Regulation of the juvenile hormone esterase gene by a composite core promoter

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Transcription from the core promoter of the juvenile hormone esterase gene (–61 to +28) requires the presence of both an AT-rich motif (TATA box) and an initiator motif for any transcription to occur, when assayed by either transcription *in vitro* with lepidopteran Sf9 nuclear extracts or by transient-transfection assay in Sf9 cells. Additional gel-shift experiments indicated that at least one additional binding site is essential for transcription to occur. Mutational analysis in the transcription-*in vitro* and cell-transfection assays demonstrated that a 14-bp region from +13 to +27 relative to the transcription start site is also essential for transcription to occur. Whereas the wild-type core promoter is highly transcriptionally active, inclusion of additional flanking sequences to position –212 reduces that activity approx. 100-fold, and inclusion of the 5' region out to position –500 reduces transcription by 200-fold. The pattern of dependence on both the AT-rich motif and the initiator for detectable transcription, and the high innate activity being

repressed by 5'-binding factors, was recapitulated in mosquito C7-10 cells. This study demonstrates that the cellular juvenile hormone esterase gene is organized as a composite core promoter, dependent on both TATA-box and initiator-binding factors, an organization that has been more commonly reported for viral promoters. This highly active composite core promoter is made more complex by the absolute dependence on the presence of a third site shortly downstream from the initiator, which is distinct from the 'downstream promoter element' described from some TATA-less genes. The juvenile hormone esterase gene thus appears to be a model of a cellular composite core promoter with a multipartite, indispensable requirement for not just both the TATA box and initiator, but also for at least a third core element as well.

Key words: enhancer, gene, initiator, TATA box, transcription.

INTRODUCTION

Regulation of transcription of eukaryotic class-II genes with developmental, tissue and hormone-sensitive specificities requires as-yet not understood organization and genome-wide co-ordination of signalling by many regulators. These regulators may be in the form of varied protein signalling molecules, or hydrophobic endocrine molecules, or even structural scaffolding of the nuclear matrix. The genome-wide co-ordinated assembly of these regulators into higher-order complexes with target genes, to effect precisely timed changes in transcriptional activity, is one of the great remaining 'black boxes' in developmental biology.

At the level of the target gene, proximal regulatory information directing the proper base(s) from which transcription will commence was considered classically to reside ≈ 25–30 bases upstream of the transcription start site, in the form of a promoter motif (TATA box; [1]) to which a TATA-box-binding protein (TBP) attaches. Subsequent studies showed that this motif could be various permutations of an AT-rich composition [2,3]. However, more recent studies have confirmed that TFIID (a TBP-associated factor) is not completely neutral to the particular AT-base sequence in this binding site, and that different permutations of the AT sequence confer different transcriptional behaviours to the promoter [4]. However, the TATA-box paradigm is not universally applicable, since many genes have been identified that do not possess such a motif in the vicinity of the transcription start site.

A second regulatory element (initiator; [5]) was subsequently found to occur at or near the actual transcription start site of many genes. Several different consensus initiator motifs have been reported, as well as individual initiators of particular genes that do not resemble the reported consensus sequences [6–8]. With the discovery of the initiator motif(s), models of transcriptional regulation necessarily incorporated scenarios in which some genes were 'TATA-box-dependent' whereas others were 'initiator-dependent' [9]. This expanded model has been challenged further by the reports of promoters that do not contain either a TATA box or an initiator motif [10]; some of these genes have multiple start sites over a relatively large region, rather than one site or several immediately adjoining sites [11]. There has also been the complicating demonstration that some genes possess a third major regulatory class of motif (downstream element; [12]), typically 25–30 bases downstream from the transcription start site. Consequently, it has been proposed that among the genes that have strictly controlled transcription start sites, some are TATA-dependent, whereas the TATA-less genes are either initiator-dependent or depend on both an initiator and downstream element [13].

Recent findings on the very tightly controlled genes have generated the notion of 'composite promoters' that have and are necessarily dependent on both a TATA box and an initiator [14]. Thus far most examples of composite promoters have been from viruses. Being dependent on usurpation of host transcriptional machinery at precisely the right moment in its life cycle and in

Abbreviations used: TBP, TATA-box-binding protein; TFIID, TBP-associated factor; DTT, dithiothreitol; JH, juvenile hormone.

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just the right host-cell biological context, some viral genes must have both TATA and initiator motifs present to exert the needed precision in transcriptional control. Although so far few cellular genes have been shown to have composite promoters, it is easy to envision developmental contexts in which similarly precise control is needed.

During insect metamorphosis, the terpenoid juvenile hormone (JH) and the steroid 20-OH ecdysone exert a genome-wide action to shift gene expression away from the larval genetic program and towards expression of metamorphic programs. One gene that is induced by JH to be expressed during metamorphosis is JH esterase, the expression of which must occur sharply within a very narrow developmental window for normal development to proceed [15]. We have isolated the JH esterase gene [16] and determined its core promoter to reside between -61 and $+28$, relative to the transcription start site [17]. We have also established both a cell-free transcription system and a cell-transfection system for functional analysis of the core promoter, and both of these systems support initiation of transcription from the authentic transcription start site, as is used *in vivo*. Using these functional assay systems, our previous studies indicated that transcription of the gene requires the presence of both a TATA-box motif and an initiator motif [18], and thus it appears to be a cellular gene with properties of a composite promoter. In the present study, we demonstrate that the transcription of this core promoter is absolutely dependent not only on the TATA box and initiator, but also on the presence of a third binding site placed 3' to the initiator. Thus the JH esterase core promoter may exhibit the functional organization of a composite cellular gene, with so high a requirement for regulatory precision that at least three discrete binding complexes in the core promoter are utilized as a multipartite composite organization.

MATERIALS AND METHODS

Sf9 cell line and nuclear extracts

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

Approximately 10^9 cells were collected 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 pelleted gently 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 homogenized with a loose A-type dounce homogenizer (two strokes), and the cell debris was then pelleted gently 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 homogenized quickly with a tight B-type dounce homogenizer (15 strokes). The sample was then stirred slowly on ice and 0.1 vols. of saturated $(NH_4)_2SO_4$ added. After 30 min of incubation, the mixture was centrifuged in an SS34 rotor at 7600 g 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 Da molecular-mass cut-off) against 150 ml of the same buffer for 8–12 h. The sample was finally centrifuged for 1 h at 150 000 g in a Ty75 Ti rotor, and the collected supernatant stored at -70 °C until use.

In vitro transcription reactions

Reaction contents in 12.5 μ l were as follows: 5 μ l of Sf9 nuclear extract, 400 μ M rNTPs, 4 mM creatine phosphate, 500 ng of DNA template and, when included, 0.1 μ g/ml α -amanitin. Re-

actions were performed at 30 °C for 1 h, and then 2 μ l were taken for primer-extension reaction, primed with a 32 P-labelled antisense oligonucleotide that was complementary to the reporter luciferase cDNA transcription product. The transcription products were annealed to the primer at 58 °C for 20 min, and then at room temperature for 5 min in 500 μ M dNTPs/10 mM $MgCl_2$ /1 mM DTT/250 mM Tris/HCl (pH 8.3)/200 mM KCl/2.5 mM EDTA. The primer extension 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 detected by autoradiography.

JH esterase core-promoter constructs

The wild-type core promoter (-61 to $+28$), and constructs including upstream sequences to either position -515 or -212 , were obtained from a genomic clone of the lepidopteran *Trichoplusia ni* as described in [16,17]. Mutations in the core promoter 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 annealed products into corresponding sites of the pGL3-basic vector (Promega). Sequences of all mutant constructs were verified. The mutant constructs prepared are shown in Figure 1. In some experiments, a positive control construct of an arylphorin core promoter, in the same vector, was included along with the tested construct, as described previously [17,19]. The basic strategy in designing these constructs was as follows. Construct 1 is the wild-type, and constructs 2–7 specifically mutate the AT-rich motif (TATA box), the initiator or both. The contribution of the upstream end of the core promoter, 5' to the AT-rich motif, was assessed by construct 9. The effect of deleting various regions or bases between the AT-rich motif and initiator was assessed by constructs 10, 13, 16 and 18, while that of moving the initiator in the 5' direction was tested using constructs 15 and 18. Finally, the role of regions and bases at the 3' end of the core promoter, downstream from the initiator, was assessed with constructs 8, 12 and 13.

Gel-shift competition assay

A 97-bp *Hind*III/*Kpn*I fragment containing the 89-bp cloned wild-type core promoter was liberated by digestion and end-labelled with 32 P. After incubation on ice for 10 min in 15 μ l containing 3 μ l of Sf9 nuclear extract (10 μ g of protein), 8.6 μ g 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 detected by autoradiography. Autoradiographic results were scanned into Adobe Photoshop and imported to Adobe Illustrator for final presentation.

Cell-transfection assay

Spodoptera Sf9 cells were maintained in Sf900 medium (Gibco-BRL) supplemented with penicillin/streptomycin. Cells were seeded into 35-mm-diameter culture wells at approx. 10^6 /ml confluency and, after equilibration for several hours, were transfected for 5 h with 3 μ l of Cellfectin (Gibco-BRL) and 3 μ g of the test construct (Figure 1) or the pGL3-basic vector alone. At 72 h after transfection, the samples were harvested and assayed for luciferase activity. It was observed that some mutant constructs showed even lower activity than the control plasmid

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 transcription reactions *in vitro*, 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 core promoter. Various mutations are indicated for the ATATAT motif (AT) or initiator motif (INR), such as deletion of the motif (del), transversion of each base of the motif (trv). Mutations to other regions in the core promoter are indicated as either deletion (Δ) of other bases or regions, or as specific mutations ($\rightarrow$) to individual bases. Exchange of the adenovirus major late promoter TATA box motif (TATAAA) for the wild-type AT-rich motif is indicated as 'TATA'.

vector. Thus for the cell lines used, the reporter expression plasmid may have a low but non-zero background activity.

RESULTS

Composite requirement of JH esterase core promoter for TATA box and initiator

The basic strategy in designing the wild-type and deletion/point mutant constructs was to assess, first, the contribution of the AT-rich motif (TATA-box), initiator or both, and then regions either 5' to the TATA box, or 3' to the initiator, or in the intervening region between the TATA box and initiator. Under *in vitro* transcription conditions, the wild-type core promoter exhibited strong, RNA polymerase II-dependent transcriptional activity (Figure 2A). When the AT-rich motif was replaced with a classical TATA-box motif (TATAAA), significant transcriptional activity was retained, although at a lower level than the wild-type motif (Figure 2A). The activity of this classical TATA motif in the JH esterase core-promoter context was verified independently by cell-transfection assay, in which the classical TATA motif exhibited nearly the same activity as the wild-type AT-rich motif (Table 1). Transversion or deletion of either the natural AT-rich motif or the initiator motif abolished any detectable transcription *in vitro* (Figure 1).

Indication of third binding site in core promoter

The cell-transfection assay showed that either transversion of the AT-rich motif, or deletion of the initiator, reduced activity of the promoter by 50–100-fold (Table 1). These results further support our previous findings that the JH esterase gene possesses a composite promoter that is dependent on the presence of both a TATA box and an initiator [18]. In gel-shift competition assays, a primary binding complex binding on to the core promoter was detected (Figure 2B, NO COMP). Gel-shift competition using wild-type and mutant core-promoter competitors demonstrated that the single complex binding to the core promoter under gel-shift conditions makes contact with the AT-rich motif and initiator motif. When the AT-rich motif in the competing fragment is replaced with the adenovirus major late promoter TATA box, its ability to compete for the radiolabelled wild-type probe is reduced (Figure 2C, 125 $\times$ TATA), compared with the unlabelled wild-type competitor (Figure 2C, 125 $\times$ SELF). Also a core-promoter competitor with the initiator mutated by a transversion was more strongly competitive than the competitor with the adenovirus major late TATA box (Figure 2C, 125 $\times$ INR TRV), but was not as fully competitive as the wild-type core-promoter competitor.

The above results show that the TATA box and initiator contribute to binding of the single primary complex detected in

Figure 2 Functional contribution of TATA box and initiator motifs to binding *in vitro* to and transcriptional activity of JH esterase core promoter driven by Sf9 nuclear extracts

(A) Deletion (DEL) or transversion (TRV) of the AT-rich motif (AT) or the initiator motif (INR) abolished detectable transcription by the core promoter (CORE). The upper arrow points to the JH esterase construct reporter transcript, while the lower arrow denotes the control transcript produced by the arylphorin promoter construct that was added to each reaction. Sf9 nuclear extract was used for each reaction. + α indicates that α -amanitin and RNA pol II inhibitor was added to the reaction. (B) Electrophoretic mobility-shift assay using the wild-type core promoter as the labelled probe, in competition with SELF or NONSELF probes, or a probe with both the AT-rich motif and initiation transverse-mutated (DBL TRV). The competitor in which both the TATA box and initiator had been mutated by transversion nevertheless was able to displace the complex that otherwise bound to the wild-type probe. (C) Electrophoretic mobility-shift assay using the wild-type core promoter as the labelled probe, in competition with the SELF or NONSELF probes, or probes with the initiator transverse-mutated (INR TRV), the AT-rich motif substituted by a classical TATA box (TATA) or with the initiator moved one base in the 3' direction (3' 1 bp INR). The competitors in which both the initiator had been transversion- or point-mutated nevertheless were able to displace the complex that otherwise bound to the wild-type probe.

the gel-shift assay. However, when the TATA box and initiator were mutated simultaneously by transversion, the resultant mutant core promoter was still strongly competitive in displacing this complex from the radiolabelled wild-type probe (Figure 2B, 100 $\times$ DBL TRV). This result indicates that there exists at least one additional site on the core promoter that is not the TATA box or initiator motif, at which a protein (or complex) binds. This result also shows that this third site is able to competitively displace from the labelled wild-type probe the single detected complex that makes contacts with the TATA box and initiator, suggesting contact between that complex and the entity binding to the additional site(s).

The third binding site is not at the 5' end of the core promoter

The third binding site appeared not to reside between bases -61 to -51 , since deletion of those bases did not have a significant

Table 1 Lepidopteran Sf9 and mosquito C7-10 cell-transfection assay of transcriptional activity of wild-type and mutant JH esterase promoter constructs

Core, wild-type promoter; TATA, exchange of AT-rich motif (AT) for adenovirus major late (Adml) TATA box; TRV, transversion; INR, initiator; Δ , deletion; pGL3, expression vector only; -212 , core promoter plus flanking sequences out to position -212 ; -515 , core promoter plus flanking sequences out to position -515 . pGL3 indicates transfection with empty reporter vector only. Reporter c.p.m. values (mean $\pm$ S.E.M., $n = 3$) have been multiplied by 10^{-6} .

Construct	Reporter c.p.m.		
	Exp. 1, Sf9	Exp. 2, Sf9	Exp. 3, C7-10
Core	13.33 $\pm$ 2.63	6.35 $\pm$ 0.72	143.2 $\pm$ 16.3
TATA	11.3 $\pm$ 0.83	8.91 $\pm$ 1.86	—
AT TRV	0.28 $\pm$ 0.02	—	—
AT Δ	—	—	18.1 $\pm$ 0.6
INR Δ	0.13 $\pm$ 0.02	—	1.3 $\pm$ 0.1
TATA Δ , -6 to -12	—	0.18 $\pm$ 0.02	—
TATA Δ , $+13$ to $+22$	—	0.29 $\pm$ 0.09	—
TATA G $\rightarrow$ A $^{+27}$	—	0.28 $\pm$ 0.02	—
pGL3	0.42 $\pm$ 0.03	0.18 $\pm$ 0.24	16.4 $\pm$ 0.9

Construct	Reporter c.p.m.	
	Exp. 4, Sf9	Exp. 5, C7-10
-61 (Core)	12.3 $\pm$ 1.92	25.0 $\pm$ 3.3
-212	0.17 $\pm$ 0.09	3.7 $\pm$ 1.8
-515	0.07 $\pm$ 0.01	2.9 $\pm$ 3.1
pGL3	0.3 $\pm$ 0.01	4.3 $\pm$ 1.0

effect on transcriptional activity *in vitro* (Figure 3A). The binding site also does not require the base T $^{-47}$, since its mutation to C did not significantly reduce either transcriptional activity (Figure 3B) or the ability of the mutant probe to compete with the wild-type core promoter in gel-shift assay (Figure 4, right-hand panel).

Requirement for sequences downstream of initiator

Sequences in the region from the initiator motif to the 3' end of the core promoter were tested for their necessity in transcriptional activity of the core promoter. Mutation of base A $^{+10}$, just 3' downstream from the initiator (situated at -1 to $+4$), had no significant effect either on transcription *in vitro* (Figure 3B, Figure 5) or in the cell-transfection assay (Table 1). Nor did this mutation significantly affect the competitiveness of the mutant probe against the wild-type core promoter in gel-shift assay (Figure 4). However, further downstream, deletion of the region $+13$ to $+22$ abolished transcriptional activity, both *in vitro* (Figure 5) and in cell-transfection assays (Table 1). Another five bases further downstream at the very 3' edge of the core promoter, mutation of C $^{+27}$ to A abolished transcriptional activity in both assays (Figure 5 and Table 1). These results implicate strongly the indispensable transcriptional participation of bases 3' to position $+13$.

Interval between TATA box and initiator

There also remains the possible contribution of the interval between the TATA box and initiator. Movement of the initiator in the 5' direction, 10 bases closer to the TATA box, by deletion of the 10 bases 5' to the initiator, abolished detectable transcription (Figure 3C). While that deletion/5' movement of the

Figure 3 Transcription mutational analysis of the sequences within the core promoter

(A–D) The indicated bases and regions of the core promoter (CORE) were mutated or deleted as indicated (see also schematic explanation in Figure 1) and then used as templates in transcription reactions *in vitro*. Larger upper arrows point to the JH esterase (JHE) construct reporter transcript, while the lower smaller arrows denote the control transcript produced by the arylphorin promoter construct that was added to each reaction, except those shown in (B). Sf9 nuclear extract was used for each reaction. +α indicates that α-amanitin was added to the reaction.

Figure 4 Competitive electrophoretic mobility-shift assay using Sf9 nuclear extracts

Effective in the competition with wild-type labelled core probe were the core promoter itself (CORE), and the mutant probes of movement of the initiator 10 bp in the 3' direction (3' 10 bp INR), 10 bp in the 5' direction (5' 10 bp INR), or of bases –47 and +10 (T → C₋₄₇, G → A₊₁₀), or of deletion of bases –6 to –12 in a probe in which the AT-rich motif had been substituted by a classical TATA box (TATA DEL –6 TO –12). The competitive ability of the transcriptionally inactive mutant probes attests to the binding of nuclear components to other regions of the competitor. See Figure 1 for schematic explanation of mutant competitors.

Figure 5 Transcription mutational analysis of the sequences within the core promoter

The indicated bases and regions of the core promoter (CORE) were mutated or deleted as indicated (see also schematic explanation in Figure 1) and then used as templates in transcription reactions *in vitro*. Arrow points to the JH esterase (JHE) construct reporter transcript, driven by Sf9 nuclear extract that was used for each reaction. Mutations to the 3' end of the core promoter, from +13 to +27 downstream from the transcription start site abolished detectable transcription, whereas the other mutations elsewhere in the core promoter did not abolish transcription. +α indicates that α-amanitin was added to the reaction.

initiator debilitated transcription, it did not prevent that mutant probe from being strongly competitive with the wild-type labelled probe. In fact, similar movement of the initiator motif by even a

Figure 6 Functional contribution of TATA box and initiator motifs to binding *in vitro* to and transcriptional activity of JH esterase (JHE) core promoter driven by Sf9 nuclear extracts

The indicated mutations at the initiator (INR) abolished detectable transcription by the core promoter (CORE). Larger upper arrow points to the JHE construct reporter transcript, while the lower smaller arrow denotes the control transcript produced by the arylphorin promoter construct that was added to each reaction. Sf9 nuclear extract was used for each reaction. + α indicates that α -amanitin was added to the reaction.

single base in the 5' or 3' directions, towards or away from the TATA box, respectively, abolished transcriptional activity *in vitro* (Figure 6) and in cell-line transfection (Table 1). As a control to check that the effect of movement of the initiator one base 5' towards the TATA box was not due to sensitivity of binding factor(s) to the single base immediately 5' to the initiator motif, a six-base sequence midway between the TATA box and initiator was deleted, with the same result of abolished transcription *in vitro* (Figure 3D) and in cell-line transfection (results not shown). However, again, the mutant probe deleted for these six bases was still strongly competitive against the wild-type probe, by gel-shift assay (Figure 4, right-hand panel). As a test of the potential explanation that the binding architecture is sensitive to even a single base shift of the initiator 5' towards the TATA box, a single intervening base, G²⁰, was deleted, with the result that transcriptional activity was retained (Figure 3B).

Upstream effects on core promoter

In view of the high activity exhibited by the wild-type core promoter, both *in vitro* and in cell-transfection assays, we sought to detect any contribution of the 5' upstream flanking sequence to the activity of the core promoter. Since there are technical difficulties in securing transcription systems *in vitro* to reflect modulating activities more than 200 bases upstream [20], we used cell-line transfection for this assessment. The JH esterase core promoter from -61 to +28 yielded activity about 30-fold higher than the pGL3-basic expression plasmid alone (Table 1). However, the inclusion of 212 additional bases of 5' flanking sequence did not enhance activity, but rather suppressed the activity by about 100-fold. When the test promoter included upstream bases out to position -515, the activity was reduced over 200-fold relative to the core promoter (Table 1).

The composite activities of the core promoter, and of the strongly suppressive upstream sequences, were tested in hetero-

logous mosquito C7-10 cells. In the C7-10 cells, deletion of the AT-rich motif alone reduced transcription down to the level of the empty vector only, as occurred for the lepidopteran Sf9 cells. Deletion of the initiator motif alone strongly reduced transcription, even below the level of the empty vector only, again as was observed for the Sf9 cells (Table 1). The high transcriptional activity of the composite core promoter was suppressed strongly by inclusion in the construct of 5' flanking sequences out to either -212 or to -515 (Table 1). Thus the qualitative profile of activity of the elements of the core promoter and its 5' flanking sequences observed in lepidopteran Sf9 cells was recapitulated in the mosquito C7-10 cells.

DISCUSSION

Initial models concerning the regulation of promoter activity and transcription centred on a TATA box motif that was found to occur in many genes. It was generally conceptualized that a basal transcription apparatus included a TFIID complex, composed of a TBP and closely associated TBP-associated factors, bound at the TATA box through the protein-DNA contacts contributed by TBP. The minimal region of the promoter, including the TATA box and transcription start site, that would support constitutive transcription from the correct transcription start site was considered as a 'core promoter' module. However, footprinting and mutational analyses detected binding of the basal complex over a much larger area than the TATA box, indicating the existence of other potentially important regulatory motifs. Further studies demonstrated that some genes do not possess a TATA box, but rather utilize an 'initiator' motif through which TFIID is anchored either directly or indirectly to the core promoter [21]. Yet other investigations have identified core promoters that contain no TATA or initiator motifs [22,23].

The occasion of existence of both of the core promoter elements of a TATA box and an initiator within a single core promoter raises questions as to occurrence and nature of interaction or joint contribution of these motifs to regulation of core promoter activity. Current paradigms posit that in cases where promoters possess both a TATA box and an initiator, either the TATA box or initiator are dominant. In most cases mutation of just one or the other site may reduce but not eliminate transcription [24-27], although their combined presence is often observed as synergistic. In promoters that do not have a TATA box but possess an initiator motif and a conserved downstream element, the latter is contemplated to provide the additional binding stability that would otherwise be contributed by binding through the TATA motif [13,28]. Ohtsuki et al. [28] identify examples where if the TATA, initiator and downstream promoter elements are all found in a single core promoter, then the regulatory influence of the TATA element is dominant over the initiator/downstream element. Yet other researchers have observed that removal of a TATA box from a promoter that also contains an initiator motif may have little effect on the strength of the promoter [29].

In the present study, we have demonstrated by transcription *in vitro* and cell-line transfection functional assays that both the TATA box (AT-rich motif) and the initiator motif are necessary in Sf9 cells for transcription to occur from the JH esterase core promoter. If either motif was removed, then the transcriptional assays did not detect significant transcription activity above background. This pattern was fully recapitulated in cell-transfection assays using mosquito C7-10 cells. These results establish that the JH esterase core promoter is a composite promoter, dependent on the presence of both a TATA box and an initiator. The absolute dependence of a core promoter on the presence of

both motifs is distinguished from the more common cellular gene organization, where if a promoter contains both motifs then the removal of one motif may reduce but not eliminate transcription, or perhaps the promoter is functionally dependent on one of the two present motifs, but not both. The essential composite dependence of a core promoter on the presence of both a TATA box and an initiator motif has been best documented for viral promoters, such as that of the adenovirus major late promoter or the human immunodeficiency virus type 1 promoter [30]. However, models of cellular composite promoters have not been as well documented, and the essentiality of both the TATA box and initiator motifs that we have shown here is one of the strongest cases of composite dependency on both motifs that has been reported for a cellular gene.

More recently, investigators have detected a third type of conserved core promoter motif, the downstream element, which is proposed to operate in promoters that possess an initiator but which are missing a TATA box [12]. This element appears distinguished from specific, more narrowly functioning motifs that occur within the core promoter but which impart tissue-specific, temporally controlled or hormone-sensitive properties to the transcriptional activity. For example, binding sites within core promoters have been detected that provide tissue or stage specificity for expression in neurons [31], bone [32], liver [33], heart [34] and T cells [35], or at post-fetal [36] or post-proliferative [32] stages. In addition, core-promoter motifs other than the TATA box and initiator may provide sites through which are transduced hormonal regulatory signals from glucocorticoids [37], retinoic acid [38–40], thyroid hormone [41–43] or bile acid ligands [44]. In contrast, the conserved downstream element is postulated to be a binding site more functionally analogous to a TATA box [45]. Under current models, this canonical downstream promoter-element motif operates along with the initiator motif at TATA-less promoters [13].

We have established that a protein complex in nuclear extracts binds two sites, the AT-rich motif (TATA box) and the initiator motif, which are both indispensable sites for transcription to be detected either *in vitro* or in cell-transfection assay (see [18] and Figure 2 in this study). In the present study, we have identified a third protein-binding region of the JH esterase core promoter that does not contain the TATA box or initiator, but which is also indispensable for transcription. This region, between +13 and +27, relative to the transcription start site, does not appear to be the conserved downstream promoter element described thus far from TATA-less promoters, since this region does not contain that downstream-element motif. The operation of this third site also appears distinguished from tissue, developmental or hormone-specific activators, since such activators that occur in core promoters that already possess both a TATA box and initiator act typically to increase the already existing basal transcription conferred by the TATA-box- and initiator-binding complexes. In the present case, removal or mutation of this third site does not merely remove the extra activation that is over the basal transcription rate; rather, no detectable transcriptional activity occurs at all. Correspondingly, the single primary complex that binds to the core promoter under gel-shift conditions interacts sufficiently with proteins contacting the TATA box and initiator that a competitor probe with debilitated TATA box and initiator, but containing an intact third site, can nevertheless still competitively displace the TATA-box- and initiator-binding complex(es) from the wild-type probe (Figure 2; [18]). These results suggest that the JH esterase core promoter may represent a multipartite composite type of core promoter in which three discrete regions (TATA box, initiator and third site) are each indispensable for detectable transcription to commence.

Some promoters appear to possess an organization in which proteins binding at the core promoter exert the regulatory action to yield very high levels of transcriptional activity, and the upstream 5' flanking sequences bind regulators that suppress what would otherwise be high innate transcriptional activity of the core promoter (e.g. the rat brain creatine kinase gene [46]; defensin 1 gene [47]; *MyoD1* gene [48]). In the present study, we have shown that the high innate transcriptional activity of the highly composite core promoter of the JH esterase gene is eliminated, and even decreased below background reporter vector-only levels, when the 5' flanking sequence is included. *In vivo*, this promoter is not constitutively active, but rather is activated in strong, steep peaks at specific stages of metamorphosis [15,16,49]. There is molecular evidence that even before activation of the *hsp70* gene, TFIID is on the TATA motif, and RNA pol II is also at the core promoter in a 'paused' state [50]. A similar situation exists for the *c-myc* gene, where deletion of the TATA motif did not disrupt the paused state, but deletion of both the TATA and initiator did [51]. Recently, Okino [52] showed that the core promoter of the inducible phosphoglycerate kinase 1 gene is constitutively occupied at the TATA box. It is been proposed that TFIID is constitutively assembled and present at the promoter on inactive, inducible genes, awaiting the final cue for rapid initiation of high transcriptional activity [53].

Our working model for transcriptional control of the JH esterase gene is as follows. The activity of this promoter must be very precisely controlled, since even small changes in the proper timing of its normal profile of activation and repression [54] can derange normal metamorphic development [15]. The core promoter is organized as a multipartite composite promoter, indispensably dependent on the co-ordinate interaction of complexes binding at the TATA box, initiator and at least a third site downstream of the initiator. This complex composite organization is related to the need for very precise control over both the sharp increase and sharp decrease in its activity that is necessary for proper metamorphosis. The strongly sharp increases that occur during metamorphosis are effected in part by the high innate activity of the core promoter that is conferred by its tripartite composite organization, and in part due to stimulation by the terpenoid JH [49,55]. The steep decreases in its activity are mediated at least in part by strongly repressive modulators that bind in the 5' flanking sequences proximal to –515. Future mechanistic studies on the architectural surface presented by complexes binding to the composite core promoter may illuminate how these surfaces confer strong innate activity yet enable such complete suppression. An understanding of the mechanistic operation of this multipartite composite core promoter may also provide insight and leads on the organizational structure of cellular composite core promoters in other organisms.

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