Locus-specific variation in phosphorylation state of RNA polymerase II in vivo: correlations with gene activity and transcript processing

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To investigate functional differences between RNA polymerases IIA and IIO (Pol IIA and Pol IIO), with hypo- and hyperphosphorylated carboxy-terminal repeat domains (CTDs), respectively, we have visualized the in vivo distributions of the differentially phosphorylated forms of Pol II on *Drosophila* polytene chromosomes. Using phosphorylation state-sensitive antibodies and immunofluorescence microscopy with digital imaging, we find Pol IIA and Pol IIO arrayed in markedly different, locus- and condition-specific patterns. Major ecdysone-induced puffs, for example, stain exclusively for Pol IIO, indicating that hyperphosphorylated Pol II is the transcriptionally active form of the enzyme on these genes. In striking contrast, induced heat shock puffs stain strongly for both Pol IIA and Pol IIO, suggesting that heat shock genes are transcribed by a mixture of hypo- and hyperphosphorylated forms of Pol II. At the insertion sites of a transposon carrying a hybrid *hsp70-lacZ* transgene, we observe only Pol IIA before heat shock induction, consistent with the idea that Pol II arrested on the *hsp70* gene is form IIA. After a 90-sec heat shock, we detect heat shock factor (HSF) at the transposon insertion sites; and after a 5-min shock its spatial distribution on the induced transgene puffs is clearly resolved from that of Pol II. Finally, using antibodies to hnRNP proteins and splicing components, we have discerned an apparent overall correlation between the presence and processing of nascent transcripts and the presence of Pol IIO.

[Key Words: RNA polymerase IIA/IIO; CTD phosphorylation; transcription factors; in situ localization; immunofluorescence microscopy; digital imaging]

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The carboxy-terminal repeat domain (CTD) of the largest subunit of RNA polymerase II (Pol II) is an unusual entity composed of multiple repeats of the 7-amino-acid consensus sequence YSPTSPS (for review, see Corden 1990; Young 1991). The number of repeats ranges from 26 in yeast, to 42 in *Drosophila*, to 52 in mammals. The CTD has been shown to carry out essential in vivo roles in yeast (Nonet et al. 1987), *Drosophila* (Zehring et al. 1988), and mammalian cells (Bartolomei et al. 1988), but precisely what those roles are is not well understood. Suggested roles for the CTD include interacting with transcription initiation factors, serving as a molecular "cowcatcher" to facilitate movement of polymerase on chromatin templates, providing a link between transcription and RNA processing, and localizing polymerase to specific nuclear compartments (Corden 1990).

Most investigations implicate the CTD in a role re-

lated to transcription initiation. For example, Pol II carrying altered CTDs responds differently to certain wild-type and mutant transcriptional regulators in vivo (Allison et al. 1988; Scafe et al. 1990; Peterson et al. 1991), suggesting that the CTD may be involved in receiving regulatory signals at certain promoters. In vivo and in vitro experiments suggest that these responses might be mediated through the TATA-binding protein (TBP) component of TFIID (Koleske et al. 1992; Usheva et al. 1992; Thompson et al. 1993). On the other hand, the precise nature of the CTD involvement in initiation is not known, and questions of its generality are raised by in vitro results demonstrating that for a number of different promoters Pol II lacking a CTD can function normally in factor-dependent promoter recognition and transcription initiation (Zehring et al. 1988; Kim and Dahmus 1989; Thompson et al. 1989; Buratowski and Sharp 1990; Zehring and Greenleaf 1990; Buermeier et al. 1992).

A notable feature of the CTD is that it can be found in vivo in a hyperphosphorylated form (for review, see Dahmus and Dynan 1992) that carries phosphates predominantly on serine, with minor amounts on threonine. The

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unphosphorylated form of the largest subunit is called subunit IIA, and the form with a hyperphosphorylated CTD is called IIO; these forms can be distinguished by their different mobilities in SDS gels, where form IIO displays a mobility markedly less than that of IIA. The enzymes containing subunit forms IIA and IIO are called Pol IIA and Pol IIO, respectively.

Evidence for functional differences between RNA Pol IIA and Pol IIO was obtained by photoaffinity cross-linking experiments in HeLa extracts or nuclei (Bartholomew et al. 1986; Cadena and Dahmus 1987). Nascent RNA chains were found to be preferentially linked to IIO rather than IIA, arguing that in HeLa cells elongating Pol II is predominantly IIO. Consistent with the cross-linking results, several *in vitro* studies indicate that Pol IIA participates in preinitiation complex formation and that before or concurrent with initiation of RNA synthesis IIA is converted to IIO by CTD phosphorylation (Laybourn and Dahmus 1990; Lu et al. 1991; Chesnut et al. 1992). This would suggest that a CTD kinase activity should be part of the preinitiation complex. A purified transcription factor called TFIIF (or BTF2) in humans (Lu et al. 1992), δ in rats (Serizawa et al. 1992), or factor b in yeast (Gileadi et al. 1992) appears to have an associated CTD kinase activity. Some of the results in these studies, however, indicate that the question of how CTD phosphorylation is related mechanistically to initiation has yet to be resolved. For example, it was found that Pol IIB, which lacks the CTD, can replace Pol IIA in the *in vitro* human system, confirming that at least for some promoters preinitiation complex formation and initiation can occur even in the absence of the CTD. Furthermore, Pol IIB still requires TFIIF; thus, this factor must perform a role or roles in addition to CTD phosphorylation (Lu et al. 1992). Finally, a recent study has functionally uncoupled CTD phosphorylation from transcription initiation (and elongation) *in vitro* (Serizawa et al. 1993).

In attempts to better understand CTD phosphorylation and how it may modulate the properties of Pol II, a number of investigators have sought to identify and purify CTD kinases. For example, we previously purified a yeast CTD kinase based on its ability to hyperphosphorylate and retard the mobility of a fusion protein carrying a full-length CTD (Lee and Greenleaf 1989). Antibodies prepared against subunits of this yeast enzyme were used to clone the *CTK1* gene that encodes its α -subunit; sequence analysis indicated that this subunit is probably catalytic (Lee and Greenleaf 1991). Gene disruption studies revealed that cells lacking a functional *CTK1* gene (and the CTK1-containing CTD kinase) display a slow-growth, cold-sensitive lethal phenotype; thus the CTK1-containing CTD kinase (CTDK-I) is essential for normal growth of yeast. Furthermore, although mutant cells growing at the permissive temperature contain phosphorylated forms of the RNA Pol II largest subunit, the phosphorylation state of the CTD appears to be abnormal (Lee and Greenleaf 1991). Although these data do not relate this kinase to a particular step in the transcription cycle, they suggest strongly that yeast CTDK-I plays a role in CTD phosphorylation *in vivo*. They suggest fur-

ther that other CTD kinases exist in yeast; one such kinase that appears to be distinct from CTDK-I might be the activity associated with transcription factor b.

Additional CTD kinases that have been purified extensively are a template-associated protein kinase from HeLa cells (Dvir et al. 1993, and references therein), CTD kinases KI, KII, and KIII from *Aspergillus* (Stone and Reinberg 1992), kinases CTDK1 and CTDK2 from HeLa cells (Payne and Dahmus 1993), and *cdc2*-containing CTD kinases E1 and E2 from mouse (Zhang and Corden 1991, and references therein). Several of these enzymes display properties suggestive of physiological roles, but none has yet been demonstrated to function in CTD phosphorylation *in vivo*. Still other CTD kinase activities have been identified in extracts from mammalian (Stevens and Maupin 1989; Legagneux et al. 1990) or plant (Guilfoyle 1989) cells.

The observations summarized above have not yet led to a clear picture of CTD function and phosphorylation, especially *in vivo*. Clarifying the situation will certainly require more information about the *in vivo* behavior of the different forms of Pol II in different cells, on different genes, and under different physiological conditions. For example, the HeLa photo cross-linking results mentioned above did not completely exclude the possibility that IIA as well as IIO can be transcriptionally active for at least two reasons. First, some RNA was cross-linked to IIA. Second, HeLa cells appear to contain very little IIA, in contrast to a number of other cell types (e.g., Kim and Dahmus 1986; Kolodziej et al. 1990; Lee and Greenleaf 1991; also see below); it is possible that similar experiments in a cell type with a higher proportion of IIA would yield more nascent RNA linked to this form of polymerase. Similarly, most of the studies relating CTD phosphorylation to initiation *in vitro* have used HeLa components and a very limited number of promoters; thus, the generality of the results is not known. Finally, there is currently insufficient information to understand the functional or structural relationships among the several CTD kinases identified in different systems.

We believed that assessing the association of RNA polymerase forms IIA and IIO with specific chromosomal sites *in vivo* could provide a needed base line of Pol II behavior and contribute to an eventual elucidation of the physiological significance of CTD phosphorylation. A good experimental system for visualizing locus-specific distributions of differentially phosphorylated forms of Pol II appeared to be the giant polytene chromosomes found in *Drosophila* larval salivary glands. Chromosomal proteins can be covalently cross-linked *in situ* to these chromosomes and observed in the light microscope by immunofluorescence or immunochemical techniques (Greenleaf et al. 1976; Silver and Elgin 1976; Zink et al. 1991; for review, see Clark et al. 1991). The patterns of transcriptional activity for many loci of the polytene chromosomes have been documented extensively, originally as manifested by cytologically visible puff formation (e.g., Ritossa 1962; Ashburner and Richards 1976) and more recently by molecular techniques (e.g., Ashburner and Bonner 1979; Burtis et al. 1990; Segraves and

Hogness 1990; Rabindran et al. 1993). This information base seemed to provide the potential for correlating the activity state of certain loci with the form of Pol II found there. In addition, comparing chromosomal distributions of Pol IIA or IIO with those of other chromosomal proteins could potentially be used to test predictions about or reveal unanticipated aspects of Pol II behavior in vivo.

In this paper we describe preparing antibodies directed against the nonphosphorylated and the hyperphosphorylated CTD and using them to detect and characterize locus-specific in vivo distributions of Pol IIA and IIO by immunofluorescence microscopy with digital imaging. We also present experiments comparing these distributions with those of several other nuclear proteins involved in transcriptional regulation and pre-mRNA processing.

Results

Phosphorylation state-specific anti-CTD antibodies

To investigate the in vivo chromosomal distributions of *Drosophila* RNA polymerase forms IIA and IIO, we affinity purified goat antibodies against the nonphosphorylated CTD and rabbit antibodies against the hyperphosphorylated CTD (Materials and methods). We tested the specificity of each antibody initially using Western blots of quickly prepared embryo extracts. These extracts contain roughly equivalent amounts of Pol IIA and IIO, as revealed by an antibody (anti-exon2) that detects both forms equally because it reacts with determinants near the amino terminus of the subunit (Fig. 1, lane 1). As shown in Figure 1 (lane 3), the anti-phosphoCTD anti-

Figure 1. Western blot of quickly prepared *Drosophila* embryo extracts. Identical membrane strips were reacted with primary antibodies as indicated. For the controls in lanes 4 and 5, the primary antibody was preincubated with excess cognate fusion protein for 1 hr at room temperature before reacting with the membrane.

bodies (anti-PCTD) are exquisitely specific for the hyperphosphorylated, slower-migrating (IIO) form of the largest Pol II subunit (note that other experiments demonstrated that they do not react significantly with any lower molecular weight polypeptides that ran off the gel shown here). Likewise, the anti-CTD antibodies (anti-CTD) are highly selective for the nonphosphorylated, faster-migrating (IIA) form, although they also react to some degree with a band at the IIO position (lane 2).

To characterize further the selectivity of the two antibody preparations, we tested their reactivities toward differentially phosphorylated forms of a CTD fusion protein. First, we treated a hyperphosphorylated CTD fusion protein with increasing amounts of alkaline phosphatase to reproduce an analog of the IIO→IIA SDS gel mobility shift (Fig. 2A). Treatment with a low amount of phosphatase (lane 2) did not cause a noticeable mobility increase, nor did treatment with 10 times as much (lane 3), whereas treatment with 25 times as much or more (lanes 4–7) caused much or all of the fusion protein to move with increased mobility. Figure 2B shows that treatment with even the lowest amount of alkaline phosphatase greatly diminished the reactivity of the anti-PCTD antibodies toward the phosphorylated fusion protein (lane 2) and treatment with 10 times as much nearly abolished the reactivity (lane 3). In neither case did the mobility of the fusion protein increase appreciably, indicating that the antibodies are reactive only toward the most highly phosphorylated forms of the CTD and, incidentally, underscoring the complex relationship between the SDS mobility of the CTD fusion protein and its level of phosphorylation. No reactivity was observed with fusion protein exposed to higher amounts of phosphatase (lanes 4–7), treatment that induced substantial or complete mobility shift.

Figure 2C demonstrates the complementary specificity of the anti-CTD antibodies. These antibodies barely reacted with the hyperphosphorylated fusion protein (lane 1), but they showed some reactivity after a partial dephosphorylation that did not result in a mobility increase (lane 2). As more phosphates were removed, and especially after the mobility of the fusion protein increased, the anti-CTD reactivity increased further.

These results indicate that the anti-PCTD antibodies are quite selective for the hyperphosphorylated form(s) of the CTD; for convenience we refer to them as anti-IIO, realizing the inadequacy of the IIO/IIA nomenclature (e.g., Corden 1990; Lee and Greenleaf 1991; Dahmus and Dynan 1992). The anti-CTD antibodies react only barely with the most highly phosphorylated forms, but they do show a reactivity toward partially dephosphorylated forms that increases as the extent of dephosphorylation increases; they react most strongly with the nonphosphorylated CTD. In this paper we refer to them as anti-IIA.

Localization of RNA Pol IIA and Pol IIO on polytene chromosomes: major developmental puffs contain IIO

To analyze the in vivo distributions of RNA polymerase

Figure 2. Phosphorylation state-dependent antibody reactivities. Phosphorylated CTD fusion protein (PCTD-FP) was treated with increasing amounts of alkaline phosphatase, and replicate samples were electrophoresed in three separate SDS gels and blotted to nitrocellulose. The relative amounts of phosphatase are indicated above the lanes. (Lane 1) 0 units; (lane 2) 0.025 units; (lane 3) 0.25 units, etc. (A) Blot reacted with rabbit anti- β -galactosidase IgG and developed with BCIP/NBT (Materials and methods). Note that for this gel only, lane 1 contained 50 ng of fusion protein, whereas all other lanes contained 25 ng. (B) Blot reacted with rabbit anti-PCTD IgGs and developed by use of chemiluminescence. (C) Blot reacted with goat anti-CTD IgGs and developed by use of chemiluminescence.

forms IIA and IIO, we first fixed chromosomal proteins in situ by treating salivary glands with formaldehyde and then made squash preparations of the fixed polytene chromosomes for reacting with the antibodies [see Materials and methods].

Control indirect immunofluorescence microscopy experiments, in which one primary antibody and both chromophore-tagged secondary antibodies were included, confirmed that we could uniquely detect each form of the enzyme and also that the image in each case was dependent on the primary antibody, with virtually no

background contributed by the secondary antibodies (not shown).

To compare directly the distribution of differentially phosphorylated forms of RNA Pol II, we carried out double-label antibody staining. As shown in Figure 3, the antibodies revealed distinct but overlapping distributions. Anti-IIA (green signal) and anti-IIO (red signal) stain many distinct sites (red or green), as well as common sites (yellow or orange) distributed widely on all chromosome arms (only the distal half of the X chromosome shown here). Given the antibody specificity tests described earlier, we infer that the red sites contain only highly phosphorylated Pol II and that the green sites contain virtually no highly phosphorylated Pol II. The sites of intermediate color presumably contain a mixture of highly phosphorylated enzyme and either unphosphorylated or incompletely phosphorylated enzyme.

The characteristic developmental puff at 2B5 stains primarily red (IIO), as do several additional sites, some of which appear puffed and some of which do not. The 2B5 puff, which is the site of the Broad Complex and is induced by the molting hormone ecdysone [e.g., DiBello et al. 1991], consistently stains with anti-IIO at puff stages when it is known to be active. At these same times, the extreme tip of the chromosome stains reproducibly only green (IIA), and there are other only green sites as well. As mentioned, there are yet other sites that stain with both antibodies, giving intermediate colors in this overlay view; the one indicated by a downward arrow, for

Figure 3. Distinct distributions of Pol IIA and Pol IIO on polytene chromosomes. Formaldehyde-fixed chromosome preparation reacted with goat anti-CTD (anti-IIA) and rabbit anti-PCTD (anti-IIO) antibodies and visualized by indirect immunofluorescence and digital imaging (see Materials and methods). This overlay of anti-CTD (green) and anti-PCTD (red) images shows principally the distal half of the X chromosome. Arrows indicate examples of red signals not obviously puffed (upward arrow), green signals (leftward arrow) and mixed signals (downward arrow). (2B5) An early ecdysone-induced puff. Bar, 5 μ m.

example, is observed very reproducibly (see Fig. 6b–e, below).

Some of the most dramatic ecdysone-induced puffs are the so-called Chinese lanterns at 74EF and 75B, which are induced by the ecdysone titer increase that initiates molting (Ashburner and Richards 1976; Burtis et al. 1990; Segraves and Hogness 1990). Part of a chromosome 3L displaying these large puffs is shown in Figure 4. Figure 4a shows that the anti-IIA antibodies are virtually unreactive toward the 74EF and 75B puffs, whereas they stain many other nonpuff sites very strongly, as seen previously; in addition, they react weakly with other ecdysone-induced puffs present at this puff stage. In marked contrast, Figure 4b shows that the 74EF and 75B puffs are stained intensely by anti-IIO antibodies, as are the other prominent puffs; as described above, many other bands are also stained.

The overlay shown in Figure 4c allows direct comparisons of the IIA and IIO patterns. This view confirms the impressions from Figure 4a and b, that the largest ecdysone puffs are stained almost exclusively by anti-IIO and the other obvious puffs are stained more strongly by anti-IIO than by anti-IIA. Where a prominent puff stains with both antibodies, it is also apparent that the two patterns are spatially distinct. The overlay view also reiterates the observation that a prevalent class of nonpuffed sites appears to contain only Pol IIA (green), another class contains both IIA and IIO (yellow or orange), and a third class contains only IIO (red).

The results to this point indicate that the most obvious developmental puffs contain primarily RNA Pol IIO. Because puffing at these loci is a consequence of transcription (e.g., Burtis et al. 1990; Segraves and Hogness 1990), it seems reasonable to conclude that the transcription at these sites is carried out by Pol IIO. (It should be pointed out here that we have not yet presented direct evidence that any particular locus was actually transcriptionally active at the moment the chromosome preparation was made). At this point, we cannot say whether the other not obviously puffed red (IIO only), green (IIA only), or orange/yellow (IIO and IIA) sites are transcriptionally active. Thus, the results do not reveal whether, for example, Pol IIA is also transcriptionally competent.

Heat shock puffs contain both Pol IIA and Pol IIO

To observe a different set of genes, known to be active at the moment of sample preparation, we chose the heat shock loci. Incubation of larvae at 37°C rapidly induces a set of nine major heat shock puffs that represent sites of newly activated transcription (for review, see Ashburner and Bonner 1979). Anti-IIA and anti-IIO staining of endogenous heat shock puffs is shown in Figure 5. In marked contrast to the developmental puffs, the heat shock puffs react strongly with both anti-IIA and anti-IIO. The uniformity of the staining in the puffs suggests that the heat shock loci are being transcribed by both

Figure 4. RNA Pol IIO and Pol IIA on major ecdysone puffs. Distal three-fourths of chromosome 3L from a preparation reacted with goat anti-IIA and rabbit anti-IIO antibodies. (a) Anti-IIA signal; (b) anti-IIO signal; (c) overlay of a and b. Major early ecdysone puffs at 74EF and 75B (Chinese lanterns) are indicated, as are examples of sites not obviously puffed that stain red (upward arrow), green (leftward arrow), and mixed (downward arrow).

Figure 5. RNA Pol IIA and Pol IIO on heat shock puffs. (a) A chromosome preparation from a wild-type larva heat-shocked for ~20 min at 37°C was reacted with goat anti-IIA (green) and rabbit anti-IIO (red) antibodies; overlay shown. Major heat shock puffs are labeled. (b) Goat anti-IIA (green) and rabbit anti-IIO (red) staining of chromosomes from transformant Bg9,61 larva heat-shocked for ~5 min at 37°C; overlay image. Puffs at insertion sites near 9E and 61A are indicated.

forms of the enzyme. Similar color and uniformity of staining are observed consistently on puffs induced for times ranging from ~4–20 min.

In addition to the endogenous heat shock puffs, most of which contain several clustered heat shock genes, we monitored two novel puffs that form at the insertion sites of a previously characterized hybrid transgene consisting of a single *hsp70* promoter and 5' portion of the *hsp70* gene linked to *lacZ* (Lis et al. 1983; Simon et al. 1985). We used the Bg9,61 transformant strain that carries this transgene inserted at two separate sites: in polytene band regions 9E and 61A. We hoped that these

transgene puffs would simplify interpretation because heat shock-induced transcription begins at a single site and proceeds unidirectionally to cause a fairly large puff. Figure 5b shows that after ~5 min of heat shock, the transgene puffs at 9E and 61A stain in a pattern resembling that of the endogenous heat shock puffs, that is, they stain strongly for both IIA and IIO, giving a uniformly yellow–orange color. Longer heat shocks result in much larger puffs that continue to stain strongly with both antibodies (not shown). Note that this transgene construct produces a stable transcript of ~11 kb and even longer readthrough products (Simon et al. 1985). These transcripts are many times longer than the endogenous heat shock gene transcripts, a fact that might explain the large size of the transgene puffs.

Pol IIA at the hsp70–lacZ transgene insertion site before induction

Previous experiments had discovered a Pol II molecule arrested at the 5' end of the uninduced *hsp70* transcription unit (Gilmour and Lis 1986), apparently having initiated an RNA chain but having stopped after polymerizing ~25 nucleotides (Rougvié and Lis 1988; Giardina et al. 1992). Like the endogenous loci, the *hsp70–lacZ* transgene construct in transformant Bg9,61 had also been shown to carry a 5'-arrested Pol II (Lee et al. 1992); therefore, we attempted to observe Pol II on the hybrid transgene before heat shock.

Figure 6 shows that in non-heat-shocked samples we do observe in the transformant strain distinct Pol IIA signals at the 9E and 61A insertion sites that are not present in wild type. For reference, Figure 6, a and a', show the banding patterns around the insertion sites; several prominent bands are labeled. Figure 6, b and b', shows wild-type chromosomes stained with anti-IIA (green) and anti-IIO (red). Transformant Bg9,61 chromosomes stained with anti-IIA and anti-IIO are shown in Figure 6, c and c'. Comparing Figure 6, b with c and b' with c', reveals transposon-dependent green (IIA) fluorescence signals at the two insertion sites; these are indicated by arrows labeled 9E and t in c and c', respectively. We also observe a Pol IIA-only signal at sites of endogenous heat shock genes in unshocked preparations (not shown), although negative controls for these observations are not conveniently available.

Previously, other work had also described the presence of bound protein on the TATA box before heat shock induction (Wu 1984; Giardina et al. 1992), and recent *in situ* localizations similar to those described here also have detected TATA-binding protein (TBP) at the 87A and 87C sites of endogenous *hsp70* genes on unshocked polytene chromosomes (Carl Wu, pers. comm.). Using antibodies to the TBP component of TFIID, we also observe a strong transgene-specific signal that colocalizes with the novel Pol IIA signal in unshocked preparations (not shown).

One interpretation of our results is that Pol II arrested on the 5' end of *hsp70* genes is form IIA; this interpretation is consistent with results of recent UV cross-link-

Figure 6. Staining patterns at *hsp70-lacZ* transgene sites without heat shock. (a, a') DAPI-stained sections of chromosomes showing regions around transposon insertion sites near 9E and 61A; images reversed to mimic classical banding pattern (most intense fluorescence appears darkest). Several prominent bands are indicated. (b, b') Goat anti-IIA (green) and rabbit anti-II0 (red) staining of non-heat-shock wild-type preparations; overlay images. (c, c') Goat anti-IIA (green) and rabbit anti-II0 (red) staining of non-heat-shock transformant Bg9,61 preparations; overlay images. In c, the arrow labeled 9E points at a novel fluorescence signal present in transformant chromosomes. In c', the arrow labeled t points at a novel fluorescence signal present in transformant chromosomes.

ing experiments that show the arrested enzyme to be Pol IIA (T. O'Brien, S. Hardin, A.L. Greenleaf and J.T. Lis, in prep.). Because the transposon also contains a partial P element and the *rosy* gene (Lis et al. 1983), however, it is possible that Pol II or TBP bound to either P or *rosy* sequences in the transposon could contribute to the signals that we observe in non-heat-shocked samples. It will be feasible to test this possibility formally in the future by using transgene constructs with altered *hsp70* promoter regions (e.g., Lee et al. 1992); meanwhile, experiments with heat-shocked chromosomes, presented in the next section, already provide support for the first interpretation.

Pol IIA and heat shock factor on induced hsp70-lacZ transgene puffs

Because heat shock induction of *hsp70* depends on bind-

ing of the heat shock factor (HSF) to the regulatory elements (HSEs) in the *hsp70* promoter (e.g., Westwood et al. 1991, and references therein), we checked for the presence of HSF at the transgene sites after short heat shocks. First, controls with wild-type chromosomes confirmed that HSF (and Pol IIA) was not present at the transposon insertion sites in the absence of the transposon (Fig. 7a,a'). In contrast, strong HSF (red) and Pol IIA (green) signals appeared at both insertion sites in transformant Bg9,61 chromosomes (Fig. 7b,b'), with the overlap of the red and green signals producing novel orangish bands labeled 9E and t in b and b', respectively. At this short induction time (90 sec), no noticeable puffs had formed. Note that in control experiments (not shown) with transformant Bg9,61 without heat shock, we observed no HSF signal at the insertion sites [consistent with expectations based on the results of Westwood et al. (1991).]

Figure 7. Pol IIA and heat shock factor at *hsp70-lacZ* transgene sites after heat shock. Rabbit anti-HSF (red) and goat anti-IIA (green) staining of chromosomes from wild-type and transformant Bg9,61 salivary glands. (a, a') Wild-type salivary glands heat-shocked in 37°C buffer for ~90 sec. Arrows labeled 9E and t point at sites in regions 9E and 61A, respectively, that lack fluorescence signals. (b, b') Transformant Bg9,61 salivary glands heat-shocked in 37°C buffer for ~90 sec. Arrows labeled 9E and t point at sites of transposon-dependent fluorescence signals in regions 9E and 61A, respectively. (c, c') Transformant larva heat-shocked for 5 min; chromosomes stained with goat anti-IIA. Arrows 9E and t indicate point at more intense signals near one edge of the induced transgene-dependent heat shock puffs in regions 9E and 61A, respectively. (d, d') Same preparations as in c and c', stained with rabbit anti-HSF; arrows as in c, c'. (e, e') Overlay of images in c and d and c' and d', respectively. Bar in e, 5 μm.

We then investigated the HSF and Pol II distributions in transgene-dependent puffs induced by slightly longer heat shocks; the results are shown in the remaining panels of Figure 7. A 5-min shock is clearly sufficient to induce substantial transgene-dependent puffing at both insertion sites, and the entirety of each puff labels strongly with anti-IIA as anticipated (Fig. 7c,c'). Note that in each case one edge of the puff displays a stripe of more intense fluorescence, as indicated by the arrows labeled 9E and t. Staining the same preparations with anti-HSF (Fig. 7 d,d') reveals a much more restricted distribution than for Pol II, with the HSF concentrated in

each case at one edge of the puff. The overlay images in Figure 7, e and e', reveal that the HSF signal appears to superimpose over the more intense stripe of the Pol IIA signal, generating an orange stripe near one edge of each puff (arrows labeled 9E and t). One interpretation of these results is that the HSF signal emanates from HSF molecules clustered at the promoter region of the transgene, whereas the Pol II signal emanates both from enzyme molecules present at high density near the 5' end of the gene and from Pol II molecules distributed at somewhat lower density along the heat shock-inducible transcription unit out to the 3'-most point reached by poly-

merases during the 5-min induction [note that the transcription unit is ≥ 11 kb long (Simon et al. 1985)]. This interpretation is consistent both with the known orientation of the transposon at each insertion site (Simon et al. 1985) and with a pause at the arrest site being rate limiting even for 37°C heat shocks (Giardina et al. 1992).

HnRNP proteins colocalize primarily with Pol IIO

To explore further the relationship between transcriptional activity and CTD phosphorylation state under non-heat-shock conditions, we double-labeled chromosomes with anti-IIO or anti-IIA and with antibodies that we assumed would label most nascent transcripts—monoclonal antibodies to hrp36 and hrp48, two major *Drosophila* hnRNP proteins (Matunis et al. 1992). The results shown in Figure 8a are typical; many sites of IIO staining (green) also stain for hrp proteins (red), such that

Figure 8. HnRNP proteins preferentially colocalize with Pol IIO. Non-heat-shock preparations were double labeled with mouse monoclonal antibodies to *Drosophila* hrp36 + hrp48 (red) and either rabbit anti-IIO (green) (a) or goat anti-IIA (green) (b); overlay images.

in the overlay image these sites are some shade of orange. Most developmental puffs stain consistently for both IIO and hrp proteins. These results thus support the idea that IIO is transcriptionally active at developmental puffs and potentially at many additional sites. Note that the staining of the chromocenter seen here is apparently a property shared by a number of anti-heterogenous nuclear ribonucleoprotein (anti-hnRNP) antibodies (S. Haynes, pers. comm.).

Unexpectedly, a number of sites that contain IIO do not stain for hrp proteins (Fig. 8a, green sites), a result that might suggest IIO at these loci is not active. Another interpretation, however, is suggested by recent studies of several *Drosophila* hrp proteins, as it now appears that active loci are not stained equally by all anti-hrp antibodies (G. Dreyfuss and E. Matunis, pers. comm.). Thus, the green sites in Figure 8a (IIO only) may represent active sites whose nascent transcripts simply contain little or no hrp36 and hrp48. We obtained very similar results with a monoclonal antibody against hrp40.

Further inspection of Figure 8a and other similar preparations indicates that there are a few sites that seem to stain for hrp proteins but not for IIO (i.e., red only). Conceivably, this type of site might represent a locus being transcribed by IIA.

We also compared directly the distributions of hrp36 + hrp48 (red) and RNA polymerase IIA (green), as shown in Figure 8b. As anticipated from the above results, we found that many sites of hrp staining (especially puffs) did not stain for IIA and many sites of IIA staining (green) did not stain for hrp. On the other hand, a few sites did stain for both IIA and hrp. Although this latter result could suggest that IIA is active at these sites, it is possible that such sites also contain IIO (as in previous Figures) and that IIO is generating the nascent transcript. This experiment cannot distinguish between these possibilities.

To see whether we could identify a IIA-only site that was transcriptionally active, we first reacted chromosomes with anti-IIA + anti-IIO as usual and identified sites with only IIA present (green). We then restained the same preparation with a mixture of monoclonal antibodies to hrp40, hrp36, and hrp48, followed by a red secondary antibody. If a IIA site carried a nascent transcript complexed with hrp proteins, it should now fluoresce an orange color. A limited attempt that scanned approximately one-half of the X chromosome failed to reveal such a site (not shown).

RNA Pol IIO colocalizes with a splicing component

Splicing in *Drosophila* has been shown to occur cotranscriptionally (Beyer and Osheim 1988; Lemaire and Thummel 1990; see also Gall 1991; Greenleaf 1993 and references therein; Jimenez-Garcia and Spector 1993; Xing et al. 1993); we therefore wondered how the distribution of a splicing component might compare with that of RNA Pol IIO or Pol IIA. Figure 9a shows chromosomes stained for the U1 70K small nuclear ribonucleoprotein (snRNP) (red) and Pol IIO (green). The major result is that

Figure 9. A splicing component preferentially colocalizes with Pol II0. Non-heat-shock preparations were double labeled with a mouse monoclonal antibody to U1 snRNP 70K protein (red) and either rabbit anti-II0 (green) (*a*) or goat anti-IIA (green) (*b*). In *b*, examples of red signals (downward arrows) and a green signal (upward arrow) are indicated; overlay images.

most sites containing II0 also contain U1 70K (yellow or orange) and that few 70K sites lack II0 (they would be red). In contrast, Figure 9b shows that there is much less correlation between the Pol IIA and U1 70K patterns (e.g., there are numerous red, U1 70K-only, sites and numerous green, IIA-only, sites); furthermore, the presence of U1 70K at IIA sites might be explained by the presence of II0 at those sites.

RNA polymerase is in interbands and puffs

Previous studies using an anti-RNA Pol II serum (Jamrich et al. 1977) indicated that the enzyme was located primarily in puffs and interbands, rather than in the bands that are more condensed and contain most of the DNA mass. To relate our current work to earlier experiments, we compared the anti-IIA and anti-II0 signals with a signal from DAPI, a fluorescent dye that binds to DNA and produces a pattern nearly identical to the classical banding pattern (e.g., see Fig. 6a). Both the IIA and II0 patterns are largely nonoverlapping with the DAPI pattern (not shown; also see Fig. 6), indicating again that the polymerases that we detected are predominantly in the least condensed regions of the polytene chromosomes, the puffs and interbands.

Discussion

We have visualized the *in vivo* distributions of differentially phosphorylated forms of RNA Pol II on *Drosophila*

polytene chromosomes, using antibodies sensitive to the phosphorylation state of the CTD of the Pol II largest subunit. We find RNA Pol IIA and Pol II0, with hypo- and hyperphosphorylated CTDs, respectively, arrayed in markedly different, locus- and condition-specific patterns representing three classes of sites. One class contains only Pol II0, another class contains only Pol IIA, and the third class contains a mixture of Pol IIA and Pol II0. To begin to understand these patterns, we have established correlations between the nature and activity of certain loci and the phosphorylation state of Pol II there, and we have monitored the colocalization of RNA Pol IIA and Pol II0 with other nuclear proteins involved in either transcriptional regulation or pre-mRNA metabolism.

One primary result is that the large developmental puffs, reflecting gene activity at loci induced by the molting hormone ecdysone, contain predominantly Pol II0. Because the anti-II0 antibodies react only with the hyperphosphorylated CTD, and because the largest puffs are virtually unstained by anti-IIA antibodies that react with the hypophosphorylated CTD, we conclude that the polymerases present on the body of these puffs are essentially only the hyperphosphorylated form. Furthermore, we suggest that these enzymes are actively elongating RNA chains. First, experiments that detect both hnRNP proteins and Pol II0 on the same puffs support the contention that we are observing active enzymes, those that were generating nascent transcripts at the moment of sample preparation. Second, the two major early puffs at 74EF and 75B exhibit a spatially uniform staining pattern, suggesting that we are observing enzyme molecules distributed along the entire length of these large, 50- to 60-kb transcription units (Burtis et al. 1990; Segaves and Hogness 1990; Thummel et al. 1990). Thus, in the case of the major ecdysone puffs, our *in vivo* observations are consistent with the idea that the elongating form of the enzyme is II0 (see introductory section and Fig. 10a).

Another clear result is that the Pol II molecules transcribing the heat shock loci differ in phosphorylation state from those transcribing the major developmental puffs. Heat shock puffs induced at 37°C stain strongly with both anti-II0 and anti-IIA antibodies. Because the anti-IIA antibodies do react with incompletely phosphorylated CTD (Fig. 2), we cannot tell whether the anti-IIA-reactive polymerases on heat shock puffs are completely unphosphorylated, partially phosphorylated, or a mixture of both. We can conclude, however, that a substantial fraction of the enzyme on these puffs is not fully phosphorylated.

Because heat shock genes are much shorter than, for example, the ecdysone-induced genes at 74EF and 75B, the ratio of promoter/initiation region to gene body is much higher for the heat shock genes. If a different form of Pol II were present on the promoter/initiation region than on the body of the gene, one could argue that the staining we see is a superposition of signals from one form of Pol II at or near the promoter [e.g., the arrested Pol II described by Lis and colleagues (Gilmour and Lis

Figure 10. Model of Pol II transcribing two different kinds of gene. (A) A gene transcribed by Pol II. A long ecdysone-induced gene, such as *E74A*, is transcribed only by polymerases with fully phosphorylated CTDs. Polymerase density is not actually known, but it is envisioned as being less than maximal, such that nucleosomes (smaller ovals) could reform between passage of polymerase molecules. HnRNP proteins and splicing components are not shown (see text). (B) Transcription of a heat shock gene. Actively elongating polymerases are either hyper- or hypophosphorylated, as indicated by many or few P's on the CTD (note, an incompletely phosphorylated CTD would stain as IIA; see text). Polymerase density on induced heat shock genes at 37°C is very high, as represented by enzyme molecules almost touching. TBP component of TFIID is

shown bound to the TATA box (rectangle within TBP). Other components of TFIID presumably present, and additional factors that may be bound at the promoter, are represented by the larger, stippled square surrounding TBP. Activated HSF trimers (Rabindran et al. 1993) are bound to the HSEs [there are actually three in the 194 bp of upstream sequence present in the *hsp70-lacZ* transgene construct (Xiao and Lis 1988)] and activate transcription of the gene (unknown mechanism indicated by open curved arrow). Presumed arrested Pol II is depicted with its ~25 nucleotide nascent transcript emanating from the transcription bubble and with its unphosphorylated CTD [see Fig. 6; Discussion] contacting components of TFIID [see introductory section].

1986; Rougvie and Lis 1988)] and another form on the gene body. We think that this is unlikely for several reasons. First, the density of polymerases on an induced *hsp70* gene is very high, ~30–40 enzymes/2.4-kb transcription unit, or ~1 enzyme/60–80 bp (O'Brien and Lis 1991). Thus one 5'-arrested Pol II would represent only ~1/40–1/30 of the total Pol II signal for an *hsp70* gene. The staining that we observe does not seem consistent with this ratio. Furthermore, the nearly uniform anti-IIA and anti-IIO staining that we observe for a heat shock-induced transgene that contains only one heat shock promoter and a longer than normal transcription unit is virtually indistinguishable from that for the endogenous heat shock puffs, which contain several promoters and short transcription units. We suggest that these observations are consistent with the proposal that the actively elongating polymerases on heat shock genes are a mixture of hyperphosphorylated (Pol II O) and hypo- or nonphosphorylated enzyme (Pol II A?).

We generated additional information relevant to interpreting the immunofluorescence images by using the transgene-dependent heat shock puffs to compare the spatial distribution of a regulatory transcription factor, HSF, with that of Pol II. In favorable preparations (e.g., Fig. 7c–e) we visualized distinct patterns for HSF and Pol II, suggesting that we can resolve signals resulting from proteins bound at or near the promoter from signals resulting from proteins bound at more 3' positions along the *hsp70-lacZ* transcription unit. The following considerations argue that this resolution should be feasible. This artificial transcription unit is ≥11 kb long (Simon et al. 1985), and after a 5-min heat shock Pol II molecules should have traveled nearly to its end, if we assume that

the elongation rate for *Drosophila* Pol II at 37°C is ~2 kb/min and that there is little lag in beginning elongation after temperature shift. Furthermore, the induced puff at the 9E insertion site in Fig. 7c–e is 3–4 μm long, which at a minimum would represent 9–12 kb of DNA, a length consistent with the distance Pol II molecules could have traversed in 5 min. Thus, the anti-Pol II staining of the body of the puff may correlate directly with the spatial distribution of polymerases arrayed along the *hsp70-lacZ* transcription unit, and the localized anti-HSF and anti-Pol II staining near one edge of the puff may correlate directly with promoter-proximal concentrations of the two proteins. Future work will test the validity of these interpretations, but the results already obtained hint that using in situ immunolocalization to study further this and other transgene constructs will be valuable in helping to fashion a better understanding of in vivo regulatory events by providing a detailed description of components present at particular chromosomal sites both before and after induction.

The experiments that we have presented suggest a picture of transcription for two different classes of gene as depicted in Figure 10. The long ecdysone-induced genes, such as those at 74EF and 75B, are transcribed by very highly (completely?) phosphorylated Pol II O (Fig. 10A). In contrast, polymerases actively elongating transcripts on heat shock genes carry, on average, fewer phosphates (Fig. 10B). [The properties of the anti-IIA and anti-IIO antibodies do not enable us to distinguish between two possibilities at present: (1) a homogeneous population of highly but not completely phosphorylated enzymes; or (2) a mixed population of highly phosphorylated, and non or slightly phosphorylated enzymes, as depicted in Fig.

10B]. Furthermore, our results with the uninduced *hsp70-lacZ* transgene are consistent with those of O'Brien, S. Hardin, A.L. Greenleaf, and J.T. Lis, (in prep.), which indicate that arrested Pol II, which has synthesized an RNA chain of ~25 nucleotides, is form IIA. For heat shock promoters, then, the initiation of RNA synthesis seems to be uncoupled from CTD phosphorylation. The transcriptional event correlated with CTD phosphorylation would then appear to be the HSF-mediated release of the arrested Pol IIA and/or the transit of the elongating polymerases through the body of the gene.

Like the easily identifiable and well-characterized heat shock genes, many currently anonymous sites also stain with both anti-IIA and anti-II0 antibodies. Whether this characteristic indicates any underlying similarity to the heat shock loci is not clear. It seems likely that the subclass of these sites with a high II0/IIA ratio is transcriptionally active, in view of the general correspondence between staining for II0 and staining for hrp proteins, presumed markers of nascent transcripts; some of these sites stain for both IIA and hrp proteins. The activity status of most sites with a low II0/IIA ratio is currently unknown.

Sites that stain exclusively for IIA appear to equal or outnumber those that stain exclusively for II0. The majority of these IIA-only sites apparently lack nascent RNAs, as they do not stain for hrp proteins. We made a very limited attempt to find a IIA-only site that also stained for hrp protein but were unsuccessful. Thus, we have no direct evidence that the IIA at any of these locations is engaged in elongating RNA chains. We suggest that at least some of the IIA-only sites represent non-heat-shock loci with an arrested Pol IIA; a number of *Drosophila* genes with an arrested Pol II have been identified previously by other approaches (Rougvie and Lis 1990; Giardina et al. 1992) and may represent a subset of the sites that we observe. Other IIA-only sites might represent stationary polymerases that are parked, stored, or sequestered at specific locations. And finally, because unrecognized technical problems might have frustrated our attempts to find an active IIA, it remains possible that some IIA-only sites contain actively elongating RNA polymerases.

We have not addressed directly the question, "What is the role of the phosphorylated CTD, given the apparent correlation between phosphorylation and RNA chain elongation?" Among previously suggested roles (e.g., Corden 1990), two seem consistent with our finding that the long, alternatively spliced ecdysone-induced genes with multiple introns are transcribed by more highly phosphorylated Pol II than the shorter, intronless *hsp70* genes. The fully phosphorylated CTD is an example of an acidic polymer that may serve to catalyze the displacement of histone H2A/H2B dimers from nucleosomes to facilitate the passage of Pol II (Hansen and Ausio 1992). This function might be less critical on (often short) genes with very high polymerase densities (e.g., heat shock genes) than on long genes with presumably lower polymerase densities (e.g., *E74* and *E75*). In the former case, nucleosomes have no chance to reform after

passage of one Pol II molecule because the next one follows too closely (as in Fig. 10B). In the latter case, each Pol II must make its way through the nucleosome barriers independently (Fig. 10A). In this scenario it is not critical to elongation efficiency that polymerases transcribing the heat shock genes be hyperphosphorylated, whereas it is essential that polymerases transcribing long ecdysone-inducible genes be fully phosphorylated.

Another proposed role for the PCTD is to serve as a docking and deployment site for certain splicing components and regulators, those with positively charged domains postulated to interact with the negatively charged PCTD (Greenleaf 1993; see also Corden 1990). Our results showing a relatively good correlation between Pol II0 and U1 snRNP 70K protein and a relatively poor correlation between Pol IIA and U1 70K are consistent with this idea, as are the results just discussed that associate II0 with long, multiple intron-containing genes (whose pre-mRNAs are subject to alternative splicing). On the other hand, these correlations do not provide decisive support for this idea because Pol II0 appears to correlate in general with transcriptional activity and would therefore also correlate with splicing activity. More critical tests of the idea might, however, result from the identification of other specific loci transcribed by IIA or IIA/II0, especially if such loci tended to be intronless or not alternatively spliced.

The model in Figure 10 can also serve to highlight several features of CTD phosphorylation that we do not understand. The following questions cover a partial list of those features. Do the ecdysone-induced genes also have an arrested Pol II before induction and, if so, is it form IIA? Does CTD phosphorylation cause or is it a consequence of the release of 5'-arrested Pol IIA? For heat shock genes, is each Pol II that commences elongation phosphorylated to the same extent or does the extent of phosphorylation change with induction time or temperature? How does the binding of HSF mediate the release of arrested Pol IIA and the phosphorylation of its CTD? What is the identity of the CTD kinase that phosphorylates the arrested Pol IIA, and does the same kinase act on subsequently initiating polymerases? Is the CTD kinase acting at heat shock promoters the same as that acting at other promoters, such as those for the major developmental puff genes?

Besides helping to answer questions about the physiology of CTD phosphorylation, the approach described in this paper should offer enough sensitivity and resolution to provide new insights into the *in vivo* properties and behavior of other components of the eukaryotic transcription apparatus and the RNA processing machinery.

Materials and methods

Antibodies

Goat antibodies directed against the second exon of RNA Pol II largest subunit (anti-exon2 IgGs) were affinity purified on a column carrying a LacZ-exon 2 fusion protein as described (Lee and Greenleaf 1991, therein referred to as anti-DmE2). Goat anti-CTD antibodies (anti-IIA) were affinity purified (Robbins et

al. 1984) from the same serum using a column carrying a LacZ–*Drosophila* CTD fusion protein (Lee and Greenleaf 1989, therein referred to as D-FP). To prepare rabbit anti-phosphoCTD antibodies (anti-II0), 100 μ g of D-FP was hyperphosphorylated with yeast CTD kinase (Lee and Greenleaf 1989), and this PCTD fusion protein was injected into a rabbit. Four weeks later, the rabbit was boosted with a similar amount of PCTD fusion protein, and blood was collected beginning 2 weeks later. Total rabbit IgGs were then purified using a protein-A column and passed through a CTD fusion protein column; from the flow-through anti-PCTD, IgGs were affinity purified using a column carrying a hyperphosphorylated glutathione *S*-transferase (GST)–CTD fusion protein.

A monoclonal antibody to U1 snRNP 70-kD protein (U1 70K) was purified from a culture supernatant, a generous gift from S. Pelsue and P. Agris (North Carolina State University, Raleigh) [the cell line used was line 2.73 from S. Hoch (Billings et al. 1982; Pelsue et al. 1993)]. Ammonium sulfate precipitation and protein A affinity chromatography yielded ~200 μ g of IgG from 500 ml of supernatant.

Rabbit anti-HSF was a generous gift from Carl Wu (National Cancer Institute, Bethesda, MD). Monoclonal antibodies to *Drosophila* hnRNP proteins were a generous gift from E. Matunis and G. Dreyfuss (University of Pennsylvania Medical School). The anti-hrp40, -hrp36, and -hrp48 antibodies were monoclonals 8D2, 5A5, and 10D5, respectively (Matunis et al. 1992). Rabbit anti-TBP was a generous gift from C. Tyree and J. Kadonaga (University of California, San Diego).

Secondary antibodies for Western blots were from Boehringer Mannheim. Secondary antibodies for immunofluorescence microscopy were affinity-purified donkey IgGs (ML grade) labeled with fluorescein, rhodamine, or Texas red, from Jackson ImmunoResearch Labs, Inc. The Jackson secondary antibodies gave very little nonspecific background or cross-species reactivity.

Quickly prepared *Drosophila* embryo extracts

Wild-type embryos were collected overnight, washed thoroughly on a nitrex filter with 0.5% NaCl/0.1% Triton X-100, dechorionated in 3% Clorox solution for 2 min, rinsed several times with 0.5% NaCl/0.1% Triton X-100, and finally rinsed with deionized H₂O. The embryos were weighed, suspended in cold (–20°C) 95% ethanol containing phenylmethylsulfonyl fluoride (1% of a saturated solution in isopropanol), and homogenized in a small glass homogenizer (e.g., Radnoti econo-grind homogenizer, Fisher Scientific 08-416C) on dry ice. After centrifugation of the homogenate at 5000 rpm and 0°C for 5 min, the pellets were dried briefly under vacuum and suspended in hot (~95°C) SDS sample buffer (50 mM Tris-HCl at pH 7.4, 5% Ficoll, 5% SDS, 25 mM dithiothreitol, 1% 2-mercaptoethanol, bromphenol blue) at a concentration of ~10 μ g/ μ l. After heating at 95°C for 5 min, the samples were pushed through a 25-gauge needle to reduce viscosity and were centrifuged at room temperature for 2 min. The supernatants were divided into several aliquots and stored at –70°C.

Western blotting

Samples were electrophoresed in SDS 4–6% polyacrylamide gels, transferred electrophoretically to nitrocellulose membranes (e.g., Weeks et al. 1982), and processed following the manufacturer's protocols (Tropix, Inc.). Membranes were dried at 37°C for 10 min, rinsed several times with wash buffer (PBS, 0.1% Tween 20, 0.02% NaN₃), blocked with blocking buffer (0.2% I-Block Reagent in wash buffer), rinsed again with wash buffer, and reacted with primary antibodies for 1–2 hr at room temper-

ature or overnight at 4°C. Bound primary antibody was detected by alkaline phosphatase-conjugated secondary antibody, followed by reaction with the chemiluminescent substrate AMPPD (Tropix, Inc.) or, in some cases, with Nitroblue tetrazolium (NBT, 100 μ g/ml) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 50 μ g/ml) in 50 mM Tris-HCl, 50 mM MgCl₂ (pH 9.6).

Phosphatase treatment of PCTD fusion protein

Twenty-five-nanogram samples of PCTD fusion protein were incubated at 37°C with varying amounts of alkaline phosphatase in dephosphorylation buffer (50 mM Tris-HCl, 0.1 mM EDTA at pH 8.5) for 2 hr; reactions were stopped by adding 15 μ l of SDS sample buffer and placing on dry ice. A control contained no phosphatase. The samples were then heated at 95°C for 5 min and subjected to Western blot analysis.

Polytene chromosome preparations

We find the following procedure to be convenient and the results obtained to be reproducible and essentially identical to those obtained with somewhat different methods (Silver and Elgin 1976; Weeks et al. 1982; Clark et al. 1991, and references therein; Zink et al. 1991). Late third-instar larvae, grown on modified Instant *Drosophila* Medium (Greenleaf et al. 1979) at room temperature (22±2°C), were dissected in PBS+0.5% Triton X-100 (PBS=10 mM sodium phosphate at pH 7.2+0.85% NaCl; all steps at room temperature unless indicated otherwise). Salivary glands were picked up in ~4 μ l of solution with a mechanical pipettor and transferred into ~0.5 ml of fresh PBS+0.5% Triton X-100 in one well of a Pyrex Spot Test Plate. The glands were then transferred to another well of the plate containing ~0.5 ml of PBS+0.5% Triton X-100+3.7% formaldehyde, fixed for ~30 sec, and then rinsed again in fresh PBS+0.5% Triton X-100. The fixed glands were transferred to another well of the plate containing 45% acetic acid+1% formaldehyde, then picked up in ~9.0 μ l and placed on a siliconized 18×18-mm glass coverslip. Chromosome spreads were prepared by placing the coverslip on a microscope slide and tapping with the eraser end of a pencil. These spreads were then squashed and placed on a block of dry ice for several minutes. The coverslip was popped off with a razor blade, and the slide was placed in PBS+0.1% Tween 20 (PBS-Tw) for ≥30 min. At this point, slides were either subjected to antibody staining or transferred to PBS-Tw/50% ethylene glycol and stored at –20°C. Note that a control experiment with Pol II, in which we varied the fixation time (in the 3.7% HCHO) from 0 to 120 sec, indicated that fixation was essentially complete (i.e., maximum fluorescence was attained) by 10 sec.

For heat shock preparations, third-instar larvae were heat shocked at 37°C (in a closed 1.5-ml microcentrifuge tube submerged in a water bath) for the desired time and then dissected quickly as described above. For heat shocks of 30–90 sec, salivary glands were removed as described above, submerged in PBS+0.5% Triton X-100 at 37°C for the desired time, and transferred immediately to fixative.

Antibody staining

The squash preparations were rinsed with PBS-Tw and then blocked for 30 min in PBS-Tw+4% normal donkey serum (NDS) (these and subsequent steps at room temperature). The slides were then rinsed briefly in PBS-Tw and incubated with the primary antibodies in PBS-Tw (dilutions between 1:10 and 1:1000; final concentrations for affinity-purified IgG usually ~1–5 μ g/ml) for 45–60 min. The slides were washed in PBS-Tw

at least three times for 10 min each time and blocked again as described above. The slides were then incubated with the secondary antibodies (see above) in PBS-Tw (generally at $\sim 1 \mu\text{g/ml}$) for 45–60 min and, after incubation, were again washed as described above. At this point, the slides were stored in PBS-Tw for short times (a few hours or less) or in PBS/50% ethylene glycol at -20°C for longer times. In cases where DAPI staining was required, after the last wash, the slides were stained with a solution of DAPI (10 ng/ml) in PBS-Tw for 30–60 sec and washed again with PBS-Tw.

Indirect immunofluorescence microscopy

A coverslip was mounted using 70% glycerol, 0.03 M Tris at pH 9 + 2.5% *n*-propyl gallate, and the chromosome preparations were viewed with a Zeiss Axiophot microscope through a Zeiss 40 $\times$ Plan-NEOFLUAR objective (oil immersion, NA 1.4). Images were captured using a Photometrics STAR I Cooled CCD Imaging System and Signal Analytics Corp. IPLab Spectrum-PMI v. 2.2.0 software running on a Macintosh Quadra 950. Subsequent image processing was carried out using Adobe Systems, Inc., Photoshop v. 2.0.1 on Macintosh hardware. Images were sharpened, scaled, pseudocolored, and overlaid; they were not edited internally. Frequently, 0.5- μm latex beads that fluoresce in all three channels used were included in the mounting medium to facilitate precise alignment of the images (e.g., Rykowski et al. 1988).

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