In vitro analysis of the *Antennapedia* P2 promoter: identification of a new *Drosophila* transcription factor

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We have investigated the transcriptional regulation of the *Antennapedia* P2 (*Antp* P2) promoter using nuclear extracts prepared from *Drosophila* embryos. Transcriptional analysis of deletion mutants reveals the presence of multiple *cis*-regulatory elements located both upstream and downstream of the start site. One of the elements appears to mediate negative regulation, since deletion of this element results in higher levels of transcription. Several factors that interact with these control elements have been detected and isolated. One DNA-binding protein, *Drosophila* Transcription Factor-1 (DTF-1), specifically recognizes the consensus sequence GCAACA^T/C^G/C that is reiterated four times within an upstream activating element. DTF-1 was purified by sequence-specific DNA affinity chromatography and identified as a doublet of ~50 kD. Purified DTF-1 enhances transcription from the *Antp* P2 promoter 7- to 15-fold in a binding site-dependent manner.

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Drosophila embryogenesis and the determination of cellular fates involves the temporally programmed and spatially restricted expression of genes that are governed by a hierarchical network of maternal, segmentation, and homeotic regulatory factors (for review, see Mahowald and Hardy 1985; Scott and O'Farrell 1986; Akam 1987; Scott and Carroll 1987). The homeotic gene *Antennapedia* (*Antp*) plays a central role in this developmental pathway and is required to establish the identities of the first and second thoracic segments of the fly. In fact, the absence of the *Antp* gene results in lethality during the early larval stages, with the embryos bearing a transformation of thoracic segments into more anterior structures (Wakimoto and Kaufman 1981). Understanding the mechanisms that mediate the regulated expression of *Antp* would provide further insight concerning the molecular events that determine cellular fates during *Drosophila* embryogenesis.

The molecular cloning and characterization of the *Antp* gene revealed an unusual structure comprising 100 kb of DNA and eight exons. Transcription initiates early during embryogenesis from two distinct promoters (designated P1 and P2) that are located 70 kb apart (Laughon et al. 1986; Schneuwly et al. 1986; Stroeder et al. 1986). Several studies have suggested that the expression of the *Antp* gene is regulated at the level of transcription. For example, there is a direct correlation between the cellular localization of *Antp* transcripts and protein in the embryo (Carroll et al. 1986; Wirz et al. 1986). Furthermore, the regions of the embryo altered by *Antp* mutations are identical to those in which *Antp* RNA is dis-

tributed (Levine et al. 1983; Harding et al. 1985; Martinez-Arias 1986). Recent studies indicate that transcripts resulting from P1 and P2 are localized in spatially distinct regions of the embryo (A. Martinez-Arias et al., pers. comm.), and that the two promoters are regulated independently by different *cis*-acting elements (Jorgensen and Garber 1987).

As an approach to understanding *Antp* transcription, we have analyzed the expression of the *Antp* P2 promoter in vitro. Genetic studies have identified many loci that regulate *Antp* gene expression (Hafen et al. 1984; Ingham and Martinez-Arias 1986). Undertaking a biochemical analysis of *Antp* transcription has the potential to reveal mechanisms by which genetically identified regulators of *Antp* mediate gene expression and at the same time lead to the identification of regulatory factors that have not been detected previously. In several cases, the isolation and biochemical characterization of new transcription factors from *Drosophila* has resulted in a greater understanding of the molecular events that mediate developmental regulation (Heberlein et al. 1985; Biggin and Tjian 1988).

It has been demonstrated that sequences within 2 kb upstream and 200 bp downstream of the *Antp* P2 start site contain elements required for spatially restricted expression of *Antp* P2 RNA in the *Drosophila* embryo (A. Boulet and M.P. Scott, pers. comm.). By studying *Antp* P2 transcription in extracts prepared from *Drosophila* embryos, we have detected transcriptionally important *cis*-acting DNA elements within 200 bp upstream and downstream of the *Antp* P2 start site. Several

sequence-specific DNA-binding proteins have been isolated that interact with these elements. One of these factors, *Drosophila* Transcription Factor-1 (DTF-1), has been purified to homogeneity. We show here that purified DTF-1 is capable of activating transcription from the *Antp* P2 promoter in a binding site-dependent manner.

Results

Transcription from the Antp P2 promoter in Drosophila embryo extracts

Previous studies demonstrated that a number of *Drosophila*, human, and viral promoters are transcriptionally active in nuclear extracts prepared from *Drosophila* embryos (Heiermann and Pongs 1985; Soeller et al. 1988). Furthermore, in vitro transcription from *Drosophila* promoters in extracts from embryos at different developmental stages mimics their temporal profile of expression in vivo (Biggin and Tjian 1988; Heberlein and Tjian 1988). Therefore, we used these extracts (prepared from embryos between 0 and 12 hr of development) to study the transcriptional control of the *Antp* P2 promoter in vitro. Our results indicate that the *Antp* P2 promoter is transcribed efficiently in these extracts (Fig. 1, lanes

Figure 1. Transcription from the *Antp* P2 promoter in vitro. In vitro transcription reactions (25 μ l) contained P2 5' Δ -600 DNA (0.5 μ g; described under Experimental procedures) and varying levels of the *Drosophila* embryo nuclear extract (24 mg of protein/ml) where indicated. α -Amanitin was added to a final concentration of 1 μ g/ml (lane 6). The *Antp* P2 start sites in vivo (lane 7) were detected by primer extension of total cellular RNA (60 μ g) isolated from *Drosophila* embryos as described (Heberlein and Tjian 1988). Primer 1 was utilized for primer extension analysis of in vitro- and in vivo-synthesized RNA and also for DNA sequencing. Primer 1 is a 23-nucleotide oligomer (5'-CGCGT GAAAC GACGG CAAA CCG-3') that is complementary to the sequence between +87 and +65 bp with respect to the *Antp* P2 start site. In vitro transcription and primer extension analysis were carried out as described (Heberlein et al. 1985). Reaction products were subjected to 8% polyacrylamide sequencing gel electrophoresis and visualized by autoradiography.

3–5). Initiation was found to occur from four start sites and was template and extract dependent. It was abolished in the presence of α -amanitin (1 μ g/ml), indicating that transcription is mediated by RNA polymerase II (Fig. 1, lanes 1, 2, and 6). Although *Antp* P2 transcription initiates from the same four start sites in vivo and in vitro (Fig. 1, lane 7), the efficiency with which these sites are utilized is somewhat different. In vivo there is one predominant start site, which we have defined as +1 and which corresponds to nucleotide 621 in exon C (Laughon et al. 1986). In vitro, three of the four sites (-4, +1, +5) are utilized with approximately equal efficiency. We estimate that the level of transcription in vitro from these four start sites combined is $\sim$ 0.3 transcripts per template per hour, which is consistent with the transcription rates of other *Drosophila* promoters in these extracts (Biggin and Tjian 1988).

Analysis of Antp P2 cis-acting control elements

Since we had determined that the *Antp* P2 promoter was active in embryo extracts, we could begin to identify the sequences surrounding the P2 start site that were required for transcriptional activity. Therefore, we constructed a series of deletion mutants lacking sequences upstream or downstream of the *Antp* P2 start site (Figs. 2B,3B). Analysis of 5' deletion mutants revealed the presence of at least two important transcriptional control regions (elements A and B; Fig. 2A, B). The progressive deletion of sequences between -170 and -62 bp resulted in a stepwise reduction in the level of transcription (Fig. 2A, lanes 3–9; Fig. 2B), suggesting that this region is composed of multiple positive control elements that contribute collectively to a high level of transcription. We have designated this region element A and conclude that it is involved in activation of transcription from the *Antp* P2 start site.

In contrast, deletion of sequences within the region between -36 and -21 bp resulted in an increase in transcription (approximately fourfold) from three out of four of the *Antp* P2 start sites (Fig. 2A, lanes 13–15), suggesting the presence of a negative control element, which we have designated as element B. To ascertain that the increase in transcription as a result of deleting element B was not due to cryptic positive-acting elements within the vector DNA that were brought into close proximity to the P2 start site, we constructed templates containing insertions of random DNA fragments (λ *Sau*3A DNA fragments) between the vector and *Antp* DNA. Transcription from these insertion/deletion mutants in vitro was at a level that is consistent with the hypothesis that there is a negative regulatory element between -36 and -21 bp of the *Antp* P2 promoter (data not shown).

To determine whether there were elements downstream of the *Antp* P2 start site (within the untranslated leader) that were important for transcription, we measured the activity of a series of 3' deletion mutants (Fig. 3A,B). Our results reveal the presence of at least one positive control element (element C) that lies downstream

Figure 2. Transcriptional analysis of a series of 5'-deletion mutants of the *Antp* P2 promoter. (A) Transcription reactions (25 μ l) contained supercoiled *Antp* P2 promoter deletion mutant templates (0.1 μ g of DNA; described in Experimental procedures) as indicated above each lane. Reactions also contained pHpHL supercoiled DNA (30 ng) as an internal control and *Drosophila* embryo nuclear extract (120 μ g of protein). pHpHL is a plasmid containing the *Drosophila* alcohol dehydrogenase (*Adh*) proximal promoter that has been deleted internally between -386 bp and -56 bp upstream of the start site, leaving the TATA box intact (Heberlein et al. 1985). This plasmid is transcribed at a basal level and does not respond to the addition of promoter selective factors. In vitro transcription, primer extension and analysis by polyacrylamide gel electrophoresis were carried out as described in the legend to Fig. 1. (B) Schematic representation of the *Antp* P2 5' deletion mutant series. The numbers on the right indicate the level of transcription observed (as measured by densitometric scanning of autoradiograms) with respect to *Antp* P2 5' Δ -620 DNA (100%), and were normalized to the internal control. All experiments were repeated at least four times.

of the P2 start site (between +39 bp and +26 bp). Deletion of element C abolished transcription from all four start sites. We conclude from the mutational analysis of sequences surrounding the *Antp* P2 start site, that there are at least three distinct elements that regulate the level of *Antp* P2 transcription in vitro: positive and negative elements upstream (elements A and B, respectively), and a positive element downstream (element C) from the *Antp* P2 start site.

Detection of factors that interact with cis-regulatory elements

The next step was to identify sequence-specific DNA-binding factors that might be involved in the regulation of *Antp* P2 transcription. Therefore, we carried out DNase I footprinting analysis of DNA fragments spanning the promoter (see legend to Fig. 4). A protein fraction derived from S300 gel filtration chromatography was used, which contained many of the DNA binding

Figure 3. In vitro transcription of a 3' deletion mutant series. (A) Transcription reactions (25 μ l) contained the *Antp* P2 promoter deletion mutants (0.1 μ g of DNA; constructed as described under Experimental procedures) as indicated above each lane. Reactions also contained plasmid pHPHL (30 ng) as an internal control and *Drosophila* embryo nuclear extract (120 μ g of protein). In vitro transcription, primer extension, and analysis by polyacrylamide gel electrophoresis were carried out as described (legend to Fig. 1). Primer 1 (legend to Fig. 1) was used for primer extension analysis of transcription reactions in lanes 1–3. Primer 2 was used for primer extension analysis of transcription reactions in lanes 4–12. The efficiency of transcription from the *Antp* P2 5' Δ +92 mutant as measured by either of these primers is equivalent. Primer 2 is a 22-nucleotide oligomer (5'-TACGA ATTCG AGCTC GGTAC CC-3') that is complementary to the polylinker in plasmid pUC118. The distance between the polylinker and the *Antp* P2 start sites decreases as *Antp* P2 DNA 3' of the start site is deleted, resulting in the production of progressively smaller extension products. (B) Schematic representation of 3' deletion mutants. The numbers on the left indicate the level of transcription observed [as measured by densitometric scanning of autoradiograms] with respect to *Antp* P2 3' Δ +658 DNA (100% using primer 1 or primer 2).

activities present in the nuclear extract (data not shown). Footprinting with this partially purified fraction resulted in DNase I protection of several distinct regions surrounding the *Antp* P2 start site (designated I–V, Fig. 4A), a subset of which coincided with elements A and B (that we have determined to be important for transcriptional activation).

Factors binding to the region downstream of the P2 start site were detected using a nuclear extract and a DNA fragment spanning the region between –62 bp and +358 bp (Fig. 4B). Approximately four protected regions were observed (designated W, X, Y, and Z; Fig. 4B, lanes 6 and 7). Interestingly, one footprint (region W) overlaps with the 3' element C, suggesting that this factor might be a transcriptional activator. These DNase I footprinting analyses indicate that several regions surrounding the *Antp* P2 promoter are recognized by sequence-specific DNA-binding proteins present in *Drosophila* embryos.

Separation of factors that bind to the *Antp* P2 promoter

Having detected multiple DNA binding activities that interact with sequences coincident or overlapping with

transcriptional control elements of the *Antp* P2 promoter, it was important to separate the various DNA-binding proteins and study their biochemical properties individually. As a first step toward characterizing these putative transcription factors, three DNA-binding proteins that recognize sequences between +1 and –110 bp were isolated using sequence-specific DNA affinity chromatography (see Experimental procedures).

One factor, designated DTF-1 (Fig. 5A, lanes 2 and 3) recognized specifically four sites within protected regions II and III. A sequence GCAACA^T/_C^G/_C was found that is reiterated at least four times throughout regions II and III, suggesting that this octamer sequence may represent a recognition element for DTF-1. In support of this idea, the putative recognition sequence was present on all three different affinity columns used to isolate DTF-1 (see Experimental procedures), but each synthetic octamer sequence was flanked by different adjacent sequences. Therefore, we conclude that this factor recognizes the sequence GCAACA^T/_C^G/_C. Interestingly, three of these consensus sequences lie within the positive transcriptional control element A.

Two other DNA-binding proteins were isolated that appear to recognize overlapping sequences within foot-

Figure 4. Detection of factors binding to the *Antp* P2 promoter. (A) Reactions contained the indicated amounts of the S300 pooled fractions (1.2 mg/ml; lanes 2 and 3). The footprinting probe used was an *MluI*–*NsiI* restriction fragment (3 fmoles, spanning the distance between +85 and –391 bp with respect to the *Antp* P2 start site) from plasmid P2 5'Δ–620. The probe was 5'-end-labeled with [γ - ^{32} P]ATP and T4 polynucleotide kinase at the *MluI* site. Brackets on the left depict the regions of DNA (I–V) that are protected from DNase I cleavage, and the numbers on the right indicate the boundaries (in bp) of each of the footprints. (B) Reactions contained the indicated amounts of the embryo nuclear extract (10–40% AS, 24 mg of protein/ml; lanes 6 and 7). The footprinting probe used was an ASP718/*NarI* restriction fragment (3 fmoles, spanning the distance between –62 and +358 bp with respect to the *Antp* P2 start site) from plasmid *Antp* P2 5'Δ–62. The probe was 5'-end-labeled with [γ - ^{32} P]ATP and T4 polynucleotide kinase at the ASP718 site. Brackets on the left depict the regions of DNA (W,X,Y,Z) that are protected from DNase I digestion, and the numbers on the right indicate the boundaries (in bp) of each of the footprints. All DNase I footprinting reactions (50 μ l) contained nonspecific DNA [1 μ g of poly(dI-dC)] and were carried out as described (Heberlein et al. 1985). Reactions were analyzed by 6% polyacrylamide sequencing gel electrophoresis and visualized by autoradiography.

printing region I. One factor was identified subsequently as *Drosophila* AP-1 (dAP-1; Fig. 5C, lanes 13 and 14), a homolog of mammalian enhancer binding factor AP-1 (K.K. Perkins and R. Tjian, submitted). The other factor, designated *Drosophila* Factor-2 (DF-2; Fig. 5B, lanes 8 and 9) has also been found to recognize elements within the *Ultrabithorax* (*Ubx*) promoter that are known to be binding sites for homeo box-containing proteins (K.K. Perkins, M. Biggin and R.J. Tjian, unpubl.), suggesting that similar interactions with homeo box proteins are also taking place on *Antp* P2.

Purification and identification of DTF-1

Since DTF-1, which appears to be a novel transcription factor, interacted with several sites located within a control element upstream from the *Antp* P2 start site, it was of interest to determine its activity as a transcription factor in vitro. Therefore, DTF-1 was purified to homogeneity from *Drosophila* embryo extracts using sequence-specific DNA affinity chromatography followed

by high-performance liquid chromatography. Fractions from each stage of the purification were assayed for DTF-1-specific DNA binding activity. Active fractions were then analyzed by SDS gel electrophoresis (Fig. 6A). We estimate that from 250 grams of *Drosophila* embryos ~40 μ g of DTF-1 were obtained. Purified DTF-1 contained two polypeptide species that migrated as a doublet of ~50 kD and was purified ~33,000-fold.

To demonstrate unequivocally that the 50-kD polypeptide doublet possessed DTF-1-binding activity, we carried out renaturation of the protein eluted from an SDS–polyacrylamide gel. For this experiment, affinity-purified DTF-1 was subjected to SDS gel electrophoresis. The gel was divided into different slices (as indicated in Fig. 6B), and protein was eluted from each slice, renatured, and assayed for DTF-1 binding activity (Fig. 6C). Only protein eluted from gel slice 4 that contained the 50-kD polypeptide doublet possessed DTF-1 binding activity (Fig. 6C, lane 12). These results confirm that purified DTF-1 resides in a protein doublet of 50 kD.

Figure 5. DNase I footprinting of isolated factors on the *Antp* P2 promoter. (A–C) DNase I footprinting was carried out as described (Heberlein et al. 1985) using the footprinting probe (3 fmoles) described in Fig. 4A. Reactions contained the indicated amount of DTF-1, DF-2, or dAP-1 isolated as described in Experimental procedures. The *Antp* P2 promoter and the various regions footprinted by the S300 pool (I,II,III) are depicted by the drawing on the left. The brackets on the right of each footprint indicate the boundaries of the various footprints.

DTF-1 activates transcription in vitro

The *in vitro* transcriptional activity of DTF-1 was measured by using a *Drosophila* extract that had been depleted of DTF-1 using the DTF-1 sequence-specific affinity resin. In the DTF-1-depleted extract, the level of transcription from templates containing either four DTF-1 binding sites or lacking all DTF-1 sites was equally low (Fig. 7A, lanes 3 and 4). However, addition of increasing amounts of purified DTF-1 resulted in a substantial increase in the level of transcription (approximately sevenfold) that was dependent upon the presence of DTF-1 binding sites. These results indicate that DTF-1 can act directly as a sequence-specific transcription factor.

Since DTF-1 binds to four sites within the *Antp* P2 promoter region, it was of interest to determine the number of binding sites required for transcriptional activation by DTF-1. Therefore, we measured the level of transcription from templates that either lacked DTF-1 sites or possessed one, two, or four DTF-1 sites. As expected, extracts depleted of DTF-1 directed transcription from all four templates at equally low levels (Fig. 7B, lanes 15–18). Addition of purified DTF-1 resulted in a

substantial enhancement of transcription from templates containing either two or four DTF-1 binding sites (Fig. 7B, lanes 19 and 20). In contrast, the addition of DTF-1 had no effect on templates that lacked DTF-1 sites or on templates that contained only the promoter-proximal DTF-1 binding site (Fig. 7B, lanes 21 and 22). However, the inability of the template containing only one DTF-1 binding site to respond transcriptionally to added DTF-1 is not due to a lack of DTF-1 DNA binding, because DNase I footprinting confirmed that the single GCAACAT^T/_C^G/_C site is fully capable of binding DTF-1 (data not shown).

Discussion

In vitro transcription from the *Antp* P2 promoter

A striking feature of the *Antp* P2 promoter is that it consists of several distinct types of regulatory elements including positive as well as negative elements that are clustered within 170 bp upstream of the start site (Fig. 8), and these elements respond efficiently to specific *Drosophila* embryo DNA-binding factors in cell-free transcription reactions. Furthermore, *Antp* P2 contains at least one downstream *cis*-acting control element that

Figure 6. Purification and identification of DTF-1. (A) Protein fractions from various stages of the purification were subjected to 10% SDS–polyacrylamide gel electrophoresis and visualized by silver staining. The left lane displays molecular weight standards (M, sizes in kD). The amount of protein in each subsequent lane is as follows: nuclear extract, 20 μ g of protein, 0.4 footprinting units; S300, 6 μ g of protein, 0.8 footprinting units; second affinity, 0.3 μ g of protein, 150 footprinting units; HPLC fractions, 0.3 μ g of protein, 150 footprinting units. (B) SDS–polyacrylamide gel analysis of second-pass affinity-purified DTF-1. The numbers and brackets on the right indicate the boundaries of each gel slice that was used for renaturation [according to the method of Briggs et al. 1986]. (C) DNase I footprinting analysis of second-pass affinity-purified DTF-1 (lane 8), and of protein eluted and renatured from the SDS–polyacrylamide gel slices (lanes 9–13, and depicted in Fig. 6B). The footprinting probe used was described in the legend to Fig. 4. Reaction products were subjected to 8% polyacrylamide sequencing gel electrophoresis and visualized by autoradiography.

lies within 40 bp of the start site. Thus, it is likely that there are various synergistic and antagonistic interactions that are combined to regulate *Antp* P2. Therefore, this promoter provides an ideal system with which to analyze the interplay of multiple transcription factors and how they contribute to complex patterns of gene expression during embryogenesis.

To begin the analysis, we have demonstrated that RNA synthesis from *Antp* P2 is accurate and efficient in *Drosophila* embryo nuclear extracts. The presence of four different initiation sites on the *Antp* P2 promoter is intriguing. It is possible that *Antp* P2 is transcribed from multiple sites because it lacks an upstream TATA homology which is thought partly to be responsible for fixing the transcription initiation site (Mathis and Chambon 1981). The *Drosophila engrailed* (*en*) and *Deformed* (*Dfd*) genes also lack TATA boxes (Regulski et al. 1987; Soeller et al. 1988). Transcriptional analysis of *en* in vitro has revealed that it also utilizes multiple transcription initiation sites. The possibility exists that

these genes contain other sequences that function to position the start site. It has been suggested that the sequence TCGCCAGCG between -9 and -1 bp upstream of the *Dfd* start site might be involved in positioning the site of transcription initiation, and that this sequence is similar to the *Antp* P2 sequence TCACTGGCG that lies between -3 and -11 bp (Regulski et al. 1987). However, the transcriptional analysis of 5' deletion mutants reported here indicates that deletion of this sequence has no apparent effect on the *Antp* P2 initiation site. The possibility remains that this sequence plays a role in the in vivo expression of *Antp* and *Dfd*, but further experiments would be required to establish this point.

The negative transcriptional control element upstream of the Antp P2 start site

Our experiments suggest that transcription of *Antp* P2 is partly under the control of a negative *cis*-acting element. Deletion or substitution within control element B re-

Figure 7. Primer extension analysis of DTF-1 transcriptional activation. (A) Transcription reactions (25 μ l) contained either *Antp* P2 5'Δ-148 supercoiled DNA (0.1 μ g, lanes 1, 3, 5, 7, and 9) or *Antp* P2 5'Δ-36 supercoiled DNA (0.1 μ g, lanes 2, 4, 6, 8, and 10), pHpHL (30 ng) as an internal control, and *Drosophila* embryo nuclear extract (120 μ g of protein, lanes 1 and 2) or extract that had been depleted of DTF-1 using a sequence-specific affinity resin (see Experimental procedures). Reactions were supplemented with the following amounts of second-pass affinity-purified DTF-1: 2 μ l (3 footprinting units/ μ l); 5 μ l; 7.5 μ l, where indicated. (B) Transcription reactions (25 μ l) contained either *Antp* P2 5'Δ-148 supercoiled DNA (0.1 μ g, lanes 11, 15, and 19) or *Antp* P2 5'Δ-79 supercoiled DNA containing two DTF-1 sites (0.1 μ g, lanes 12, 16, and 20) or *Antp* P2 5'Δ-62 supercoiled DNA containing one DTF-1 site (0.1 μ g, lanes 13, 17, and 21) or *Antp* P2 5'Δ-36 supercoiled DNA lacking DTF-1 binding sites (0.1 μ g, lanes 14, 18, and 22). Reactions contained either *Drosophila* embryo nuclear extract (120 μ g of protein) as indicated, or nuclear extract that had been depleted of DTF-1 using a sequence-specific affinity resin (see Experimental procedures) as indicated. Reactions in lanes 19–22 were supplemented with 5 μ l (15 footprinting units) of second-pass affinity-purified DTF-1.

sulted in an increase in the level of transcription from the *Antp* P2 promoter. We speculate that this negative element could function on its own to repress *Antp* P2 during certain stages of development or in different cell types in which the positively acting factors are absent. Alternatively, the negative element could interact with the positive control elements of *Antp* P2 to fine-tune the expression of *Antp*. Several cases involving negative control of transcription in eukaryotic cells have been reported (Rio et al. 1980; Goodbourn et al. 1986; Jones 1986; Sassone-Corsi et al. 1987). Our studies have, thus far, not led to the identification of negatively acting transcription factors. Two of the factors we have isolated, *Drosophila* Factor-2 (DF-2) and *Drosophila* AP-1, recognize sequences that overlap with the negative element. However, the role of these factors in the regulation of *Antp* P2 expression is not yet clear. Remarkably, we have found that dAP-1 is the functional *Drosophila* homolog of the mammalian nuclear *c-jun* proto-oncogene product (K.K. Perkins, G.M. Dailey, and R. Tjian, in prep.). In addition, preliminary experiments suggest that DF-2 may be a homeo box-containing factor that also recognizes regulatory elements of the *Ubx* promoter (K.K. Perkins et al. unpubl.).

Positive transcriptional control elements of the *Antp* P2 promoter

We have demonstrated the presence of a positive *cis*-acting control element upstream from the *Antp* P2 start site (element A). Progressive deletion of sequences within element A resulted in a gradual decrease in the level of transcription, suggesting that element A is composed of multiple elements that contribute collectively to the overall level of transcription. For example, transcription factor DTF-1 binds tandem GCAACAT^T/C^G/C sequences within element A in a manner reminiscent of Sp1 binding to multiple sites in the 21-bp repeats of the SV40 promoter (Dynan and Tjian 1983). Moreover, we have purified DTF-1 to homogeneity and demonstrated that this sequence-specific factor can activate high levels of *Antp* P2 transcription in a reconstituted reaction. Since embryo extracts depleted of DTF-1 transcribed P2 at a very low basal level and transcription is fully restored by purified factor, we conclude that DTF-1 must play a major role in directing the transcription of *Antp* P2 *in vitro*. The importance of DTF-1 in the regulation of *Antp* P2 expression during embryogenesis can best be addressed after we have isolated the gene encoding DTF-1.

Why is the DTF-1 binding site reiterated several times within element A? One possibility is that the binding of clustered DTF-1 protomers is required to activate fully the *Antp* P2 promoter. Consistent with this hypothesis is the finding that the promoter-proximal DTF-1 site alone is not sufficient for transcriptional activation even though DTF-1 binds efficiently to this site. Our data suggest that at least two DTF-1 sites are required for DTF-1-mediated transcriptional activation, and that a synergistic interaction of multiple DTF-1 protein molecules may be required to trigger transcription efficiently. Further analyses of DTF-1 binding sites by generating substitution mutants may shed light on the mechanism of DTF-1 activation and its interaction with this complex array of *cis*-control elements.

Preliminary DNase I footprinting experiments using nuclear extracts from embryos harvested at different developmental stages suggest that DTF-1 is present early

in embryogenesis (between 0 and 4 hr) and during the intermediate stages of embryogenesis (between 8 and 12 hr), but is not detectable late in embryogenesis (between 16 and 20 hr; K.K. Perkins and R. Tjian, data not shown). This correlates with the established temporal pattern of expression from the *Antp* P2 promoter in the embryo (Schneuwly et al. 1986). These findings raise the possibility that DTF-1 contributes to the temporal regulation of the *Antp* P2 promoter as well as to the regulation of other genes expressed during the early to middle stages of embryogenesis. However, at present we have not detected the consensus sequence $GCAACA^{T/C}G/C$ on other characterized promoters.

It is possible that in addition to DTF-1 there are other factors not yet detected by our experiments that interact with element A to contribute to transcription from *Antp* P2. For example, we have found that purified GAGA transcription factor that interacts with both the *Ubx*

Figure 8. Summary of transcriptional control elements and factor binding sites. (A) The nucleotide sequence of the *Antp* P2 promoter region is shown. The numbers (–250 to +40) refer to the distance from the transcription initiation site (+1). The arrows refer to the four start sites. The shaded boxes refer to the transcriptional control elements (A, B, and C). The DTF-1 consensus sequence $GCAACA^{T/C}G/C$ is displayed in boxes. The brackets refer to footprinting regions recognized by proteins in the S300 fraction and nuclear extract (FP I, II, III, IV, V, W). Regions X, Y, and Z are not within the boundaries of sequences shown. (B) A schematic diagram of the *Antp* P2 promoter is shown. The arrow represents the transcription start site. Protein factors (depicted as ovals) bind to specific transcriptional control elements (as represented by the shaded boxes and identified below each box). The identities of the factors that interact with element C are presently unknown.

promoter (Biggin and Tjian 1988) and the *en* promoter (Soeller et al. 1988) also weakly interacts with sequences within element A (K.K. Perkins and R. Tjian, unpubl. data). It is possible that the GAGA factor interacts with DTF-1 to coordinate the level of transcription from the *Antp* P2 promoter. Further experiments are required to elucidate the role, if any, of the GAGA factor in *Antp* P2 transcription.

Interestingly, we have detected a transcriptional control element with the 5'-untranslated leader of *Antp* P2 (element C) that is active in vitro. Although *cis*-acting elements located downstream of the transcription initiation site are unusual in mammalian and viral genes, they appear to be more common in *Drosophila* promoters. For example, the *en* (Soeller et al. 1988), *Ubx* (Biggin and Tjian 1988), and 74EF ecdysone-responsive (C. Thummel, pers. comm.) promoters all contain important downstream regulatory sequences. This raises the possibility that these promoters share similar mechanisms for transcriptional activation. It has been found that both *Ubx* and *en* contain a consensus sequence with their downstream transcriptional control element that is recognized by homeo-domain-containing proteins (Biggin and Tjian 1988). However, the consensus sequence that is found on *Ubx* and *en* is not present within the 3' transcriptional control element of *Antp* P2. This suggests that the regulation of *Antp* P2 by the downstream transcriptional regulatory element we have defined here is mediated by proteins distinct from those regulating *en* or *Ubx*. In the future, we hope to purify the factors that interact with the downstream regulatory sequences of *Antp* P2. Together with the transcription factors we have already isolated that interact with the upstream regulatory elements, it should be possible to initiate a more complete study of the biochemical mechanisms underlying the regulation of *Antp*.

Experimental procedures

Plasmid DNA

The *Antp* P2 promoter recombinants were derived from an *EcoRI* subclone of λ phage 448 (448 R4.6; Laughon et al. 1986). *Antp* P2 5'-620 contains sequences from the *HindIII* site at -620 bp to the *EcoRI* site at +658 bp (with respect to the *Antp* P2 start site) inserted into a pUC118 vector previously digested with *HindIII* and *EcoRI*. The *Antp* P2 5' and 3' deletion mutations were generated by *Bal31* nuclease digestion of templates previously cut with *NsiI* (-391 bp) or *NarI* (+358 bp). *KpnI* linkers were ligated to the deletion end points. *Antp* P2 5' deletion mutants contain sequences from the *EcoRI* site (+658 bp) to the *KpnI* site at the deletion end point inserted into an *EcoRI*-*KpnI*-digested pUC118 vector. *Antp* P2 3' deletion mutants contain sequences from the *HindIII* site (-620 bp) to the *KpnI* site at the deletion end point inserted into a *HindIII*-*KpnI* digested pUC118 vector. All procedures were carried out according to Maniatis et al. (1982).

Drosophila embryo nuclear extracts

Canton-S wild-type flies were maintained in population cages at 25°C. Embryos were collected on yeasted molasses-agar

trays for 12 hr and stored for up to 3 days at 4°C, as described (Biggin and Tjian 1988). Nuclear extracts were typically prepared from 125 grams of embryos essentially as described (Soeller et al. 1988), except that nuclei were not pelleted by centrifugation in sucrose. The final ammonium sulfate pellet was resuspended in a volume corresponding to one-fourth the weight of the starting material (31 ml) in a buffer containing 25 mM HEPES-KOH (pH 7.6), 40 mM KCl, 12.5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol (0.04 M/HEMG). The resulting suspension was dialyzed and centrifuged as described (K.K. Perkins and R. Tjian, submitted). The resulting nuclear extract (~24 mg of protein/ml) was stored at -70°C. Nuclear extracts utilized in reconstituted reactions were depleted as follows. An aliquot of extract (500 μ l) was mixed in a 1-ml plastic tube with DTF-1 sequence-specific affinity resin (250 μ l; described below), and centrifuged (K.K. Perkins and R. Tjian, submitted). The depleted extract was stored at -70°C.

Separation of *Antp* P2 DNA-binding proteins

All steps were carried out at 4°C. To separate *Antp* P2 promoter DNA-binding proteins, the nuclear extract was prepared from 0- to 12-hr embryos (125 grams of embryos) as described above, except that the supernatant from the ammonium sulfate nuclear lysis was brought to 40% saturation by the addition of 0.17 g/ml of solid ammonium sulfate (K.K. Perkins and R. Tjian, submitted). The resulting fraction (10-40% AS, 24 mg of protein/ml) was stored at -70°C and retained 100% of the *Antp* P2 promoter DNA-binding activities for at least 1 year.

Two 10-40% AS fractions were pooled (32 ml, 24 mg of protein/ml) and fractionated by heparin-agarose chromatography (heparin fraction 33 ml, 11 mg of protein/ml) followed by Sephacryl S300 gel filtration chromatography (K.K. Perkins and R. Tjian, submitted). The fractions from the S300 column containing *Antp* promoter DNA-binding activity were pooled (S300 fraction 310 ml, 1.2 mg of protein/ml), supplemented with Nonidet P-40 (NP-40, 0.1%) and poly(dI:dC) (3.9 mg), stirred for 10 min at 4°C, and then centrifuged at 10,000 rpm for 10 min in a Sorvall SS34 rotor.

The *Antp* P2 promoter DNA-binding proteins were then separated using 1-ml sequence-specific DNA affinity columns (Kadonaga and Tjian 1986) previously equilibrated with 0.1 M/HEG [25 mM HEPES-KOH (pH 7.6), 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 0.1 M KCl] containing 0.1% NP-40. All first affinity column buffers contained 0.1% NP-40. The S300 fraction was applied sequentially to four different affinity columns (1-4) so that the flowthrough from one column was immediately applied to the next column. Affinity columns 1-4 each consisted of DNA sequences of a distinct region upstream of the *Antp* P2 start site. Each column was eluted with three column volumes of 1 M KCl/HEG and assayed for *Antp* P2 promoter DNA-binding. The complementary oligodeoxynucleotides used for the columns were as follows. Column 1 consisted of sequences between -83 bp and -58 bp within footprinting region III (containing the complementary oligonucleotides 5'-GATCC CGCAG CAGCA ACATG TTGCC GGCGG-3', and 5'-GATCC CGCCG GAACA TGTTG CTGCT GCGG-3'). Column 2 consisted of sequences between -116 bp and -85 bp within footprinting region III (containing the complementary oligonucleotides 5'-GATCG GTGGC CGTGT TGCTG CTG CT CTTGA TGTTC C-3' and 5'-GATCG CAACA TCAAG AGCAG CAGCA ACACG GCCAC C-3'). Column 3 consisted of sequences between -55 bp and -27 bp within region II (containing the complementary oligonucleotides 5'-GATCG CCATT GGCAA CATGC GATGT TTACG AGC-3' and 5'-GATCG CTCGT AAACA TCGCA TGTTC CCAAT GGC-3').

Column 4 consisted of sequences between -24 bp and $+5$ bp within footprinting region I (containing the complementary oligonucleotides 5'-GATCG GCTCA TTGTG ATTCA CTGGC GTTCA GTT-3' and 5'-GATCA ACTGA ACGCC AGTGA ATCAC AATGA GCC-3'). The activity that eluted from column 1 (DTF-1) which recognized sequences between -83 bp and -58 bp (within footprinting region III) also recognized sequences between -116 bp and -83 bp and between -55 bp and -27 bp according to DNase I footprinting experiments, although no DNA-binding activity eluted from columns 2 and 3.

To separate the two overlapping DNA-binding activities that eluted from column 4 and that recognized *Antp* promoter sequences between $+5$ and -29 bp, the S300 fraction (310 ml, 1.2 mg of protein/ml) was instead applied directly to three DNA affinity columns (containing the sequences of column 4) that had previously been equilibrated with 0.1 M/HEG. The column was washed with 10 volumes of 0.1 M/HEG and then step eluted with HEG containing 0.2 M, 0.5 M, and 1.0 M KCl (3 ml each step). The dAP-1 footprinting activity was detected in the 0.5 M eluate; activity was pooled (first affinity, 8 ml, 80 μ g of protein/ml) diluted to 0.1 M KCl/HEG and mixed with poly(dI:dC) (16 μ g) as described above, and applied to a second DNA affinity column (column 5; 1 ml) previously equilibrated with 0.1 M/HEG containing 0.1% LDAO. (0.1% LDAO was used instead of NP-40 in all second-pass affinity column buffers). The complementary oligodeoxynucleotides used to prepare the resin (column 5) were 5'-GATCG TGACT CAGCG CG-3' and 5'-GATCC GCGCT GAGTC AC-3'. The column was washed with 10 volumes of 0.1 M/HEG, step-eluted with HEG containing 0.2 M and 0.5 M KCl (3 ml each step), and then eluted with a step gradient containing 1 ml each of 0.6 M, 0.7 M, 0.8 M, 0.9 M, and 1 M KCl in HEG. dAP-1 activity was obtained in the 0.7 M and 0.8 M KCl eluates (second affinity dAP-1, 2 ml, 10 μ g of protein/ml), and DF-2 was obtained in the 0.2 M KCl eluate (second affinity DF-2, 2 ml, 100 μ g of protein/ml).

Purification of DTF-1

DTF-1 was isolated as described above, with the following modifications. The S300 fraction was applied to an affinity column (column 6) previously equilibrated with 0.1 M KCl/HEG. The oligonucleotide used for column 6 was the palindromic sequence 5'-GATCG CAACA TGTTG C-3'. The column was eluted with three column volumes each of HEG containing 0.2 M, 0.3 M, 0.4 M, 0.5 M, and 1.0 M KCl. The DTF-1 footprinting activity that eluted with 0.4 M KCl was pooled (first affinity 6 ml, 100 μ g of protein/ml), diluted to 0.1 M KCl/HEG and subjected to a second cycle of DNA affinity chromatography using column 6. The second affinity column was eluted with three column volumes each of HEG containing 0.2 M, 0.3 M, and 1.0 M KCl. DTF-1 footprinting activity was obtained in the 0.3 M KCl eluate (second affinity 4 ml, 10 μ g of protein/ml). The protein concentration in affinity-purified fractions was estimated by SDS-polyacrylamide gel electrophoresis and silver staining. The fraction was stored at -70°C .

Purification of DTF-1 by HPLC was carried out as described for dAP-1 [K.K. Perkins and R. Tjian, submitted] except that 100 μ l of a second affinity fraction was used as the starting material.

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