Pin1 modulates the structure and function of human RNA polymerase II

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The C-terminal domain of the RNA polymerase (RNAP) II largest subunit (CTD) plays critical roles both in transcription of mRNA precursors and in the processing reactions needed to form mature mRNAs. The CTD undergoes dynamic changes in phosphorylation during the transcription cycle, and this plays a significant role in coordinating its multiple activities. But how these changes themselves are regulated is not well understood. Here we show that the peptidyl-prolyl isomerase Pin1 influences the phosphorylation status of the CTD in vitro by inhibiting the CTD phosphatase FCP1 and stimulating CTD phosphorylation by cdc2/cyclin B. This is reflected in vivo by accumulation of hypophosphorylated RNAP II in *pin1*^{-/-} cells, and of a novel hyper-hyperphosphorylated form in cells induced to overexpress Pin1. This hyper-hyperphosphorylated form of RNAP II also accumulates in M-phase cells, in a Pin1-dependent manner, and associates specifically with Pin1. Functionally, we find that Pin1 overexpression specifically inhibits ongoing transcription of mRNA precursors in vivo and both transcription and RNAP II-stimulated pre-mRNA splicing in cell extracts. Pin1 thus plays a significant role in regulating RNAP II CTD structure and function.

[**Keywords:** Pin1; RNA polymerase II; RNAP II CTD; RNA splicing; transcription; prolyl isomerase]

Received July 22, 2003; revised version accepted September 17, 2003.

The multisubunit RNA polymerase II (RNAP II) has long been known to be responsible for transcription of mRNA-encoding genes. More recently, it has become clear that the enzyme participates in posttranscriptional events as well (for review, see Hirose and Manley 2000; Maniatis and Reed 2002; Orphanides and Reinberg 2002). Key to all these functions is the CTD (C-terminal domain), which consists in mammals of 52 tandem repeats of the consensus heptapeptide YSPTSPS. The CTD can be extensively phosphorylated, especially at the Ser 2 and Ser 5 positions, which results in two distinguishable forms of RNAP II, the hypophosphorylated IIA and hyperphosphorylated IIO (for review, see Dahmus 1996). RNAP IIA is preferentially recruited to promoters during the formation of the transcription preinitiation complex, in which the CTD contributes to the response to different signals from activators or repressors (Myers and Kornberg 2000). The transition from initiation to elongation is accompanied by CTD phosphorylation, predominantly on Ser 5 by the CDK7 component of the general transcription factor TFIIF (Komarnitsky et al. 2000; Schroeder et al. 2000). During elongation, the phosphorylation pattern changes toward Ser 2 phosphorylation (Cho et al. 2001). This involves distinct cyclin-dependent kinases, such as the CDK9 subunit of the elongation factor p-TEFb (Price 2000). Although several

CTD kinases have been identified, only one CTD phosphatase, FCP1, has been characterized (Chambers and Dahmus 1994; Cho et al. 1999). FCP1 presumably helps to recycle RNAP II at the end of the transcription cycle by converting RNAP IIO into IIA for another round of transcription.

The involvement of RNAP II, and specifically its phosphorylated CTD, in splicing of mRNA precursors is supported by several studies (see Manley 2002 and references therein). For example, mRNA precursors synthesized in transiently transfected cells by an RNAP II with a truncated CTD are inefficiently spliced (McCracken et al. 1997). Splicing can also be significantly stimulated in vitro in the absence of transcription by purified RNAP IIO, but not IIA, which in fact inhibits splicing (Hirose et al. 1999). Splicing stimulation during transcription may involve two mechanistic phases. First, the interaction of splicing factors with CTD-associated factors facilitates recognition of splice sites on the nascent pre-mRNA. For instance, the elongation factor TAT-SF1 can recruit splicing factors, including snRNPs, to the nascent RNAs during in vitro transcription, and this interaction was found not only to stimulate splicing but also to promote transcription elongation (Fong and Zhou 2001). Second, the CTD likely participates directly in the assembly of the spliceosome by interacting with specific splicing factors. An interesting example is provided by Prp40, an evolutionarily conserved component of U1 snRNP (Kao and Siliciano 1996; Neubauer et al. 1997), which interacts with the phosphorylated CTD via a proline-recognizing WW domain (Morris and Greenleaf 2000).

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Article published online ahead of print. Article and publication date are at <http://www.genesdev.org/cgi/doi/10.1101/gad.1135503>.

The coordinated assembly and disassembly of both transcription and RNA processing machineries would appear to require a flexible and dynamic CTD structure. The structural flexibility of the CTD is suggested by recent crystal structure of RNAP II, even though the structure of the CTD was not resolved (Cramer et al. 2001). The CTD is located near the exit point of the nascent transcript from the body of the polymerase, and a linker sequence, which could serve to provide important flexibility, connects the CTD. Once the nascent transcript emerges from RNAP II, it would be easily accessible to factors assembled on the CTD. Thus, regulation of CTD structure could be an important aspect in mRNA production. Because the CTD undergoes dynamic changes in phosphorylation during transcription, such regulation could be modulated by changes in phosphorylation and/or dephosphorylation. Unique to the CTD is not only the tandem heptad repeats, but also the presence of multiple SP dipeptides, which constitute a conserved phosphorylation motif important in many cellular processes. Significantly, *cis-trans* isomerization of prolyl peptide bonds is known to be a rate-limiting step in protein folding (Fisher 1994). The multiple repeats of the SP motif suggest that reconfiguration of the CTD may involve additional factors to catalyze peptidyl-prolyl isomerization. One candidate for this is the peptidyl-prolyl isomerase Pin1, which has a specificity for phosphorylated S/T-P dipeptides (Yaffe et al. 1997).

Human Pin1 was initially identified as an evolutionarily conserved protein required for proper mitotic progression (Lu et al. 1996). Many recent studies have indicated that Pin1 functions not only in cell cycle regulation, but also in several other cellular processes (for review, see K.P. Lu et al. 2002). For instance, Pin1 can interact with transcription factors such as phosphorylated c-Jun and β -catenin and increase their activities in transcription of the cyclin D1 gene (Ryo et al. 2001; Wulf et al. 2001). The phosphorylated CTD may also be a target of Pin1, as the CTD interacts with human Pin1 *in vitro* (Albert et al. 1999), and with the yeast homolog of Pin1, Ess1, *in vitro* and *in vivo* (Morris et al. 1999; Wu et al. 2000). Unlike other substrates, which have only one or two SP motifs, the CTD contains multiple potential sites for Pin1 binding and catalysis. Pin1 colocalizes with the splicing factor SC35 (Lu et al. 1996), suggesting that Pin1 may play a role in transcription and/or pre-mRNA processing. Consistent with this, in *Saccharomyces cerevisiae*, mutation of *ESS1* resulted in defects in pre-mRNA 3'-end formation (Hani et al. 1999).

In this paper, we show that the interaction of Pin1 with the CTD modulates the phosphorylation status of the CTD. Pin1 inhibits CTD dephosphorylation by FCP1 and stimulates its phosphorylation by *cdc2/cyclinB* *in vitro*. Confirming the *in vivo* significance of these findings, the level of CTD phosphorylation is reduced in *pin1*^{-/-} cells, especially in M phase, and inducible Pin1 overexpression results in accumulation of a hyper-hyperphosphorylated form of RNAP II. We demonstrate dynamic phosphorylation changes of RNAP II during cell cycle progression, and show that the hyper-hyperphos-

phorylated RNAP II accumulates exclusively in M phase. We also show that Pin1 overexpression inhibits both RNAP II transcription and pre-mRNA splicing. Our data indicate that Pin1 plays a significant role in regulating the activities of the CTD.

Results

Pin1 inhibits RNAP II CTD dephosphorylation by the CTD phosphatase FCP1

The close relationship between phosphorylation and CTD structure and function suggests that Pin1 could play a direct role in modulating CTD function by influencing phosphorylation or dephosphorylation. In fact, genetic data in yeast as well as biochemical data point to a potential interaction among FCP1, Pin1, and RNAP II (Wu et al. 2000; Kops et al. 2002). We first wished to test the effect of Pin1 on the ability of FCP1 to dephosphorylate the CTD, and to this end purified human Pin1 (as a GST fusion protein from *Escherichia coli*), human FCP1 (as an his-tagged protein from recombinant baculovirus-infected insect cells), and human RNAP IIA and RNAP IIO (from HeLa cells; see Fig. 1A). As an initial experiment, two different amounts of FCP1 were incubated with RNAP IIO in the presence or absence of increasing amounts of GST-Pin1, and CTD dephosphorylation was assayed by Western blotting with an antibody (8WG16) that detects the CTD regardless of phosphorylation status. The results (Fig. 1B) show that, as expected, FCP1 was able to convert the IIO isoform to the dephosphorylated IIA form (Fig. 1B, cf. lanes 1 and 2,6). In each case, addition of GST-Pin1 inhibited dephosphorylation (Fig. 1B, lanes 3–5,7–9). To provide evidence that the inhibition was specific, we expressed and purified two Pin1 mutant proteins as GST fusions (see Fig. 1A,C). One, Y23A, alters a key residue in the N-terminal WW domain, which is responsible for substrate binding (Lu et al. 1999), and the other, R68,69A, disrupts catalytic activity (Shen et al. 1998). These two mutant proteins, together with wild type, were then used in an experiment similar to that in Figure 1B. GST-Pin1 again strongly inhibited dephosphorylation (Fig. 1D, cf. lanes 2,3 and 4–6). However, both mutants were defective, Y23A (Fig. 1D, lanes 7–9) partially so and R68,69A (Fig. 1D, lanes 10–12) was completely inactive. The results indicate that the interaction between Pin1 and RNAP II inhibits dephosphorylation of the CTD by FCP1.

*Pin1 stimulates CTD phosphorylation by *cdc2/cyclin B**

We next set out to investigate whether Pin1 might also influence phosphorylation of RNAP II. Several CTD kinases have been identified, but they have different phosphorylation specificities, and function during different stages of the transcription cycle and/or under different physiological conditions (for review, see Lin et al. 2002). For example, the mitotic kinase *cdc2/cyclinB* can phosphorylate the CTD on both Ser 2 and Ser 5, but the significance of this is unknown (Cisek and Corden 1989;

Figure 1. Dephosphorylation of RNAP IIO by FCP1 is inhibited by Pin1 in vitro. (A) Proteins used in this study. Silver staining of 7.5% SDS-PAGE gel with RNAP IIA (lane 1) and IIO (lane 2) purified from HeLa cells. Coomassie staining of 7.5% SDS-PAGE gel with GST-Pin1 (lane 3), GST-Pin1 Y23A (lane 4), and GST-Pin1 R68,69A (lane 5) purified from *E. coli*. Coomassie staining of 7.5% SDS-PAGE gel with his-tagged FCP1 purified from recombinant baculovirus-infected SF9 cells (lane 6). Sizes of marker proteins are indicated. (B) Inhibition of RNAP IIO dephosphorylation by Pin1. Purified RNAP IIO (50 ng) was preincubated with GST-Pin1 (10, 100, 200 ng; lanes 3–5, 7–9, respectively) at 30°C for 10 min followed by addition of 5 ng (lanes 2–5) and 10 ng (lanes 6–9) of FCP1. Reaction mixtures were incubated at 30°C for 20 min. Reactions were terminated by addition of SDS loading buffer and proteins were analyzed by 7.5% SDS-PAGE. Blots were probed with mAb 8WG16. The positions of RNAP IIA and IIO are indicated. (C) Diagram of human Pin1 structure. Positions of the two Pin1 mutations are shown. (D) Mutation in the Pin1 catalytic domain abolishes inhibition. Dephosphorylation reactions were performed as in B with 10, 100, or 200 ng GST-Pin1 (lanes 4–6), GST-Pin1 Y23A (lanes 7–9), or R68,69A (lanes 10–12). Reaction mixtures were incubated at 30°C for 30 min. The blot was probed with polyclonal antibody N20.

Gebara et al. 1997). Given the fact that Pin1 is a known mitotic regulator, we reasoned that there could be a link between Pin1 and cdc2 in modulating phosphorylation of the CTD during, or as cells enter, M phase. To examine the potential role of Pin1 in controlling CTD phosphorylation by cdc2/cyclin B, we first used purified RNAP IIA as a substrate. Because Pin1 binding is phosphorylation-dependent, there is no detectable interaction between Pin1 and RNAP IIA (Morris et al. 1999; data not shown). However, once cdc2 begins to phosphorylate the CTD, the partially phosphorylated CTD can be recognized by Pin1, and this interaction could possibly affect subsequent phosphorylation. To test this, RNAP IIA was incubated with purified recombinant cdc2/cyclin B in the presence of increasing amounts of GST-Pin1 or the R68,69A mutant. As expected, cdc2/cyclin B phosphorylated the IIA subunit, as observed by the mobility change compared with the input IIA (Fig. 2A, cf. lanes 2 and 3,4). Strikingly, addition of GST-Pin1 stimulated phosphorylation (Fig. 2A, lanes 5–7), but Pin1 R68,69A was without effect (Fig. 2A, lanes 8–10). Similar results were obtained in a direct phosphorylation assay using [γ - 32 P]ATP (Fig. 2B). Incorporation of 32 P into RNAP IIA was stimulated by GST-Pin1 (Fig. 2B, lanes 4–6) but not by the R68,69A mutant (Fig. 2B, lanes 7–9). The data suggest that binding of Pin1 to newly phosphorylated sites enhances subsequent CTD phosphorylation.

We next tested whether Pin1 might also stimulate phosphorylation of RNAP IIO by cdc2/cyclin B. Although RNAP IIO is considered to be hyperphosphorylated, it can be further phosphorylated, on both Ser 2 and Ser 5, and indeed such a hyper-hyperphosphorylated form accumulates in M-phase cells (see below). To investigate the possible effect of Pin1 on RNAP IIO CTD phosphorylation, purified RNAP IIO was preincubated with or without GST-Pin1 or the R68,69A mutant followed by addition of cdc2/cyclin B. CTD phosphorylation was monitored by Western blotting with H5 and H14 antibodies, which allow detection of Ser 2 and Ser 5 phosphorylation, respectively (Fig. 2C,D, respectively). The RNAP IIO subunit CTD was, indeed, further phosphorylated, on both Ser 2 and Ser 5 (Fig. 2C,D, cf. lanes 2 and 3,4). Most importantly, GST-Pin1 significantly stimulated phosphorylation on Ser 2 and Ser 5 in a dose-dependent manner (Fig. 2C,D, lanes 4–6), whereas the R68,69 mutant was essentially without effect (Fig. 2C,D, lanes 7–9). Thus our data together indicate that Pin1 can enhance CTD phosphorylation in vitro both by inhibition of FCP1-mediated dephosphorylation and by increasing phosphorylation by cdc2/cyclin B.

Pin1 modulates RNAP II CTD phosphorylation in vivo

The above results indicate that Pin1 has the potential to modulate RNAP II phosphorylation levels. But it was

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Figure 2. Pin1 stimulates RNAP II phosphorylation by cdc2/cyclin B in vitro. (A) Purified RNAP IIA (20 ng) was incubated with 200 ng GST (lane 4), GST-Pin1 and GST-Pin1 R68,69A (10, 100, 200 ng; lanes 5–7, 8–10, respectively) in the presence of cdc2/cyclin B at 30°C for 30 min. Reactions were terminated by addition of SDS loading buffer and proteins analyzed by 7.5% SDS-PAGE. (Lane 1) HeLa whole-cell extract (5 μ g). Blots were probed with mAb 8WG16. Positions of RNAP IIA and IIO are indicated. (B) Reactions were performed as in A, but with 20 μ Ci [γ - 32 P]ATP at 30°C for 20 min. (C) Purified RNAP IIO (20 ng) was preincubated with GST (lane 3), Pin1 and R68,69A mutant (10, 100, 200 ng; lanes 4–6, 7–9, respectively) at 30°C for 10 min, followed by addition of 10 ng of cdc2/cyclin B. Mixtures were incubated at 30°C for 30 min. Reactions were terminated by addition of SDS loading buffer, and proteins were analyzed by 7.5% SDS-PAGE. The blot was probed with mAb H14. The positions of RNAP IIO are indicated. (D) Reactions were performed as in C, but probed with mAb H5.

important next to determine whether this actually occurs in vivo. To this end, we first examined the phosphorylation status of RNAP II, by Western blotting, under conditions in which Pin1 was inactivated. If a function of Pin1 is to reduce FCP1 activity and to increase cdk activity toward the CTD, then the amount of RNAP IIO should decrease in the absence of Pin1 activity. We initially asked whether incubation of cells with the Pin1 inhibitor juglone affected RNAP IIO accumulation in HeLa cells. The results (Fig. 3A) reveal a concentration-dependent increase in gel mobility and decrease in the amount of the IIO subunit, as expected if Pin1 is inhibited and therefore unable to down-regulate FCP1 and/or up-regulate CTD kinase activities. Note that the amount of RNAP IIA did not increase correspondingly, suggesting that the excess unphosphorylated IIA subunit was degraded. To examine more directly the role of Pin1 in determining RNAP II phosphorylation levels, we used

mouse embryonic fibroblasts (MEFs) derived from *pin1*^{−/−} mice (Liou et al. 2002). We first compared the phosphorylation status (i.e., gel mobility) of the IIO subunit in wild-type and mutant MEFs and for comparison in NIH 3T3 cells (Fig. 3C). As in the juglone-treated cells, the IIO isoform detected in the *pin1*^{−/−} cells displayed significantly greater gel mobility than that in either of the other two cell lines (Fig. 3C, lanes 1–3). Strongly supporting the possibility that the effect of juglone on RNAP II phosphorylation was mediated by Pin1, wild-type MEFs displayed a similar decrease in RNAP IIO accumulation in response to juglone as observed in HeLa cells (Fig. 3B, lanes 6–10), but RNAP IIO in the *pin1*^{−/−} MEFs was unaffected by the inhibitor (Fig. 3B, lanes 1–5).

To extend these findings, we next examined RNAP II phosphorylation status in cells overexpressing Pin1. To this end, HeLa cells were stably transfected with plas-

Figure 3. Pin1 enhances RNAP II phosphorylation in vivo. (A) The Pin1 inhibitor juglone decreases RNAP II phosphorylation. HeLa cells were treated with juglone for 8 h (0.05, 0.125, 0.625, 1.25 μ M, lanes 2–5). Total cell lysates were separated by 7.5% SDS-PAGE, and blots were probed with mAb H14 (upper), 8WG16 (middle), and anti-actin antibody (lower). (B) RNAP II phosphorylation levels in *pin1*^{−/−} MEFs are unaffected by juglone. *pin1*^{−/−} (right panel) and wild-type (left panel) MEFs were treated with juglone (0.05, 0.125, 0.625, 1.25 μ M, lanes 2–5, 7–10, respectively) for 8 h. Total cell lysates were separated by 7.5% SDS-PAGE and probed with mAbs H5 and H14 (upper), mAb 8WG16 (middle), and anti-actin (bottom). The positions of RNAP IIA/O and actin are indicated. (C) Western blot analysis of total cell lysates from NIH 3T3 cells (lanes 1, 4) and *pin1*^{−/−} (lanes 2, 5) and wild-type (lanes 3, 6) MEFs, in the absence (lanes 1–3) or presence of 100 ng/mL nocodazole for 12 h (Noco-treated; lanes 4–6). Equivalent amounts (~5 μ g) of the lysates were separated by 7.5% SDS-PAGE and probed with mAb H14 (upper panel) and mAb 8WG16 (lower panel).

mids expressing flu epitope-tagged wild-type or R68,69A Pin1 from a minimal tetracycline (tet)-responsive promoter and a tet-activatable tet-VP16 fusion protein. Cell lysates were prepared at different times after addition of doxycycline and analyzed by Western blotting, first with H14 antibodies. The results show that as flu-Pin1 levels increased (Fig. 4A, top), the amount of the Ilo subunit appeared to increase, and its mobility decreased (Fig. 4A, bottom). At the same time, RNAP Ila levels decreased, consistent with its conversion to Ilo. At the longest time of induction (19 h), flu-Pin1 levels declined, probably because of toxicity, and, significantly, so did levels of hyperphosphorylated RNAP Ilo. In contrast, Pin1 R68,69A expression had no detectable effect on RNAP II phosphorylation even though it was expressed at least as well as wild-type Pin1 (Fig. 4B). Pin1-induced RNAP II hyperphosphorylation also occurred on Ser 2, because enhanced phosphorylation was detected in Western blots probed with H5 antibodies (Fig. 4A). It is noteworthy, however, that Ser 5 phosphorylation appears to contrib-

ute more to the reduced mobility of RNAP Ilo (see below). Taken together, our results indicate that Pin1 helps to determine the phosphorylation status of RNAP II in vivo.

Pin1-dependent RNAP II hyperphosphorylation occurs during M phase

Pin1 was initially identified as a potential mitotic regulator, and considerable data support the idea that this is, indeed, an important aspect of its function (K.P. Lu et al. 2002). We therefore wished to investigate whether the Pin1-dependent hyperphosphorylation of RNAP II we detected might be heightened in M phase. To this end, we first assessed the phosphorylation status of RNAP II at different stages of the cell cycle, again by Western blotting. An initial experiment examined HeLa cells arrested by a double thymidine block at G1/S phase and then released, allowed to continue through the cell cycle and to accumulate in M phase. Cell lysates were probed first with the 8WG16 antibody to detect both the Ilo and Ila isoforms (Fig. 5A, top). Strikingly, not only did the amount of Ilo appear to increase, but more significantly, its mobility decreased in the lysates from cells accumulated in M phase (Fig. 5A, lanes 9,10). In contrast, the levels of Ila remained similar, except in M phase, where they were slightly reduced. To extend this analysis, blots from a similar experiment were probed with either H14 (Fig. 5A, middle panel) or H5 (Fig. 5A, bottom panel). The H14 blot shows a striking accumulation and mobility change of the hyperphosphorylated Ilo subunit, consistent with the analysis with 8WG16, whereas the H5 blot revealed almost no accumulation of this hyperphosphorylated form. Together, these results indicate that RNAP II becomes very highly phosphorylated during M phase, and that this occurs principally on Ser 5. Although a form of RNAP II that appears to possess an intermediate level of CTD phosphorylation has been described (RNAPIIm; Bonnet et al. 1999), such a hyper-hyperphosphorylated form of RNAP II has not, and we refer to it as RNAP IIOO.

We next wished to determine whether the M-phase-specific hyperphosphorylation was dependent on Pin1 activity. A first experiment extended the analysis of the *pin1*^{-/-} MEFs to examine Ilo phosphorylation in lysates from mitotic (nocadazole-treated) cells. The results (Fig. 3C, lanes 4–6) reveal first that the Ilo subunit from mitotic wild-type cells (3T3 and MEFs) displayed reduced mobility relative to that from asynchronous cells (Fig. 3C, cf. lanes 1 and 4, 3 and 6), consistent with the results obtained with HeLa cells (Fig. 3C). Most striking, however, was the significant underphosphorylation (i.e., lack of hyperphosphorylation) of the Ilo subunit from the nocadazole-arrested *pin1*^{-/-} MEFs (Fig. 3C, lane 5). These results indicate that Pin1 is necessary for M-phase-specific hyperphosphorylation of the RNAP II CTD. This likely reflects its effects on FCP1 and cdc2/cyclinB, although additional factors may also be involved.

The above results strongly suggest that Pin1 interacts with RNAP IIO in vivo, as it does in vitro. To examine

Figure 4. Inducible overexpression of Pin1 increases RNAP IIO hyperphosphorylation in vivo. (A) HeLa cells stably expressing inducible flu-tagged Pin1 were cultured in the absence (lane 2) or presence (lanes 3–6) of 1 μ g/mL doxycycline, and total cell lysates were prepared at the times indicated. Lysates were also prepared from the parental (tet-on, lane 1) cells. Proteins were fractionated by 7.5% SDS-PAGE, and blots were probed with α -flu and anti-Pin1 antibodies, and mAb H14, H5, and 8WG16 (from top to bottom, respectively). Flu-Pin1, Pin1, and RNAP IIO and IIA are indicated. (B) Similar experiments as in A but using cells inducibly expressing R68,69 Pin1.

Figure 5. Hyperphosphorylated RNAP IIO accumulates in M phase and preferentially associates with Pin1. (A) HeLa cells were arrested at the G1/S boundary and then released. Nocodazole was added 8 h after release, and cells were incubated for another 4 or 6 h (Noco+, lanes 9,10). G1 cells (Noco+/-, lane 11) were obtained by washing the nocodazole-treated cells (6 h) and incubating in media without nocodazole for another 4 h. At the indicated time points, cells were harvested and total cell lysates were fractionated by 7.5% SDS-PAGE. Blots were probed with mAb 8WG16 (upper), H14 (middle), H5 (lower), and anti-SR protein mAb104 (bottom). The positions of RNAP IIO and IIA are indicated. (B) HeLa cells were transfected with an expression vector encoding Flag-epitope-tagged Pin1. Cell extracts were prepared after 48 h. Aliquots were then immunoprecipitated with anti-Flag mAb (lane 4), anti-mouse IgG (lane 3), and protein-A-conjugated beads alone (lane 2). Pellets were washed, dissolved in sample buffer, and fractionated by 7.5% SDS-PAGE. Blots were probed with mAbs 8WG16 plus H14 (upper) and mAb 104 (lower).

this directly, we performed coimmunoprecipitation (coIP) experiments to assess the association between the two proteins. HeLa cells were transiently transfected with an expression vector encoding Flag-epitope-tagged Pin1, and extracts were prepared and immunoprecipitated with anti-Flag antibodies. Blots were probed with 8WG16 and H14 or, as a control, an anti-SR protein splicing factor antibody. The results (Fig. 5B) show that a significant fraction of RNAP IIO, but not RNAP IIA or SR proteins, was specifically coIPed (Fig. 5B, lane 4). Remarkably, only a very slow mobility form of the IIO subunit, which appears identical to the hyperphosphorylated mitotic form described above (RNAP IIOO), was detected. This interaction was very specific and efficient.

Only a small amount of this form could be detected in the input (Fig. 5B, lane 1), and it was absent in the IP flowthrough (Fig. 5B, lane 5) but recovered in high yield in the IP (Fig. 5B, lane 4). Together these results indicate that not only is Pin1 required for M-phase-specific accumulation of a hyperphosphorylated form of RNAP IIO, but also that this likely reflects a strong and specific interaction between the two proteins.

Pin1 inhibits RNAP II transcription in vivo

We next wished to investigate possible functional consequences of Pin1-dependent hyperphosphorylation of RNAP II. Gene expression is known to be silenced during M phase in metazoan cells, and it seemed feasible that Pin1 might contribute to this via its effect on RNAP II. To test this, we first used the Pin1-inducible cell lines described above and measured synthesis of nascent mRNAs precursors by pulse labeling following induction of wild-type or mutant Pin1. Wild-type and the R68,69A mutant Pin1-expressing cells were treated with doxycycline exactly as above, and at the indicated times cells were exposed to [³H]uridine for short pulses (~10 min). Total RNA was extracted from the cells, and, to assess mRNA transcription, poly(A)⁺ RNA was purified by oligo(dT) column chromatography. Both poly(A)⁺ RNA and the corresponding poly(A)⁻ RNA fractions were quantitated by scintillation counting and by slot blotting on membranes that were then exposed to X-ray film (see Materials and Methods for details). By both measurements, the amount of newly transcribed poly(A)⁺ RNA was significantly reduced after treatment with doxycycline in the wild-type Pin1-expressing cells (Fig. 6A, lane 1, B, lanes 1–4), whereas poly(A)⁻ RNA synthesis was not detectably affected (Fig. 6A, lane 2, B, lanes 9–12). In contrast, both poly(A)⁺ and poly(A)⁻ RNA synthesis in the cells expressing mutant Pin1 was unaffected (Fig. 6A, lanes 3,4, B, lanes 5–8,13–16). Significantly, poly(A)⁺ RNA synthesis increased slightly after 19 h of doxycycline treatment in the wild-type expressing cells, which is consistent with the observed decrease in levels of RNAP IIOO and Pin1 at this time (see Fig. 4A). These results, coupled with our characterization of RNAP II phosphorylation in these same cells, suggest that Pin1-mediated hyperphosphorylation of RNAP II leads to inhibition of mRNA synthesis.

The above experiments relied on oligo(dT) selection to identify RNAP II products. Because this, in turn, relied on efficient polyadenylation, and because Pin1 (Ess1) has been suggested to influence 3'-end formation in yeast (Hani et al. 1999), we wished to verify the effect of Pin1 overexpression on transcription by an independent method. To this end, we prepared nuclear extracts (NE) from cells induced to express wild-type or R68,69A Pin1 for the same times indicated in Figure 4A, and assayed their transcriptional potential with a linearized plasmid containing the CMV promoter (Fig. 6C). The NEs from the cells overexpressing flu-Pin1 displayed decreased transcriptional activity as Pin1 accumulated, but the presence of the mutant Pin1 was without effect. These

Figure 6. Inducible overexpression of Pin1 reduces RNAP II transcription in vivo and in vitro. (A) Pulse-label analysis of nascent poly(A) RNA synthesis. Wild-type and R68,69A Pin1 cell lines were incubated with or without doxycycline for the indicated times. Cells were exposed to [3 H]uridine for ~10 min. Total cellular RNA was purified and poly(A) RNA was oligo(dT)-selected. Poly(A)⁺ and poly(A)⁻ RNAs were slot-blotted, and the filter was soaked in EN3HANCE for 1 h and exposed to X-ray film. (B) Poly(A)⁺ and poly(A)⁻ RNAs from the wild-type and R68,69A cell lines as in A were spotted on filters and the incorporation was determined by scintillation counting. Lanes 1–4, 5–8, 9–12, and 13–16 represent cells without and with doxycycline for 6, 9, and 19 h, respectively. (C) NEs from wild-type (right) and mutant R68,69A (left) Pin1-expressing cells with or without doxycycline treatment for the times indicated were used for in vitro transcription. A linearized CMV promoter-containing plasmid was used as template in the presence of [α - 32 P]GTP. RNAs were resolved by 7 M urea–6% PAGE. The positions of DNA markers are indicated. The expected RNA transcript is 523 nt.

results correlate well with the accumulation of hyperphosphorylated RNAP II over the same time course (Fig. 4A), and with the inhibition observed in the in vivo pulse-label experiment, except that for unknown reasons, in vitro transcription did not recover at the 19-h time point. These results confirm that RNAP II transcription can be inhibited by Pin1 overexpression.

Pin1 inhibits pre-mRNA splicing in an RNAP II-dependent manner

RNAP II can also function to enhance pre-mRNA splicing, and this activity requires a phosphorylated CTD. For example, we showed previously that purified RNAP IIO, but not IIA, can stimulate splicing when added to extracts lacking RNAP II (Hirose et al. 1999). To determine if Pin1 might influence this activity, we first tested whether addition of purified recombinant Pin1 to HeLa NE affects splicing of a model (β -globin) pre-mRNA. The results (Fig. 7A, lanes 1–4) indicate that Pin1 inhibited splicing in a concentration-dependent manner. Inhibition was specific, as the R68,69A mutant (Fig. 7A, lanes

5–7) did not affect splicing. Inhibition occurred at an early stage in the reaction, as analysis of spliceosome assembly showed that splicing was blocked exclusively at assembly of the A complex (Fig. 7B), which is the same step at which RNAP IIO stimulates splicing (Hirose et al. 1999).

To determine directly whether RNAP IIO might be the target of Pin1 in splicing inhibition, we used a splicing assay dependent on RNAP IIO (Hirose et al. 1999). This involves use of HeLa S100 extract, which contains all factors required for splicing except SR proteins and also lacks RNAP II. Splicing can be activated by addition of SR proteins, and under limiting conditions, stimulated by RNAP IIO. In the presence of very low levels of the SR protein ASF/SF2, sufficient to induce only barely detectable levels of splicing (Fig. 7C, cf. lanes 1 and 2), increasing amounts of RNAP IIO significantly stimulated splicing (Fig. 7C, lanes 3–5). As with NE (Fig. 7A), addition of Pin1 effectively inhibited splicing (Fig. 7C, lanes 6–8). However, when splicing was stimulated to a comparable level solely by addition of a higher concentration of ASF/SF2 (Fig. 7C, lane 9), addition of Pin1 was without effect

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Figure 7. Pin1 inhibits RNAP II-stimulated pre-mRNA splicing in vitro. (A) In vitro splicing of β -globin pre-mRNA in HeLa NE in the presence of increasing amounts (10, 100, or 200 ng) of GST-Pin1 (lanes 2–4) or GST-Pin1 R68,69A (lanes 5–7). The positions of precursor RNA and splicing products are indicated. (B) Spliceosome formation in NE supplemented with buffer (lanes 2–8) or 100 ng of GST-Pin1 (lanes 9–15). Reaction mixtures were incubated at 30°C for the times indicated. The positions of splicing complexes are indicated. (C, right) In vitro splicing of β -globin pre-mRNA in S100 supplemented with limiting ASF/SF2 (12.5 ng; lane 2), ASF/SF2 plus increasing amounts of purified RNAP IIO (5, 10, 20 ng; lanes 3–5) or 20 ng of RNAP IIO preincubated with increasing amounts of GST-Pin1 (10, 50, 100 ng; lanes 6–8). (Left) Similar splicing reaction but supplemented with 50 ng of ASF/SF2 (lane 9) plus increasing amounts of GST-Pin1 (10, 50, 100 ng; lanes 10–12). (D) In vitro splicing of β -globin pre-mRNA in NEs prepared from the inducible wild-type (left panel) and R68,69A (right panel) Pin1-expressing cell lines, as in Figure 6C. Cells were incubated with or without doxycycline for the indicated times. RNAs were resolved by 7 M urea–6% PAGE. The positions of precursor RNA and splicing products are indicated.

(Fig. 7C, lanes 10–12). These results indicate that Pin1 specifically inhibits splicing enhanced by RNAP IIO.

To obtain further evidence that Pin1 can affect pre-mRNA splicing, we again made use of the inducible Pin1 cell lines. Nuclear extracts were prepared from the wild-type and R68,69A Pin1-expressing cells after treatment with doxycycline as described above, and splicing was measured with the β -globin pre-mRNA (Fig. 7D). Splicing efficiency of NEs from the wild-type Pin1-expressing cells was reduced as time of treatment with doxycycline increased (Fig. 7D, lanes 1–5). Splicing inhibition correlated with the levels of RNAP II hyperphosphorylation, except that as with transcription splicing remained inhibited at 19 h. In contrast, splicing efficiency was unaffected by doxycycline treatment in NEs prepared from the R68,69A Pin1-expressing cells (Fig. 7D, lanes 6–10). These results indicate that elevated Pin1 levels in cells interfere with their competence in splicing, likely via effects on RNAP II phosphorylation.

Discussion

The data presented here provide biochemical and genetic evidence that Pin1 helps to control the phosphorylation status of the RNAP II CTD. We also showed that, in addition to the known phosphorylation changes of the CTD during the transcription cycle, the CTD undergoes significant phosphorylation changes during cell cycle progression. Specifically, we found that a hyperphosphorylated form of RNAP II, RNAP IIOO, accumulates in M-phase cells in a Pin1-dependent manner. Pin1 overexpression is sufficient to induce the formation of RNAP

IIOO, and this likely underlies the observed inhibition of both transcription and splicing. Our data support a model in which elevated Pin1 activity induces RNAP IIO hyperphosphorylation, and this represses RNAP II function in both transcription and pre-mRNA splicing.

Pin1 appears to have numerous substrates, including many proteins phosphorylated in M phase (for review, see K.P. Lu et al. 2002). This is in keeping with its role as a mitotic regulator, and our results are consistent with this property. Gene expression is known to be silenced during mitosis in metazoan organisms, and our data suggest that Pin1 contributes to this by stimulating hyperphosphorylation, and hence inactivation, of RNAP II. But our finding that this reflects direct inhibition of FCP1 activity and stimulation of cdc2/cyclin B activity toward the CTD points to a novel mechanism whereby Pin1 not only inhibits dephosphorylation but also activates phosphorylation of a substrate. Exactly how this occurs is unknown, but it is consistent with previous findings showing that Pin1 often regulates the function of its substrate via multiple mechanisms leading to the same consequences. Well-studied examples include a mitotic phosphatase, cdc25c (e.g., Zhou et al. 2000; Stukenberg and Kirschner 2001) and a G1 cyclin, cyclin D1 (Wulf et al. 2001; Liou et al. 2002). Cdc25c contains a cluster of more than a dozen SP dipeptides at its N terminus that are targets for at least two mitotic kinases, cdc2/cyclin B and plk (Izumi and Maller 1993; Kumagai and Dunphy 1996). As with the CTD, Pin1 binds to phosphorylated cdc25c and can have different effects on its activity. One is interference with the activity of the phosphatase PP2a, which naturally dephosphorylates

cdc25c (Lee et al. 1991). This may involve stoichiometric interaction between *cdc25c* and Pin1, which could block access to PP2a (Zhou et al. 2000; Stukenberg and Kirschner 2001), and/or catalytically alter *cdc25c* phosphatase activity even in the absence of PP2a (Stukenberg and Kirschner 2001). Significantly, the response to Pin1 in vitro can vary depending on the phosphorylation status of *cdc25c*: If the phosphatase was phosphorylated by *cdc2* alone, then its activity is inhibited by Pin1. But if it was phosphorylated by both *cdc2* and *plk*, then its activity is enhanced (Stukenberg and Kirschner 2001). These findings illustrate how a substrate can respond to Pin1 in different ways, and thus the ability of Pin1 to both increase phosphorylation and decrease dephosphorylation of the CTD is not unreasonable. This would allow the cell to turn off the function of the phosphorylated CTD with high efficiency and precise timing during mitosis.

The CTD has a remarkable, even unique, primary structure that makes it an exceptional and unusual target for Pin1. Whereas other characterized substrates typically have a small cluster of phosphorylatable S/T-P dipeptides, the CTD has from 50 (in yeast) to >100 (in mammals) stretched out in an essentially linear array. Given this complexity, it has not been possible to elucidate exactly which of these S/T residues, or even how many, are phosphorylated at different times. All that is really known is that the pattern changes during the transcription cycle (e.g., Komarnitsky et al. 2000; Cho et al. 2001). Pin1 may, on the one hand, help facilitate such changes, for example, by isomerizing a specific prolyl-peptide bond and thereby enhancing phosphorylation of nearby S, T, or even Y residues. On the other hand, Pin1 could be affected by these changes in phosphorylation so that the effects of Pin1 binding and/or catalysis may change, analogous to the simpler situation observed with *cdc25c*. One example may be provided by earlier results suggesting that Pin1, or its yeast homolog ESS1, can in fact stimulate CTD phosphorylation by FCP1. Kops et al. (2002) used a biochemical assay to show a modest stimulation of dephosphorylation of an in vitro phosphorylated GST-CTD fusion protein, whereas genetic studies in yeast indicated that overexpression of FCP1 could rescue a lethal *Ess1* mutation (Wu et al. 2000). These results could reflect different phosphorylation states of the CTD such that Pin1 has a positive effect on FCP1 activity as opposed to the negative effect we reported here. There are, however, alternative explanations. First, it may be that the use of GST-CTD phosphorylated in vitro (as opposed to purified RNAP II) together with a mixture of yeast and human proteins (Kops et al. 2002) may have produced results that differ from the in vivo situation, and the genetic effects in yeast need not reflect direct interactions. A second and more intriguing possibility is that the interaction involving the three proteins has different consequences in yeast and mammals, stimulating dephosphorylation in the former and repressing it in the latter. This could reflect the fact that the yeast CTD is half the size of the mammalian CTD, and lacks the sequence divergence found in the C-terminal half of the mammalian CTD. Teleologically, because yeast cells do

not silence gene expression in M phase, there is no need to inhibit RNAP II activity, and in fact this would likely be detrimental.

Our experiments have shown that overexpression of Pin1 can be deleterious to cell growth. In addition to the effects on gene expression we documented, HeLa cells overexpressing wild-type but not mutant Pin1 were difficult to isolate and maintain, could only produce modestly elevated, unstable levels of Pin1, and displayed somewhat reduced growth rates (data not shown). It is interesting to contrast these properties with those of other cells reported to overexpress Pin1. These include various cancer tissues and transformed cell lines, including a derivative of a breast cancer cell line (MCF-7) capable of inducibly expressing exogenous Pin1 (Ryo et al. 2001; Wulf et al. 2001). These cells do not appear to display properties of the Pin1-overexpressing cells we described. Although this could reflect any of multiple differences between these cell types, one interesting possibility reflects the fact that a proposed critical Pin1 target protein, β -catenin, which plays a significant role in activating genes such as cyclin D1 that are involved in transformation, is not expressed in HeLa cells (Denk et al. 1997). Perhaps under these conditions, the inhibitory effects we have documented here, which would otherwise play a significant role only in mitosis, are dominant.

A subpopulation of RNAP II is located in 20–50 nuclear bodies called speckles, which contain both transcription and pre-mRNA processing factors (Lamond and Spector 2003). However, speckles are not the active site either for transcription or RNA processing; instead, they appear to be storage sites for components of the transcription and RNA processing machineries. In actively transcribing cells, RNAP II is dispersed in the nucleoplasm, but in transcriptionally inactive cells it localizes preferentially in the speckles (Zeng et al. 1997). RNAP II in speckles is highly phosphorylated and can be recognized by both H5 and H14 antibodies (Bregman et al. 1995), and importantly, is not transcriptionally active (Zeng et al. 1997). To maintain highly phosphorylated RNAP II in nuclear speckles likely requires additional factors, because FCP1 also appears to be located in these domains (Huang and Spector 1996). Significantly, Pin1 has been shown to colocalize with the splicing factor SC35, a marker for speckles (Lu et al. 1996), indicating that Pin1 has the potential to play a role in maintaining high levels of RNAP II phosphorylation in these domains.

The Pin1-dependent formation of RNAP II_{OO} in M-phase cells provides evidence that Pin1 plays a role in controlling RNAP II activity during mitosis. It is well known that transcription is inhibited during M phase, and in the case of RNAP II this involves phosphorylation of components of the general transcription factors TFIID and TFIIF (Akoulitchev and Reinberg 1998; Long et al. 1998). It has also been observed that RNAP II_{OO} accumulates at the expense of IIA (Akoulitchev and Reinberg 1998), which would contribute to inhibition of new rounds of transcription, as IIA is the initiating form of

RNAP II. However, elongation is also blocked, and elongating polymerases are released, during M phase (Shermoen and O'Farrell 1991; Parsons and Spencer 1997). As the IIO isoform is the elongating form of the enzyme, we suggest that the IIOO form reflects a totally inactive, M-phase-specific isoform. In keeping with this, it is intriguing that IIOO is characterized by excessive Ser 5 phosphorylation. As mentioned above, Ser 5 phosphorylation naturally decreases as elongation proceeds. In addition, structural studies suggest that Pin1, via its WW domain, preferentially interacts with Ser 5 (Verdecia et al. 2000), consistent with our observation that Ser 5 is preferentially overphosphorylated in RNAP IIOO. Our model suggests that Pin1 activity should be elevated in M phase. Although Pin1 levels do not change during the cell cycle (Shen et al. 1998), Pin1 undergoes dephosphorylation in M phase, which may reflect removal of an inhibitory phosphate in the WW domain (P.J. Lu et al. 2002).

The inhibition of RNAP IIO-stimulated splicing by Pin1 also likely reflects the silencing of gene expression that occurs during M phase. We have recently shown that splicing, like all other steps in metazoan gene expression, is repressed in extracts of mitotic cells (Shin and Manley 2002). This inhibition requires a newly characterized SR protein, SRp38, but a role for RNAP II, and Pin1, is also consistent with available data. RNAP IIO is not an essential splicing factor, but appears to play an important stimulatory role. An attractive model is that RNAP IIO functions to recruit splicing factors to sites on nascent RNAs to facilitate cotranscriptional splicing, and that elevated Pin1 activity prevents these interactions. Although it will be important in the future to determine whether it is Pin1 binding, catalytic activity, or both that is responsible for its effects on RNAP II, our results indicate that Pin1 plays a significant role in modulating gene expression at multiple levels via its interaction with RNAP IIO.

Materials and methods

Protein purification and in vitro RNAP II dephosphorylation and phosphorylation assays

Purification of RNAP IIA and IIO from HeLa cells was carried out as previously described (Lu et al. 1991). Recombinant GST-Pin1 and the mutant Y23A and R68,69A were purified from *E. coli* as described (Xiao and Manley 1997). Recombinant histagged FCP1 was expressed in baculovirus-infected SF9 cells and purified as described (Cho et al. 1999). Flu-epitope-tagged cdc2/cyclin B was purified from Sf9 insect cells coinfecting with human p34cdc2 and cyclin B recombinant baculovirus (Wang and Prives 1995). RNAP IIO dephosphorylation analysis was performed in buffer P containing 20 mM HEPES (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 10% glycerol, and 0.2 mM PMSF at 30°C (Cho et al. 1999). RNAP II phosphorylation was conducted in a buffer containing 60 mM KCl, 50 mM Tris (pH 7.8), 10 mM MgCl₂, 0.5 mM DTT, 1.5 mM sodium azide, 1.5 mM NaF, and 0.2 mM PMSF at 30°C.

RNAP II phosphorylation analysis during the cell cycle

HeLa cells were arrested at the G1/S boundary by double thymidine block, and cells were then released into the cell cycle

(Whitfield et al. 2000). To obtain pure populations of mitotic cells, nocodazole (50 ng/mL, Sigma) was added at 8 h after release, and cells were incubated for another 4 h or 6 h. G1 cells were obtained by washing the nocodazole-treated cells (6 h) and incubating in media without nocodazole for another 4 h. At the indicated time points, cells were harvested and total cell lysates were prepared in 1× SDS sample buffer. A fraction of cells from each time point was analyzed by flow cytometry using FACS-Calibur (Becton Dickinson). All nocodazole-treated cells were >85% G2/M phase.

Cell culture, total cell extract, juglone treatment, and stable Pin1 cell line

HeLa, NIH3T3 cells, and *pin1*^{-/-} and wild-type MEFs were cultured in DMEM supplemented with 10% FBS. Total cell extracts were prepared by solubilizing the cells in TD buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 50 mM sodium fluoride, and protease inhibitor cocktail (Sigma). Extracts were rocked for 15 min, then centrifuged at 14,000 rpm at 4°C for 15 min. HeLa, *pin1*^{-/-}, and wild-type MEF cells were treated with the indicated concentrations of juglone (Sigma) at 37°C for 8 h, and total cell extracts were prepared in TD buffer. The flu-tagged Pin1 constructs were generated by subcloning wild-type and R68,69A mutant Pin1 cDNA into the pTRE-HA plasmid. Tet-on cells were generated by transfecting HeLa cells with a pTet-on plasmid expressing a fusion protein of reverse Tet repressor and VP-16 activation domain (Gossen and Bujard 1995). Stable cell lines inducibly expressing Pin1 were obtained by cotransfecting Tet-on cells with pTRE-HA-Pin1 plasmid and a pTK-Hyg selection plasmid. Cells were cultured in DMEM media containing 10% tet-free FBS (Clontech). Expression of wild-type and mutant Pin1 was induced by addition of 1 µg/mL doxycycline to the media for appropriate times.

Immunoprecipitation

A plasmid expressing Flag-tagged Pin1 was generated by subcloning Pin1 cDNA into the plasmid p3Xflag-CMV. HeLa cells were transfected with 10 µg of the plasmid by calcium phosphate precipitation and after 48 h, total cell extract was prepared in TD buffer. Anti-Flag mAb, or anti-mouse IgG was coupled to protein A-agarose beads at 4°C for 12 h. After washing, beads were incubated with extract in 500 µL of TD buffer at 4°C for 40 min, washed four times, and then resuspended in SDS loading buffer and fractionated by SDS-PAGE. Proteins were detected by Western blotting.

In vitro transcription, splicing, and spliceosome formation

Nuclear extract was prepared from HeLa cells (~3 × 10⁷) as previously described (Kleiman and Manley 2001). Cytoplasmic S100 was prepared and His-tagged ASF/SF2 was purified from baculovirus-infected SF9 cells as described (Tacke and Manley 1995). In vitro transcription was carried out in a buffer containing 20 mM HEPES (pH 7.9), 10% glycerol, 50 mM KCl, 3 mM MgCl₂, 0.5 mM DTT, 0.2 mM EDTA, rNTP mixture (400 µM ATP, 400 µM CTP, 400 µM UTP, 16 µM GTP, and 10 µCi [α -³²P]GTP) in the presence of ~20 µg of NE protein and 100 ng of DNA template (a p3Xflag-CMV derivative linearized with *Bam*HI). Reaction mixtures were incubated at 30°C for 60 min. Reactions were terminated by adding stop solution containing 0.3 M Tris-HCl (pH 7.5), 0.3 M sodium acetate, 0.5% SDS, 2 mM EDTA, and 3 µg/mL tRNA, and then were extracted with

phenol and chloroform. Splicing reactions with NE or S100 supplemented with ASF/SF2 and purified RNAP IIO and spliceosome assembly assays were performed essentially as described (Hirose et al. 1999). RNA products were analyzed by denaturing PAGE.

Pulse labeling and poly(A) RNA purification

Wild-type and mutant Pin1-expressing cell lines were grown in media with or without doxycycline (1 μ g/mL) for the times indicated in the figures. After adjusting the cell concentration ($\sim 10^6$ /mL), cells were pulse-labeled with 50 μ Ci/mL [3 H]uridine (NEN) for ~ 10 min for each cell culture. Total RNAs were isolated with Trizol and poly(A)⁺ RNA was purified by oligo(dT) selection (Oligotex, QIAGEN). The poly(A)⁺ RNAs were slot-blotted onto Nylon filters. Filters were soaked in liquid scintillation cocktail for 1 h, and then exposed to X-ray film. Incorporation of [3 H]uridine in RNA was also quantitated by scintillation counting using a Micro-Betaplate reader.

Acknowledgments

We are grateful to S. Kaneko for purified RNAP IIO, A.D. Reinberg for the FCP1-expressing baculovirus, J. Prasad for ASF/SF2, and K. Ryan for help in the early stages of the study. We thank I. Boluk for help preparing the manuscript. This work was supported by NIH grants GM37971 to J.L.M. and GM58556 to K.P.L.

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Genes Dev. 2003, **17**:

Access the most recent version at doi:[10.1101/gad.1135503](https://doi.org/10.1101/gad.1135503)

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