift assays. Deletion or transversion of the initiator-binding motif (−1 to +4 bp) abolished detectable transcription either *in vitro* or in transfected cells. An AT-rich motif (ATATAT; −28 to −23 bp) serves another transcription factor-binding site. Mutation of the AT-rich motif to a canonical TATA-box preserved transcription, while either its deletion or complete transversion abolished or significantly reduced detectable transcriptional activity. These results indicate that, under these conditions, the functional operation of this core promoter approaches that of a composite promoter in which both the TATA- and initiator-binding protein complexes are necessary, even for basal transcription. On the other hand, these debilitating mutations to either the TATA box or initiator motif did not prevent the ability of the corresponding gel-shift competitive probes to compete with the wild-type promoter for binding by the transcription factors. Even a double transversion of both the AT-rich motif and the initiator-binding motif was able to competitively displace the protein complex that bound to the labelled wild-type probe. These data strongly indicate the presence of (an) additional core-promoter-associated transcription factor(s) (that is not the 'downstream element') that contact(s) the AT-binding complex and/or initiator-binding factor with sufficient avidity to remove them from binding to the competing wild-type promoter sequence.

INTRODUCTION

The transcription of class II eukaryotic genes typically requires the properly choreographed binding of transcription factors to at least a core promoter. In 'classical' cases the TATA-box-binding protein (TBP) binds to a TATAAA (or similar) motif, thereby bringing into association with the promoter a number of TBP-associated factors (TAFIIs) that, together with TBP, constitute the TFIID complex. These TAFIIs may themselves touch the promoter DNA [1] and/or interact with other DNA-binding transcriptional regulators, including hormone receptors (e.g. TAFII28 and RXR; [2]). In a number of cases there also appears to be specific binding by factors other than TBP to an 'initiator' motif downstream from the TBP-binding site [3–12]. There is increasing evidence that particular DNA sequences in the core promoter, other than the TBP- and initiator-binding motifs, play a pivotal role in controlling whether a particular TBP/TAFII complex [13] or other non-TFIID factor (e.g. see [14]) binds to the given core promoter. In some cases, hormone receptors may in fact bind at either the immediately proximal side of the TATA box or over the initiator site, sterically blocking transcription complexes from binding to the promoter [15–18]. The regulation of transcription of either hormone-sensitive or other genes is considered to be to a large extent determined by its character as a TATA-box-dominated or initiator-dominated promoter, since most eukaryotic genes appear to be sufficed by the presence or action of only one of these two elements. In contrast, some viral promoters appear to be true 'composite promoters', dependent on the participation of both a TATA box and an initiator [19].

Transcription of the juvenile hormone (JH) esterase gene is rapidly induced by JH during the metamorphic developmental transition of insects from the juvenile to the pupal form [20]. The gene has been isolated and structurally characterized, and a core promoter functionally identified to reside within bp −61 to +28, relative to the transcription start site [21]. The core promoter contains a consensus arthropod transcription initiator element at −1 to +4 bp [22], and an AT-rich motif 28 bases upstream of the transcription start site [21]. However, the functional contributions of these motifs to transcriptional activation have not been determined. We demonstrate here that the promoter of this temporally, spacially and hormonally controlled gene is dependent on both a functional initiator motif and the ATATAT motif.

MATERIALS AND METHODS

Sf9 cell line

Sf9 cells were maintained as monolayers at 28 °C in Grace's supplemented media (Gibco BRL, Gaithersburg, MD, U.S.A.) with 10% (v/v) fetal bovine serum (FBS).

Sf9 nuclear extracts

Approx. 10^9 cells were collected by centrifugation, and processed as follows at 4 °C. The cells were washed in 10 mM Tris/HCl (pH 8.0)/10 mM NaCl/3 mM $MgCl_2$/0.5 mM dithiothreitol (DTT), and then gently pelleted and resuspended in 9 ml of 10 mM Tris/HCl (pH 8.0)/1 mM EDTA/2 mM DTT for 20 min on ice. The cells were then homogenized with 2 strokes of a loose

Abbreviations used: TBP, TATA-box-binding protein; TAFIIs, TBP-associated factors; DTT, dothiothreitol; JH, juvenile hormone; FBS, fetal bovine serum.

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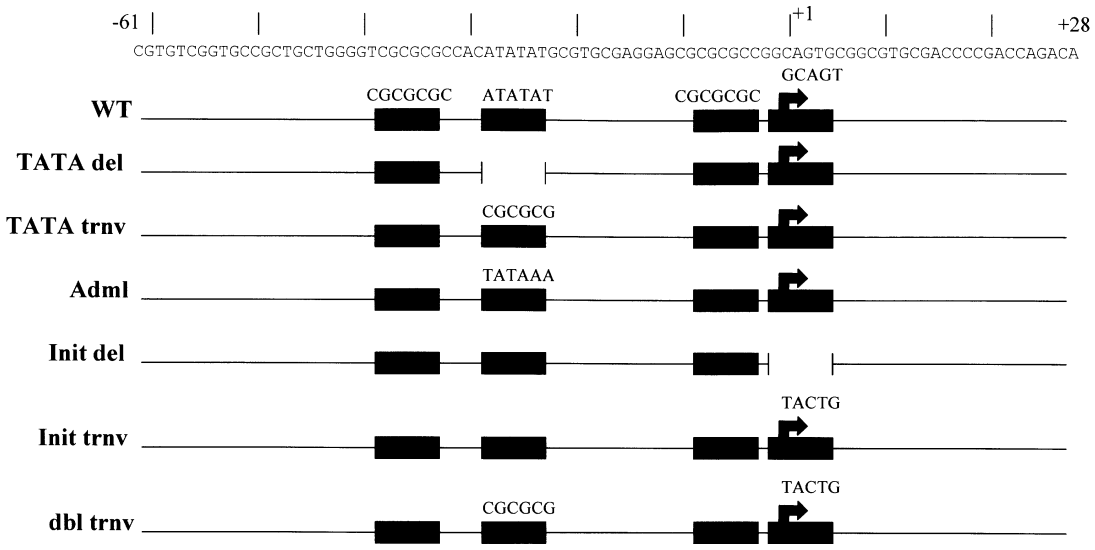

Figure 1 Wild-type and mutant minimal JH esterase promoter constructs developed in the pGL-2 reporter vector

These constructs were used as templates for *in vitro* transcription reactions, as well as unlabelled competitors against the radiolabelled wild-type probe in gel-shift assays. Above the schematic representations is the base sequence of the wild-type (WT) core promoter. Various mutations to the ATATAT motif are deletion of the motif (TATA del), transversion of each base of the motif (TATA trnv) and swapping for it the adenovirus major-late promoter TATA box (Adml). Various mutations to the transcription initiator motif include deletion of the motif (Init del), transversion of each base of the motif (Init trnv) and the double mutation of transversion of both the ATATAT motif and the initiator motif (dbl trnv).

A-type pestle dounce homogenizer, and the cell debris was then gently pelleted and resuspended in 10 vol. of 40 mM Tris/HCl (pH 8.0)/5 mM $MgCl_2$/12.5% (w/v) sucrose/25% (v/v) glycerol/3 mM DTT. The suspension was quickly homogenized with 15 strokes of a tight B-type pestle Dounce homogenizer. The preparation was slowly stirred on ice and 0.1 vol. of saturated $(NH_4)_2SO_4$ then added. After 30 min incubation on ice, the preparation was centrifuged for 3 h at 175000 ***g*** (46000 r.p.m.) using a Ty75 Ti rotor, and 330 mg of $(NH_4)_2SO_4$ was added to the supernatant. After 30 min incubation, the mixture was centrifuged in an SS34 rotor at 7600 ***g*** (8000 r.p.m.) for 20 min. The pellet was resuspended in 1 ml of 20 mM Hepes (pH 7.9)/100 mM KCl/12.5 mM $MgCl_2$/0.1 mM EDTA/17% (v/v) glycerol/2 mM DTT and dialysed (6000–8000 molecular-mass cut-off) against 150 ml of the same buffer for 8–12 h. Finally, the preparation was centrifuged for 1 h at 150000 ***g*** (40000 r.p.m.) in a Ty75 Ti rotor, and the collected supernatant stored at −70 °C until use.

In vitro transcription reactions

Reaction contents in 12.5 μl were as follows: 5 μl of Sf9 nuclear extract, 400 μM rNTPs, 4 mM creatine phosphate, 500 ng of DNA template and, when included, 0.1 μg/ml α-amanitin. Reactions were performed at 30 °C for 1 h, and then 2 μl were taken for primer-extension reaction using a ^{32}P-labelled, antisense oligonucleotide complimentary to the reporter luciferase cDNA transcription product. The transcription reaction products were preannealed to the primer at 58 °C for 20 min, and then at room temperature for 5 min in 500 μM dNTPs/10 mM $MgCl_2$/1 mM DTT/250 mM Tris/HCl (pH 8.3)/200 mM KCl/2.5 mM EDTA. The primer reaction was initiated by addition of 15 units of reverse transcriptase, and incubated at 37 °C for 30 min. The reaction was terminated with 80% (v/v) formamide and heating to 85 °C for 2 min, and the products then loaded on to a urea 7% polyacrylamide gel. Labelled extension products were revealed by autoradiography.

JH esterase core promoter constructs

The wild-type core promoter (−61 to +28) was obtained from a genomic clone of the lepidopteran insect *Trichoplusia ni* as described previously [21]. Mutations in the core promoter sequence were prepared by annealing appropriate synthetic oligonucleotides encoding the entire mutant core promoter with overhanging *Kpn*I and *Bgl*II sticky ends, and ligating the annealing products into corresponding sites of the pGL3-basic vector (Promega). All mutant constructs were verified by sequencing. The constructs prepared with various mutations in the ATATAT motif, the initiator motif or at other sites are as shown in Figure 1. In some reactions, a positive control construct of an arylphorin core promoter in the same vector was included along with the tested construct, as described previously [23].

Gel-shift competition assay

A 97-bp *Hin*dIII/*Kpn*I fragment containing the 88-bp cloned wild-type core promoter was liberated by digestion and end-labelled with ^{32}P. After incubation on ice for 10 min in a 15 μl vol. containing 3 μl of Sf9 nuclear extract (10 μg of protein), 8.6 μg of salmon sperm DNA, and, when included, mutant competitor fragment, the sample was loaded on to a 4% native polyacrylamide gel. After electrophoresis the gel was dried and the radiolabelled probe revealed by autoradiography.

Analysis

Autoradiographic results were scanned into Adobe Photoshop and then transferred into Microsoft Powerpoint for final preparation.

Cell transfection assay

Drosophila K_c cells (generously given by Jim Henderson and Peter and Lucy Cherbas) were maintained in Grace's medium (Gibco BRL) supplemented with 10% FBS and penicillin/streptomycin. Medium (1 ml aliquots) containing 10^6 cells was seeded into each 35-mm well, and after equilibration overnight, these aliquots were transfected for 6 h with 5 μl of lipofectin (Gibco BRL) and 3 μg of the test construct (Figure 1) and 3 μg of a construct expressing β-galactosidase under the control of a hsp70 promoter. Also included in each experiment was the transfection of an actin–luciferase construct [22] as a control against which to calculate the relative activity of each experimental JH esterase promoter construct. At 6 h after transfection, 0.5 ml of Grace's medium supplemented with $3\times$ FBS containing 100 units penicillin and 100 μg/ml streptomycin (pen-strep) was added to each well, and a further ml of medium/10% FBS/pen-strep was added at 24 h. At 72 h, the samples were harvested and assayed for luciferase activity, as according to manufacturer's instructions.

RESULTS

Functional necessity of the initiator motif for *in vitro* transcription

The JHE core promoter supported α-amanitin-sensitive transcription *in vitro*, directed by nuclear extracts from insect Sf9 cells (Figure 2; lanes 1 and 2). When the initiator motif GCAGT spanning bp -1 to $+4$ (Figure 1) was either deleted from the core promoter or completely transverse-mutated, no *in vitro* transcription was detectable (Figure 2; lanes 3 and 4). These results thus indicate that binding of (an) initiator protein(s) to this motif is prerequisite for detectable transcription to commence.

Functional necessity of the initiator motif for *in vivo* transcription

These results *in vitro* demonstrating a requirement for the initiator motif for transcription were also seen in the cell transfection assay. Either transversion or deletion mutation of the initiator motif drastically lowered transcriptional activity, in comparison with the wild-type core promoter (Table 1).

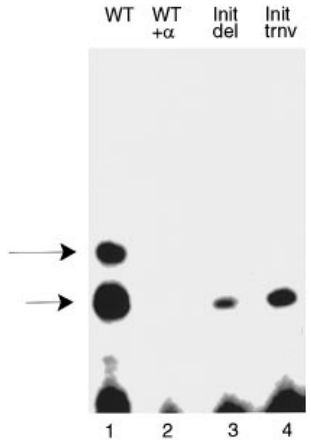

Figure 2 Effect of mutation of initiator, or of initiator and ATATAT motif of promoter, on the functional ability of the JH esterase core promoter to support *in vitro* transcription driven by Sf9 nuclear extracts

Either deletion (init del) or transversion (init trnv) of the initiator motif at -1 to $+4$ eliminated detectable transcription by the template promoter. The large arrow shows position of reporter transcript for this promoter, whereas the smaller arrow shows the position of the reporter transcript for internal control included in each transcription reaction (arylphorin gene core promoter). $+\alpha$, $+\alpha$-amanitin.

Table 1 Activities of wild-type core promoter and transversion or deletion mutants of the initiator or TATA-like motif in insect K_c cells

Abbreviations used: Core, wild-type promoter; Init. Trnv and Init. Del., transversion or deletion mutants of the initiator respectively; TATA Del, deletion of the ATATAT motif; Dbl. Trnv., transversion of both the initiator and ATATAT motif. (n) represents the number of independent replications. Values shown ($\bar{X}$) are normalized to the activity of an actin–luciferase construct.

Promoter Construct	$\bar{X}\pm$S.D.	(n)
Core	1.04 ± 0.30	3
Init. Trnv.	0.04 ± 0.01	3
Init. Del.	0.02 ± 0.01	2
TATA Del.	0.63 ± 0.16	2
Dbl. Trnv.	0.04 ± 0.01	3

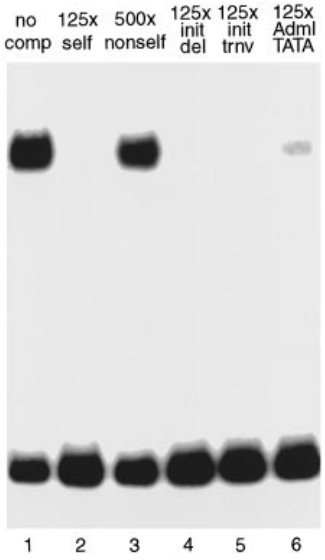

Figure 3 Gel-shift competition assay comparing wild-type competitor with various mutants in the ATATAT motif

Effective in competition with the wild-type labelled probe are competitors in which the transcription initiator motif was either deleted (del init) or transversed (init trnv), or in which the ATATAT motif is exchanged for the Adml TATA box.

Participation of core promoter sequence other than the initiator

In competition experiments, sequences in the core promoter other than the initiator motif supported sufficiently stable binding to substantially dislodge the transcription apparatus from the wild-type probe. Promoter probes containing either the deletion or transversion mutation of the initiator motif were effective as competitors in electrophoretic mobility-shift assays (Figure 3; lanes 5 and 6), though not as effective as the wild-type core promoter (Figure 3; lanes 1–3). Thus these results demonstrate a contribution of the protein complex–initiator motif interaction to the total binding affinity of the transcription complex for the probe. However, these results also indicate that stable binding of

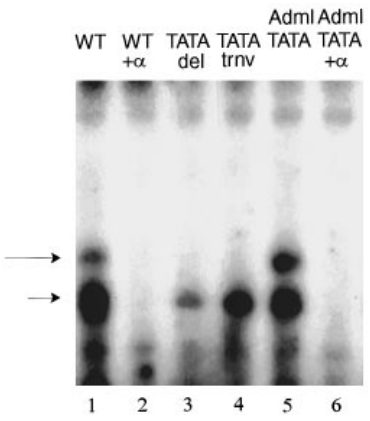

Figure 4 *In vitro* transcription driven by Sf9 nuclear extracts and supported by the wild-type (WT) core promoter of the JH esterase promoter, which is α-amanitin-sensitive

The large arrow shows position of the reporter transcript for this promoter, while the smaller arrow shows the position of the reporter transcript for internal control included in each transcription reaction (arylphorin gene core promoter). Either deletion of the ATATAT motif (TATA del) or transversion of the motif (TATA trnv) eliminated any detected transcription, whereas the adenovirus major-late promoter (AdmI) TATA motif functioned to effect α-amanitin-sensitive transcription when exchanged for the wild-type motif. +α, +α-amanitin.

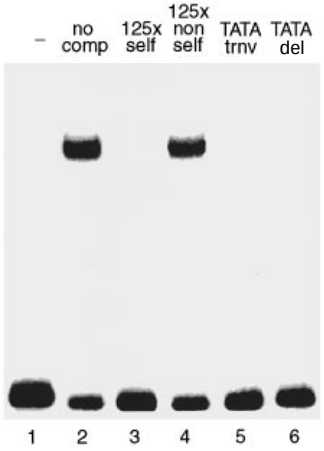

Figure 5 Competition of mutant JH esterase promoter sequences with the labelled wild-type sequence used as a probe in electrophoretic gel-mobility shift assay

In comparison with the wild-type probe alone (—), the Sf9 nuclear extract yielded a single major protein–DNA complex (no comp), which was specifically in competition with the self competitor (125 × self), but not the unrelated non-self competitor (125 × non self). Effective in competition were the probes in which the ATATAT motif was either transversed (TATA trnv) or deleted (TATA del).

the transcription complex to the initiator motif requires the binding of another factor to the core promoter at some site other than the initiator, but does not yet distinguish whether that other site is the ATATAT motif alone or possibly a third binding site on the core promoter.

Functional necessity of AT-rich motif for *in vitro* transcription

Either deletion of the ATATAT motif or its complete transversion resulted in no detectable transcription, thus demonstrating the requisite participation of this motif in transcription under the cell-free, *in vitro* conditions (Figure 4; lanes 3 and 4). The function of the ATATAT motif as a TBP-binding site is further supported by the fact that mutation of the motif to the putative canonical TBP-binding motif of TATAAA also resulted in α-amanitin-sensitive transcriptional activity (Figure 4; lanes 5 and 6). Correspondingly, electrophoretic mobility-shift assay detected that both the self (wild-type) sequence (Figure 5; lane 3) and the promoter with the ATATAT motif replaced by TATAAA (Figure 3; lane 6) competitively displaced the binding of the transcription complex to the wild-type probe.

Functional participation of ATATAT motif *in vivo*

The participation of the ATATAT motif in transcription was also observed in the cell line transfection assay. As shown in Table 1, deletion of the ATATAT motif significantly decreased transcriptional activity in comparison with that of the core promoter.

Participation of site(s) other than ATATAT motif

Competition experiments further indicated that under the *in vitro* transcription conditions, the ATATAT motif is not the only transcription factor-binding site on the core promoter. A competitive promoter probe, from which the ATATAT motif was deleted, was as nearly as effective at displacing the binding complex from the labelled wild-type probe as was the wild-type sequence itself (Figure 5; lane 6). The same result was obtained when the competitive probe contained a completely transversion-mutated ATATAT (Figure 5; lane 5). These mutant competitors were not as fully competitive as the wild-type probe (seen upon long autoradiographic exposures), again reflecting the tangible contribution of the interaction of the protein complex and the TATA-like motif to the total binding affinity of the total protein complex with the core promoter probe. However, the ability of an ATATAT-less competitor probe to substantially dislodge TBP binding to the wild-type ATATAT motif indicates that stable binding of the TBP to the wild-type probe involves requisitely TBP association with another nuclear factor(s) that binds the core promoter at a site other than the ATATAT motif.

Binding of additional nuclear factor to composite core promoter at site other than ATATAT motif and initiator sites

When both the ATATAT motif and the initiator were disabled by transversion, the 'double transversion' core promoter, as expected, failed to drive cell-free transcription *in vitro* (Figure 6; left panel). However, the double-transversion probe was still strongly effective in competing for binding to the complex in nuclear extracts that bound to the wild-type core promoter (Figure 6; right panel). These results indicate that stable binding of the TBP and initiator nuclear factors to the core promoter probe also required associative binding by another factor(s) to some location in the core promoter other than the TBP-binding site and the initiator site; that is, on the condition that a third nuclear factor could instead be binding to the core promoter probe independently of the binding of the TBP and initiator factors, then competitive titration of that third factor on to the excess, double-transversion mutant probe would not have competitively eliminated detectable binding by the TBP and initiator factors to the wild-type probe. This result also indicates that the third (or more) factor(s) is/are able to bind to the remaining

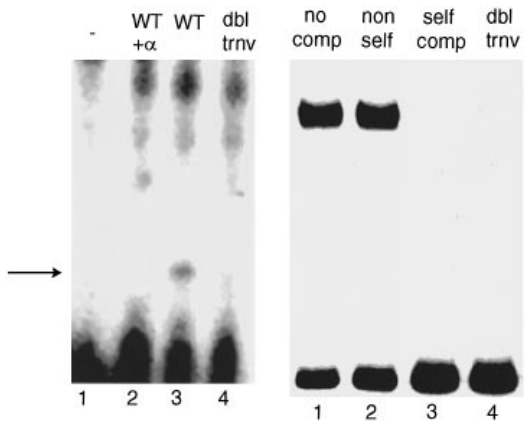

Figure 6 Binding, but not transcriptional activity, of double transversion of both the TATA box and initiation factor

Double transversion (dbl trnv) of the eliminated detectable α-amanitin-sensitive ($+\alpha$) transcriptional activity is shown (left panel). Also shown is the double-transverted probe (dbl trnv; right panel), which retains the ability to effectively compete with the wild-type probe in the gel-shift assay.

wild-type part of the core promoter with sufficient energy that, when this double-transversion promoter is in standard competitive excess, it/they can fully displace from the pure wild-type promoter the complex containing TBP- and initiator-binding protein.

DISCUSSION

While ongoing research continues to modify thinking on the components of a 'core promoter', an early paradigm was that a 'TATA'-box motif (for attachment of TBP) is necessary for basal rates of transcription [24]. Additional studies demonstrated that in some genes, typically housekeeping genes, there was not a TATA box, but there did exist a motif at or near the site of transcription initiation ('initiator'). The situation has become more complicated than these two simple alternative models. With respect to the initiator, several families of conserved motifs have emerged [25–28]. On the other hand, in other genes where an initiator-binding site has been functionally detected no motif resembling a conserved site is apparent (e.g. see [29]). A number of studies have identified various proteins as binding at or near the initiator motif, such as RNA polymerase II [3], YY1 [30], E2f(HIP1) [5], TAFII150 [1], USF [6], TFIIA [7], TFII-I [8,9] or TFIID itself [10–12], but the actual mechanisms of joint interaction of these proteins with the initiator (binding protein) and TFIID are obscure.

The role and form of the TATA box are also more complicated than originally conceived. While in some yeast and human genes the canonical TATAAA motif can functionally survive mutation to other AT-rich arrays [14,19,31,32], in other genes, such mutations either abolish transcriptional function or move the start site [33,34]. There is also the situation in which a TATA box is present as the wild-type motif and can bind TBP or TFIID, but yet is dispensable for significant rates of transcription [35,36]. Of further complexity is the discovery of a 'downstream element', typically 20–30 bases downstream from the transcription start site [37,38]. This motif, either weakly or strongly conserved in a number of genes, is apparently a binding site for another category of proteins and is essential for the quality and quantity of transcription in some genes [39], but the protein(s) binding to this site is/are even less understood than those binding at the initiator. In marked contrast to the above are those genes that apparently function without either a TATA box or an initiator element [40].

The present study on a JH-sensitive gene has demonstrated the functional existence of a TATA-like box and an initiator. Under the cell-free *in vitro* conditions, both of these motifs were essential for transcription. Many genes shown to possess both a TATA- (or TATA-like) box and an initiator are such that at least one of these two motifs is sufficient to drive substantial transcription if either motif is mutationally disabled (e.g. [34,38,41–44]). We also observed that in cell lines, transcription was essentially abolished when the initiator was debilitated and it was decreased when the ATATAT motif was removed. An important result observed here is that mutation of the initiator, whether by deletion of the central initiator motif, or by its transversion, completely abolished detectable transcription from any start site. This result is thus distinct from those obtained in a number of other studies [33,41] in which such mutations did not prevent transcription, but instead caused changes in the site of initiation. As reviewed recently by Novina and Roy [19], 'composite promoters' in which both the TATA box and initiator are essential for transcription to occur are most commonly found in viral promoters and less frequently in cellular promoters.

It has been postulated that the requirement for operation of both a TATA box and an initiator is related to increasing the regulatory complexity at the core promoter, and thereby increasing the specificity of transcriptional regulation that occurs [19,42]. In this regard, the JH esterase gene studied here is very tightly regulated temporally, because its induction in response to JH [20,45] at precisely the right time during the dramatic changes of metamorphosis is crucial for development, and its temporal misexpression is disastrous [46,47]. JH, an invertebrate structural relative of retinoic acid, binds to a nuclear receptor, ultraspiracle [48], that is a member of the nuclear hormone receptor family. Some nuclear hormone receptors activate transcription by acting through proteins binding to the initiator element [49], whereas in other genes the receptor may repress transcription by binding directly across the initiator element [50]. It is thus possible that the functional organization of the JH esterase gene approaches that of a composite promoter, in relation to its very tightly regulated temporal expression and/or its sensitivity to hormone induction.

However, additional complexity of the JH esterase core promoter is indicated here by the fact that the TATA/initiator double-mutant competitive oligonucleotides detected (an)other factor(s) that bind(s) at the core promoter at a site other than the TATA box or initiator. 'Tethering factors', which bind at the initiator of TATA-less promoters that have been proposed to hold TFIID at the core promoter [51]. Although the binding of additional transcription factors to core promoters at sites other than the TATA box or initiator has been shown in other genes (e.g. AGCE1 binding at bp -26 to -9 of the angiotensinogen gene; [14]), the results obtained here are distinguished from similar experiments with other TATA/initiator-containing core promoters, in which a third (or more) factor(s) was/were not sufficiently associated to the basal apparatus to dislodge it from a wild-type core (e.g. [52]).

The third binding factor that is responsible is probably not that which binds to the 'downstream element' described from a number of TATA-containing [37] and TATA-less promoters, including some insect genes ([39]; A/GGA/TCGTG). The JH esterase gene does possess a similar motif with close similarity to that element, and it is appropriately positioned about 30 bp downstream from the transcription start site ($_{+24}$AGACATG$_{+30}$). However, this 7-bp element was not included in either the

transcription templates or the gel-shift probes and competitors. The fact that transcription both occurred, and was initiated at the correct start site, in the absence of this motif indicated that it was not necessary to support basal transcription or correct positioning of initiation.

The quality of transcriptional initiation from the JH esterase gene promoter appears to be tightly controlled, with properties approaching that of a composite promoter and with contribution from regulators binding at core promoter sites that include more than the TATA box and consensus initiator. The presence of these and other putative regulatory elements at or near the basal-transcription-competent core promoter suggests a high degree of complexity of regulators controlling the precise temporal and hormone-sensitive expression of this gene. Given that most research to date on composite promoters has been on viral genes, the identification of the above features of this cellular promoter offers a model system for determining further mechanisms of function of cellular and hormone-sensitive genes.

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