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Insensitivity of the Preset hsp26 Chromatin Structure to a TATA Box Mutation in Drosophila*

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The role of the TATA element in establishing the chromatin structure and inducible transcription of the Drosophila melanogaster hsp26 gene has been analyzed. An hsp26/lacZ fusion gene with a mutant promoter, in which the TATA box sequence TATAAA was changed to CCCAAA, was introduced into Drosophila by P-element transformation. The mutation had little effect on formation of the preset chromatin structure observed prior to induction. However, the mutation dramatically reduced transcription levels following heat shock. Northern analysis indicated that weak, inducible expression of hsp26/lacZ fusion gene was limited by the transcriptional machinery and regulatory factor TBP (TATA-binding protein) and the GAGA factor, respectively (9–11). Mutations in the TATA box of the hsp70 promoter have been shown to mediate ATP-dependent disruption of nucleosomes upon addition of GAGA factor (15).

The interaction of TFIID and RNA polymerase II with the hsp26 promoter implies a potential role of the TATA box in formation of an appropriate chromatin structure prior to induction. DNA footprinting has revealed that the conformation of the TFIID-DNA complex differed significantly from that of the normal promoter. These results indicate that alterations in the conformation or the stability of the TFIID-DNA complex drastically reduce the level of induction, but do not dramatically affect chromatin structure formation. Formation of the requisite chromatin structure is either independent of, or highly tolerant of, changes in the TFIID-DNA complex.

In eukaryotic cells, almost all of the DNA is packaged with histones into ordered arrays of nucleosomes. Biochemical and genetic analyses have established that the assembly of DNA into nucleosomes not only packages DNA effectively, but also provides an important means of transcriptional control. In many cases, nucleosomes appear to repress transcription by limiting the access of the transcriptional machinery and regulatory factors to the DNA template (1–5).

The heat shock genes of Drosophila are a good model system for understanding the relationship between chromatin structure and gene activation. These genes are rapidly induced by heat shock as for the normal promoter, suggesting that TBP was associated with the mutant promoter prior to heat shock. Biochemical analysis showed that the mutant promoter still bound TFIID in vitro, but with 3–5-fold less affinity than the normal promoter. DNase I footprinting revealed that the conformation of the TFIID-DNA complex differed significantly from that of the normal promoter. These results indicate that alterations in the conformation or the stability of the TFIID-DNA complex drastically reduce the level of induction, but do not dramatically affect chromatin structure formation. Formation of the requisite chromatin structure is either independent of, or highly tolerant of, changes in the TFIID-DNA complex.

Of assays have revealed that RNA polymerase II is associated with the promoter region of heat shock genes prior to induction (6–9). The polymerase has initiated transcription but is paused in elongation in a region 20–40 base pairs downstream of the transcription start site. Genomic footprinting has revealed that the TATA box and the (CT)₃(GA)₉ repeats are constitutionally bound by proteins, presumably TFIID (the TATA-binding protein complex) and the GAGA factor, respectively (9–11). Mutation in the TATA box has been shown to mediate ATP-dependent disruption of nucleosomes upon addition of GAGA factor (15).

The interaction of TFIID and RNA polymerase II with the hsp26 promoter implies a potential role of the TATA box in formation of an appropriate chromatin structure prior to induction. Biochemical analyses have revealed further support. If nucleosomes are reconstituted onto an hsp70 promoter, subsequent addition of nucleic acids from heat-shocked Drosophila embryos does not result in transcription. If yeast TATA-binding protein (TBP) is bound prior to the reconstitution, addition of the nuclear extract leads to transcription. Hence, TBP establishes the transcriptional potential of the promoter in this model reaction (16). While these reconstitution experiments provide support for a simple model in which TBP alone might drive formation of the necessary chromatin structure, the situation is more complicated in vivo. Experiments with modified transgenes have shown that the TATA box and downstream sequences alone are insufficient to generate the DH sites in vivo (14). In addition, TBP is only one subunit of TFIID in higher eukaryotes. The remaining subunits are called TAFs (17–19). TAFs are required to reconstitute activator-dependent transcription (20–22). TAFs also appear to recognize specific sequences located downstream of the TATA element in the heat shock and histone promoters of Drosophila (23–25).

To better understand the function of the TATA box and TFIID in chromatin assembly and transcription of the heat shock genes, we have analyzed the consequences of mutating the TATA box in the Drosophila hsp26 heat shock gene promoter. We have examined the effects of this mutation on formation of the DNase I-hypersensitive sites at the promoter and on heat shock induction of the gene in vivo. We have also analyzed the effects of the mutation on the binding of purified TFIID in vitro. Our results indicate that the wild type TATA box of the Drosophila hsp26 gene promoter is essential for correct binding of

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§ The abbreviations used are: TBP, TATA-binding protein; DH site, DNase I-hypersensitive site; TAF, TBP-associated factor; kb, kilobase pair(s); HSE, heat shock element.

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TFIID and for inducible expression, but not for formation of the DNase I hypersensitive sites.

**EXPERIMENTAL PROCEDURES**

**P-Element Plasmids and Transformation**—The construction of pCarX has been described (11,36). CarXmTATA was constructed as follows. The XbaI/SacI fragment (−51 to +489) of Drosophila hsp26 was cloned in XbaI treatment of nuclei, DNA purification, and Southern blot analyses. hsp26 sequences in the constructs used here (Fig. 1) were normalized by this method. The percent accessibility of the proximal XbaI site was quantitated in each construct; all of them contained single inserts. The effect of the mutation on the promoter was evaluated by measuring the levels of β-galactosidase activity that were induced by a 90-min heat shock at 37 °C. The TATA mutation severely inhibited promoter activity; the heat shock-induced level of β-galactosidase activity was, on average, 3% that of the wild type control (Fig. 2). However, the mutant gene was heat shock-inducible compared to the non-heat shock controls (see also the Northern analysis below), implying that some level of TBP must still interact with the promoter either before or during heat shock induction.

While mutation of the TATA box clearly has an effect on transcription per se, it is also of interest to determine whether or not this change affects establishment of the normal preset chromatin structure. Consequently, we assessed the effect of the TATA mutation on the DNase I hypersensitivity of the promoter. Nuclei were isolated from third instar larvae of transformed lines and each preparation was treated with a range of DNase I concentrations. As shown in Fig. 3A, two DH sites were detected in the promoter region of both the wild type and mutant promoters. The patterns of cleavage in both cases were quite similar to that detected for the endogenous hsp26 gene (11, 36). The proximal DH site extended from the XbaI site.
at −51 to the EcoRI site at +7, covering the TATA box region. The similarities of these patterns indicated that the TATA box was not required to form the DNase I-hypersensitive sites.

Although the overall pattern of DNase I hypersensitivity was similar for the two promoters, it appeared that the level of sensitivity to DNase I in the mutant transgene was decreased compared to that of the wild type control CarX. We have previously established that the accessibility of a DH site can be quantitatively assessed by measuring the accessibility of a restriction site located within the DH site (12, 14, 26); there is an XbaI site located in the proximal and the distal DH sites of the hsp26 promoter region (see Fig. 1). The accessibility of the proximal XbaI site (−51) and of the distal XbaI site (−351) in transgene CarXmTATA was determined to be 64 and 81% that of the wild type control transgene CarX, respectively (Fig. 3, B and C). Previously, we have shown that when the preset chromatin structure at hsp26 is severely altered due to mutations at the (CT)9,(GA) repeats, the accessibility of the proximal and the distal XbaI sites is reduced to 9 and 18% that of the wild type control, respectively (12). The results here indicate that the TATA box is not essential for the formation of DH sites at this promoter. On the other hand, mutation of the TATA box alters the accessibility of both of the DH sites, implying that proteins that bind (or are dependent on) the TATA box, such as TFIID, are contributing to DH site formation, particularly to the formation of the proximal site.

Given that the TATA mutation severely affected transcription from the promoter, we decided to examine the kinetics of induction for the wild type and mutant promoters. We reasoned that if TFIID were absent from the mutant promoter, it might exhibit a delayed response upon induction. The wild type promoter, already having a poised polymerase, would immediately begin synthesis of a transcript upon heat shock. As shown in Fig. 4, the mutant promoter responded to heat shock as rapidly as did the wild type template. In both cases, transcripts were detected within 5 min, although the detected level in CarXmTATA is slightly lower. If we allow 1 min for the temperature to rise in the chamber containing the flies and 3 min for elongation of the 3.5-kb fusion gene (6, 37), the appearance of transcript in 5 min suggests that the TFIID did interact with the promoter before induction in both the normal and mutant cases. The alternative, that TFIID is rapidly recruited to the promoter, is less likely because the binding of TFIID is a slow process (38). Since the hsp26 promoter is preset before induction, the congruity of the kinetics argues that the RNA polymerase II is also transcriptionally engaged and paused at the mutant promoter. While the time of induction required for transcriptional activation is similar in both transgenes, it is notable that the amount of transcript that accumulates over time is much less for the mutant transgene.

Given that mRNA from both CarX and CarXmTATA was detected 5 min after heat shock, we thought that TFIID might interact with the promoter prior to activation, even though the TATA box had been mutated. Recent work has shown that sequences downstream of the TATA element are recognized by TFIID, and these interactions play a major role in the assembly of TFIID on the template (23–25). Thus, we tested directly whether or not TFIID still bound to the TATA mutant promoter. Antibody against the TBP subunit was used to purify TFIID from a protein fraction derived from a Drosophila nuclear ex-

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**Fig. 1. Map of the hsp26/lacZ construct and the probes used for chromatin structure analyses.** The structure of CarX is shown. hsp26 sequences from −1917 to +632 (with the exception of sequences from −371 to −352, which are deleted) are fused in-frame to the E. coli lacZ gene. Restriction sites shown on the top line are those sites giving marker fragments or those used for mapping the chromatin structure in indirect end-labeling experiments. Probes used in chromatin structure analyses are indicated. The partial restriction map of hsp26 sequences (−1917 to +632) is enlarged below with the (CT);(GA), repeats, the accessibility of the proximal and distal sites, and the TATA box (stippled box), regions (striped boxes), the TATA box (stippled box), and two required HSEs (HSE1-2 and HSE6, filled boxes) diagramed. Chromatin structural features of the hsp26 gene are marked below. Dra, DraI; RI, EcoRI; RV, EcoRV; Hind, HindIII; Hpa, HpaI; S, SmaI; Xba, XbaI.

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**Fig. 2. Expression and chromatin structure of the transgenes.** Transgenes are diagramed on the left side of the figure. A filled box labeled T indicates the wild type TATA box in CarX; an open box indicates sequence alterations in the TATA box in CarXmTATA. The number of independent transformed lines used to determine heat shock-inducible β-galactosidase activity is shown. The percentage values represent average levels of heat induced activity and accessibility of the XbaI sites for each transgene, shown normalized to the values obtained for CarX. Within the bar graph, the top bar (определен) for each transgene shows relative levels of heat shock-induced β-galactosidase activity; the middle bar (определен) shows relative values of accessibility of the proximal DH site (from results shown in Fig. 3B); the bottom bar (определен) shows relative values of accessibility of the distal DH site (from results shown in Fig. 3C). The actual percentage of XbaI cleavage in chromatin is also shown.
A genomic DNA was subsequently purified and cleaved with EcoRV. After increasing amounts of DNase I, as indicated by the 1.1-kb agarose gel and transfer to nylon membrane, the DNA was probed with the XbaI site within the distal DH site. Nuclei were treated with XbaI.

The genomic DNA was purified and restricted to completion with EcoRV. After size fractionation by electrophoresis through a 1% agarose gel and transfer to a nylon membrane, the DNA was probed with a 0.6-kb DNA fragment from the hsp26 promoter (24, 25). We presented different amounts of the immunopurified TFIID with a constant mixture of end-labeled DNA fragments corresponding to the wild type and mutant hsp26 promoters.

The binding of the hsp26 TATA mutant template (pCarXmTATA) than the wild type promoter template (pCarX) has an affinity for TFIID that was comparable to two wild type constructs of hsp70 (+61 and +43).

The above analysis shows that TFIID can still associate with the protein G-Sepharose. Previously, it had been found that the immunopurified TFIID retained its capacity to bind specifically to DNA fragments containing the hsp70, hsp26, or histone H3 promoters (24, 25). We presented different amounts of the immunopurified TFIID with a constant mixture of end-labeled DNA fragments corresponding to the wild type and mutant hsp26 promoters. As shown in Fig. 5, TFIID binds 3–5-fold less of the hsp26 TATA mutant template (pCarXmTATA) than the wild type template (pCarX).

The Northern blot analysis shows that this result combined with the rapid response upon induction leads us to propose that TFIID still participates in setting up the promoter for rapid induction. This hypothesis is further supported by analysis of other mutations in the hsp26 footprint on the CCCAAA-containing mutant promoter, but one that was strikingly different from that observed for the wild type promoter. The sharp boundaries of the −44 and +35 regions were entirely absent, and the hypersensitive sites that appear within the normal footprint were missing. Instead, regions of protection lacking sharp boundaries were evident over the CCCAAA sequence, the start site, and around +25 (Fig. 6).

The above analysis shows that TFIID can still associate with the hsp26 promoter even though the TATA box has been mutated. This result combined with the rapid response upon induction leads us to propose that TFIID still participates in setting up the promoter for rapid induction. This hypothesis is further supported by analysis of other mutations in the hsp26 footprint on the CCCAAA-containing mutant promoter, but one that was strikingly different from that observed for the wild type promoter. The sharp boundaries of the −44 and +35 regions were entirely absent, and the hypersensitive sites that appear within the normal footprint were missing. Instead, regions of protection lacking sharp boundaries were evident over the CCCAAA sequence, the start site, and around +25 (Fig. 6).

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and hsp70 promoters. In the case of the hsp26 promoter, the region between −135 and +7 is not sufficient for the formation of DH sites. This region contains a GAGA element (−135 to −85), TATA box, and the transcription start site. Addition of the region from −351 to −136, which contains a GAGA element, or of sequences from +8 to +62, which would provide the downstream contacts of TFII D (23, 25), restored the capacity for formation of the DH sites (14). In the case of the hsp70 promoter, deletion of sequences downstream of the TATA element leads to reduced levels of paused polymerase when the sequences upstream of the TATA element extend to −89 (13). Insertion of additional copies of the GAGA element from the region between −38 and −89 caused a significant increase in the level of paused polymerase (13). All of these results point to the possibility that multiple interactions involving the GAGA factor and TFII D act in concert to ensure the transcriptional potential of the heat shock gene promoters.

The dramatic effect of the mutation on the level of transcription following heat shock could then be inferred to be due to alterations in the TFII D-DNA interactions per se. Differences in the DNase I footprint formed by immunopurified TFII D suggest that the conformation of the complex is quite different on the wild type and mutant promoters. The cocrystal structure of the TBP and the TATA element indicates that the DNA is dramatically distorted causing the DNA to be sharply angled by 100° (39, 40). Mutation of the TATA box in the Drosophila hsp26 gene may result in a failure of TFII D to induce the correct conformational changes in the DNA complex. This might disrupt protein-protein contacts at some stage in the transcription process. In addition, the stability of the TFII D-DNA complex might be reduced. The CCCAA mutation could weaken the association of the TFII D-complex so that fewer rounds of initiation occur before the TFII D dissociates and a new molecule must be recruited. It is interesting that transcripts are detected within 5 min for the mutant promoter (Fig. 4). We have recently suggested that the downstream contacts of TFII D could function primarily to set up the promoter (25, 41). The elongation of RNA polymerase II from the transcription start might disrupt many of the downstream contacts of TFII D leading to a situation in which the retention of TFII D for multiple rounds of initiation becomes largely dependent on upstream contacts of TFII D such as those involving the TBP subunit.

The effect of an altered TATA box on expression has been studied for a number of genes in vitro and in vivo. Studies

![Fig. 5. TFII D binds with specificity to the hsp26 mutant promoter. Binding of immunopurified TFII D with a collection of hsp26 and hsp70 promoter fragments. The +61, +43, and ATATA hsp70 fragments are derivatives of the hsp70 promoter. The pCarXmTATA and pCarX are hsp26 derivatives. Binding reactions were set up with increasing amounts of immobilized TFII D associated with 2.5, 5, 10, and 20 μl of settled beads. The lane labeled Input indicates the end-labeled fragments before TFII D binding; the lane labeled Bound indicates the TFII D-bound fragments. Equal radioactive counts are loaded on the lanes, although increasing amounts of DNA were recovered with increasing amounts of TFII D.

Fig. 6. DNase I footprinting reveals an altered TFII D-DNA complex on the hsp26 mutant promoter. DNase I footprinting of the complexes formed between immobilized TFII D and either the wild type (pCarX) or mutant (pCarXmTATA) hsp26 promoter fragment. Lanes labeled with a + or a − indicate the presence or absence of TFII D in the reactions; duplicate samples are included. Arrows labeled with numbers indicate nucleotide positions relative to the transcriptional start site.

including analysis of the chromatin structure of altered genes include those of the hsp82 gene (42), the PHO5 gene (43) and the SUC2 gene (44) from yeast. The effects of TATA mutation on transcription and chromatin structure of the above yeast genes are consistent with what we have observed in studying the hsp26 gene in Drosophila. That is, a dramatic reduction in transcription is observed, with little effect, if any, on chromatin structure formation. Whether or not the yeast TFII D can still bind to the mutant promoter in each of the above cases has not been determined.

Taken together, our analysis of TFII D is consistent with biochemical analysis by others (16, 45, 46) showing that TFII D is involved in establishing chromatin structure of gene promoters and the transcriptional potential. Further, our study indicates that the TATA element is not the only sequence in the promoter recognized by TFII D; contacts made downstream may allow TFII D to interact with the mutant TATA box. Mutation of the TATA box may alter the conformation of TFII D, which apparently does not dramatically interfere with its function in chromatin structure formation, but severely reduces the level of induced transcription.

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