

# Evidence that P-TEFb alleviates the negative effect of DSIF on RNA polymerase II-dependent transcription *in vitro*

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**Recently, a positive and a negative elongation factor, implicated in 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB) inhibition of transcription elongation, has been identified. P-TEFb is a positive transcription elongation factor and the DRB-sensitive kinase that phosphorylates the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (Pol II). PITALRE, a member of the Cdc2 family of protein kinases, is the catalytic subunit of P-TEFb. DSIF is a human homolog of the yeast Spt4–Spt5 complex and renders elongation of transcription sensitive to DRB. DRB sensitivity-inducing factor (DSIF) binds to RNA Pol II and may directly regulate elongation. Here we show a functional interaction between P-TEFb and DSIF. The reduction of P-TEFb activity induced by either DRB, antibody against PITALRE, or immunodepletion resulted in a negative effect of DSIF on transcription. DSIF acts at an early phase of elongation, and the prior action of P-TEFb makes transcription resistant to DSIF. The state of phosphorylation of CTD determines the DSIF–RNA Pol II interaction, and may provide a direct link between P-TEFb and DSIF. Taken together, this study reveals a molecular basis for DRB action and suggests that P-TEFb stimulates elongation by alleviating the negative action of DSIF.**

*Keywords:* DRB/DSIF/P-TEFb/transcription elongation

## Introduction

The elongation stage of eukaryotic mRNA transcription is an important target for the regulation of gene expression (Bentley, 1995; Uptain *et al.*, 1997). Analyses of the density of RNA polymerase II (Pol II) in cells have permitted the identification of many genes, including *hsp70* (Rougvie and Lis, 1988), *c-myc* (Krumm *et al.*, 1992; Roberts and Bentley, 1992; Storbl and Eick, 1992), *c-myb* (Bender *et al.*, 1987), *c-fms* (Yue *et al.*, 1993), adenovirus (Kessler *et al.*, 1989), SV40 (Kessler *et al.*, 1991) and human immunodeficiency virus (HIV) (Jones and Peterlin, 1994 and references therein), that have promoter-proximal pause sites, and are thus potentially regulated at the level of elongation. Some transcriptional activators, such as heat shock factor (Lis and Wu, 1993),

Gal4-VP16, Gal4-E1A (Yankulov *et al.*, 1994; Blau *et al.*, 1996) and the HIV-encoded Tat (Marciniak and Sharp, 1991; Kato *et al.*, 1992), are capable of stimulating the rate of elongation. These activators probably target a rate-limiting step of transcription elongation, thereby increasing the overall efficiency of elongation. However, the general components and the mechanism of such a regulatory step are poorly understood.

The nucleoside analog 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB) is a classic inhibitor of transcription elongation by RNA Pol II. DRB potently inhibits the synthesis of long transcripts, while it does not affect, and even sometimes enhances the synthesis of short transcripts *in vivo* and *in vitro* (Sehgal *et al.*, 1976; Tamm and Kikuchi, 1979; Roberts and Bentley, 1992; Meulia *et al.*, 1993). DRB is unique in that it has no effect on random, promoter-independent RNA Pol II transcription (Zandomeni *et al.*, 1982; Chodosh *et al.*, 1989; Wada *et al.*, 1998), and on transcription reconstituted by purified general transcription factors (GTFs) and RNA Pol II (Wada *et al.*, 1998). These findings have predicted the presence of general components that control elongation in a DRB-sensitive manner, and are different from GTFs and RNA Pol II.

Recent studies involving DRB have led to the discovery of the positive and negative elongation factors, positive transcription elongation factor b (P-TEFb) and DRB sensitivity-inducing factor (DSIF). P-TEFb was originally identified as an activity that releases paused RNA Pol II in a DRB-sensitive fashion (Marshall and Price, 1992, 1995). P-TEFb has been shown to be a cyclin–Cdk complex whose kinase activity is sensitive to DRB (Marshall *et al.*, 1996; Mancebo *et al.*, 1997; Zhu *et al.*, 1997; Peng *et al.*, 1998; Wei *et al.*, 1998). The catalytic component is a Cdc2-related kinase PITALRE (it is also termed Cdk9, but here we use the term PITALRE), and the regulatory component is cyclin T (Peng *et al.*, 1998; Wei *et al.*, 1998). The most likely target of phosphorylation by P-TEFb is the C-terminal domain (CTD) of the largest subunit of RNA Pol II (Marshall *et al.*, 1996; Zhu *et al.*, 1997). The intact CTD is required absolutely for P-TEFb-dependent elongation stimulation (Marshall *et al.*, 1996). Interestingly, HIV Tat strongly associates with P-TEFb and requires P-TEFb kinase activity for its function (Mancebo *et al.*, 1997; Zhu *et al.*, 1997). Despite the accumulating evidence for its critical role in elongation, the way in which P-TEFb controls elongation remains elusive. On the other hand, DSIF was originally identified as an activity that represses transcription in the presence of DRB (Wada *et al.*, 1998). DSIF is composed of two subunits, p160 and p14, which are human homologs of the yeast transcription factors Spt5 and Spt4 (Wada *et al.*, 1998). Immunodepletion of DSIF p160 from HeLa cell nuclear extracts results in DRB-insensitive transcription

and add-back of DSIF induces DRB-sensitivity, demonstrating its essential, negative role in DRB action (Wada *et al.*, 1998). Several lines of evidence strongly support the argument that DSIF works as a negative elongation factor. First, DSIF and Spt4/Spt5 physically and genetically interact with RNA Pol II (Hartzog *et al.*, 1998; Wada *et al.*, 1998). Secondly, even in the absence of DRB, addition of excess amounts of DSIF to a partially purified transcription system represses transcription (Wada *et al.*, 1998). Thirdly, DSIF p160 shows a structural similarity with the bacterial elongation factor NusG (Hartzog *et al.*, 1998; Wada *et al.*, 1998). However, the physiological function of DSIF, and the way in which DSIF activity is related to DRB remain unclear.

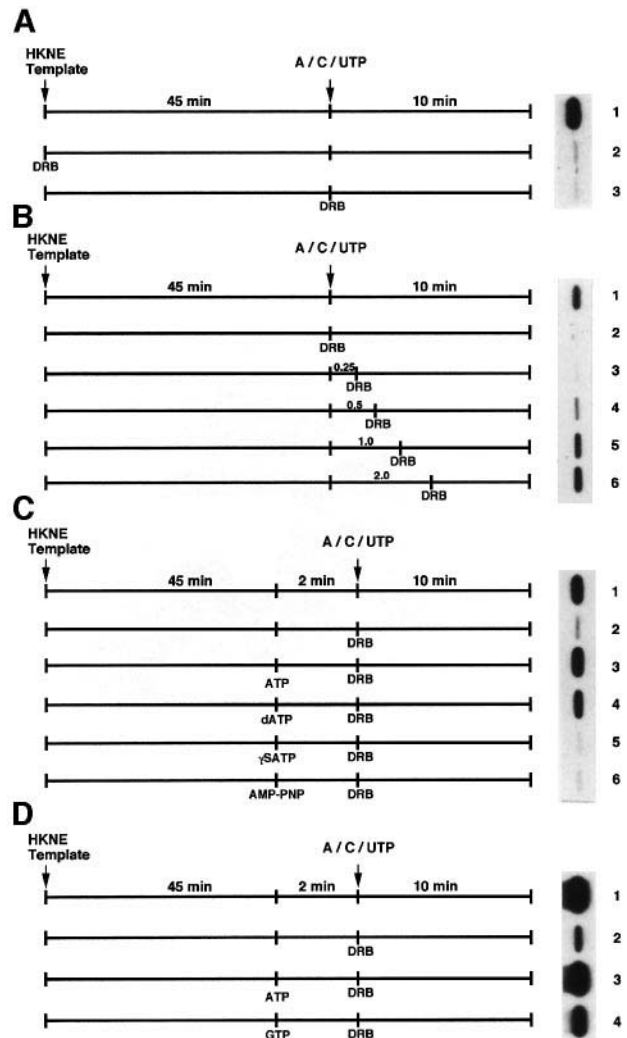
Since P-TEFb and DSIF positively and negatively regulate elongation, and both are essential components for DRB-sensitive elongation machinery, we have presented a model which indicates that P-TEFb and DSIF act antagonistically (Yamaguchi *et al.*, 1998). Based on this model, we have performed a combination of immunodepletion and add-back experiments, and have found that P-TEFb stimulates elongation by alleviating the negative action of DSIF. Kinetic analyses have established that DSIF acts at an early phase of elongation, and the prior phosphorylation of the CTD of the largest subunit of RNA Pol II by P-TEFb probably makes transcription resistant to DSIF. This study will provide insight into the mechanism of action of P-TEFb, DSIF and DRB.

## Results

### **DRB inhibition analysis using a kinetically synchronized transcription assay *in vitro***

To understand the mechanism of transcription inhibition by DRB, an *in vitro* transcription reaction was divided into two steps: a preincubation step with hexokinase-treated HeLa cell nuclear extracts and a DNA template for 45 min, followed by an initiation/elongation step in the presence of A/C/UTP for 10 min, a period that was sufficient to produce full-length 380 nucleotide G-free transcripts (data not shown). We used plasmid pTF3-6C<sub>2</sub>AT DNA as the template because transcription from this template was efficiently inhibited by DRB (Wada *et al.*, 1998). When 50  $\mu$ M DRB was present throughout the reaction, transcription was inhibited by 90% (Figure 1A, lane 2). When DRB was added to reactions concomitantly with A/C/UTP, the synthesis of RNA transcripts was also inhibited by 90% (Figure 1A, lane 3). These results indicate that DRB inhibits transcription after preinitiation complex (PIC) formation.

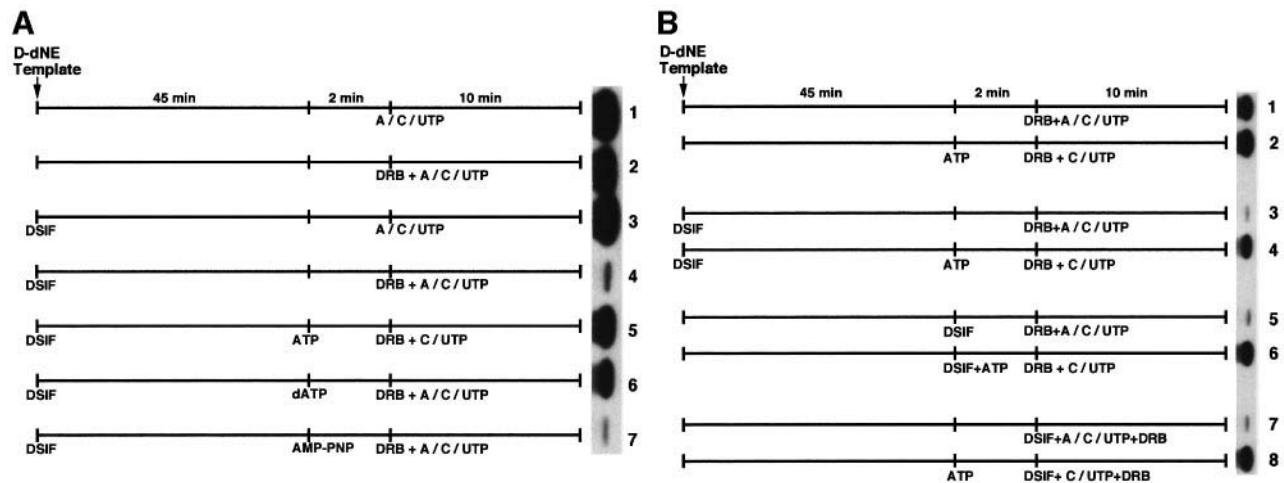
Next, we added DRB to a transcription reaction at different intervals following the addition of A/C/UTP. The extent of the inhibition was reduced as the time interval increased (Figure 1B). Inhibition was not observed when the interval was  $>2$  min (Figure 1B, lane 6), indicating that the PIC becomes resistant to DRB during the 2-min incubation in the presence of A/C/UTP. To define which ribonucleoside triphosphates in the A/C/UTP mixture are responsible for the alleviation of the inhibitory effect of DRB, the PIC was incubated with a single nucleotide for a period of 2 min between the 45 min preincubation and the 10 min initiation/elongation steps. After the 2-min incubation, the remaining ribonucleoside triphosphates,



**Fig. 1.** Kinetic analysis of the mechanism of DRB inhibition. G-free transcripts (of 380 nucleotides) were analyzed. (A) Hexokinase-treated HeLa cell nuclear extracts (HKNE) (Wada *et al.*, 1998) and the template pTF3-6C<sub>2</sub>AT were used in a kinetically synchronized protocol. DRB was added as indicated to a final concentration of 50  $\mu$ M. (B) Reactions proceeded essentially as described in (A), except that DRB (50  $\mu$ M) was added at the indicated times (min) after A/C/UTP addition. (C) Reactions proceeded using the 2-min protocol (see Materials and methods). DRB (50  $\mu$ M) was added as indicated. The ATP analogs (at a final concentration of 240  $\mu$ M each) indicated in the drawing were added during the 2-min period of incubation. (D) The 2-min protocol was performed. ATP and GTP (at a final concentration of 240  $\mu$ M each) were added during the 2-min period of incubation.

including [ $\alpha$ -<sup>32</sup>P]UTP, were added and incubation was continued for an additional 10 min in the presence or absence of DRB (Figure 1C). Addition of ATP during the 2-min incubation relieved the inhibitory effect of DRB (Figure 1C, lane 3). However, addition of CTP, UTP, or even the two together had no effect on DRB inhibition (data not shown).

To determine how ATP conferred DRB-resistance during the 2-min incubation, we examined the effect of the ATP analogs dATP, ATP- $\gamma$ S or AMP-PNP on DRB inhibition (Figure 1C). Addition of dATP during the 2-min incubation decreased DRB inhibition to the same extent as ATP (Figure 1C, lanes 3 and 4). Neither AMP-PNP nor ATP- $\gamma$ S could overcome the inhibition by DRB (Figure 1C,



**Fig. 2.** Transcription *in vitro* with DSIF-depleted nuclear extracts. G-free transcripts (of 380 nucleotides) were analyzed. (A) ATP and dATP can relieve the inhibitory effect of DRB on transcription. (B) ATP treatment and DSIF. Reactions were undertaken using the 2-min protocol, except DSIF-depleted nuclear extracts (D-dNE) were used. DRB (50  $\mu$ M) and renatured DSIF p14 (4 nM) plus p160 (4 nM) were added as indicated (Wada *et al.*, 1998). The ATP analogs (each at a final concentration of 240  $\mu$ M) indicated in the drawing were added during the 2-min period of incubation.

lanes 5 and 6). We also tested the effect of GTP on DRB inhibition, since GTP has a  $\gamma$  phosphate bond that can be utilized by some protein kinases (Serizawa *et al.*, 1995; Dikstein *et al.*, 1996; Marshall *et al.*, 1996; Wada *et al.*, 1996). As shown in Figure 1D, GTP had the same effect as ATP on DRB action. dATP can be utilized for a necessary activation step in transcription initiation but cannot be used as a nucleotide substrate for elongation (Sawadogo *et al.*, 1984). Thus, dATP (and probably GTP) must relieve DRB inhibition by a mechanism that does not involve incorporation of nucleotides into the RNA transcript. Other experiments (not shown) indicated that DRB did not have any effect on promoter opening or formation of the first phosphodiester bond. Since DRB is an ATP analog, it seemed reasonable to assume that an essential phosphorylation event of the transcription apparatus by ATP was the target of DRB inhibition. A likely target of DRB is P-TEFb, a recently identified protein kinase and elongation factor (Marshall *et al.*, 1996; Mancebo *et al.*, 1997; Zhu *et al.*, 1997). Thus, phosphorylation by P-TEFb at this step may play a critical role in subsequent elongation (see below).

#### **Putative phosphorylation event alleviates the negative effect of DSIF**

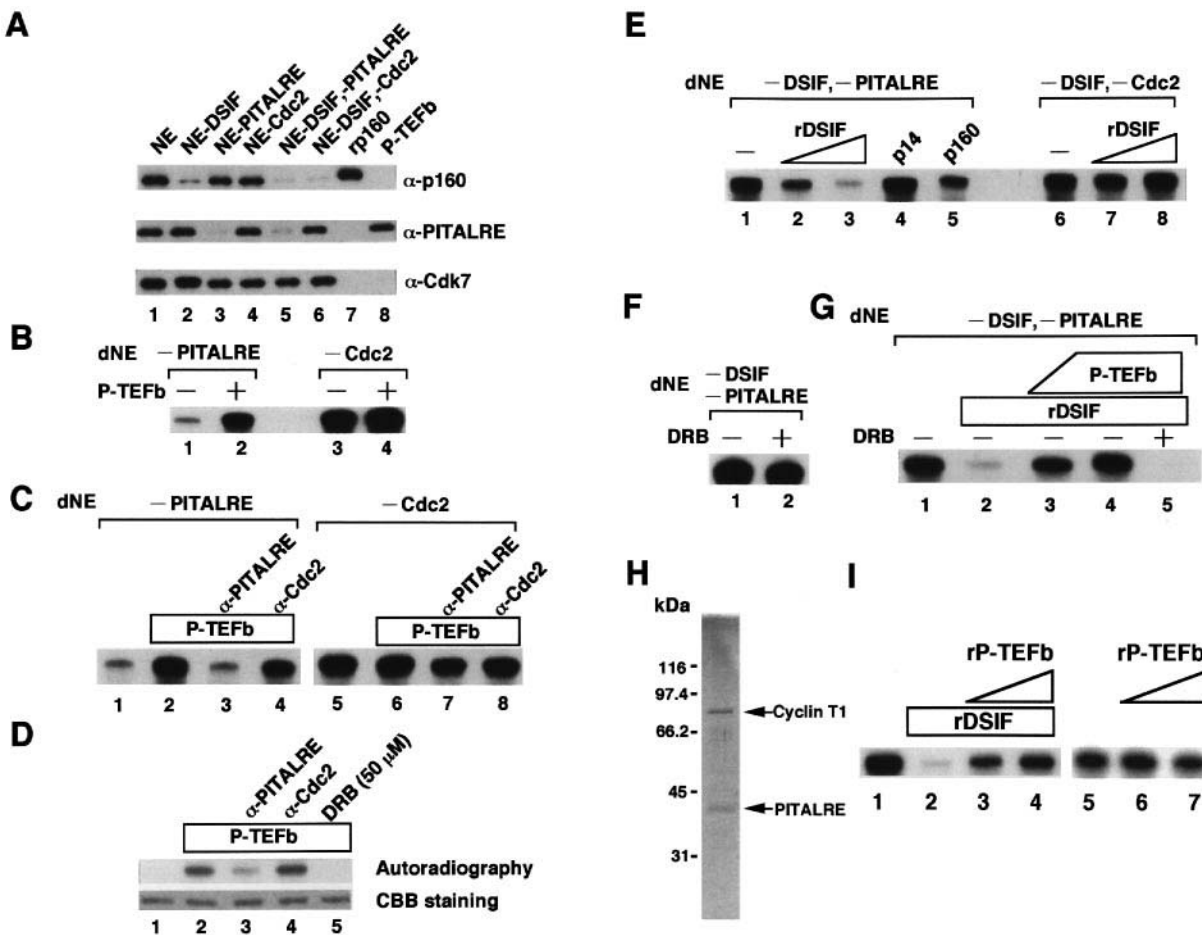
We have shown previously that DSIF is essential for the negative effect of DRB (Wada *et al.*, 1998). DSIF is composed of two subunits, p160 and p14, which are homologs of the yeast Spt5 and Spt4 proteins. To elucidate the way in which the inhibitory event identified in Figure 1 is related to DSIF, we utilized DSIF-immunodepleted HeLa cell nuclear extracts (D-dNE) and recombinant DSIF (rDSIF) (Wada *et al.*, 1998). Transcription in the presence of D-dNE was insensitive to DRB, but DRB sensitivity could be recovered through the addition of rDSIF to the depleted extracts, in agreement with previous results (Figure 2A, lanes 1–4; Wada *et al.*, 1998). Under these conditions, DSIF represses transcription in the presence of DRB and has little effect in its absence (Figure 2A, lanes 1 and 3). When the preincubation mixture containing rDSIF was treated with ATP for 2 min before initiation,

repression by DRB was almost completely reversed (Figure 2B, lanes 3 and 4). However, the 2-min treatment of the depleted extract with ATP in the absence of DSIF had no effect on subsequent inhibition by DRB (Figure 2B, lanes 1 and 2). Therefore, the putative phosphorylation event somehow alleviated the negative effect of DSIF. In agreement with the results shown in Figure 1, dATP, but not AMP-PNP, could be utilized for the reaction in place of ATP (Figure 2A, lanes 4–7).

As a possible explanation for the data presented above, DSIF might be directly inactivated by phosphorylation. To test this possibility, we added DSIF to D-dNE and the DNA template at different time points. When DSIF was added 2 min prior to initiation or at initiation, transcription became similarly sensitive to DRB (Figure 2B, lanes 5 and 7). Thus, DSIF does not require any particular incubation period for its function. This observation allowed us to test the requirement for DSIF in the 2-min ATP treatment. When DSIF was added concomitantly with NTPs and DRB, i.e. after the 2-min ATP treatment, a prior ATP treatment still relieved the repression (Figure 2B, lanes 6 and 8). Thus, the phosphorylation/DRB-sensitive reaction does not require the presence of DSIF, and the phosphorylation of some factor(s) other than DSIF most probably confers resistance to the negative effect of DSIF on transcription.

#### **P-TEFb alleviates the negative effect of DSIF**

Next we sought to address the possible involvement of P-TEFb in this process. For this purpose, we prepared P-TEFb-immunodepleted HeLa nuclear extracts (P-dNE) using an antibody against PITALRE, a Cdc2-related kinase and the catalytic component of P-TEFb (Mancebo *et al.*, 1997; Zhu *et al.*, 1997). Efficient and specific depletion of PITALRE from the extracts were confirmed by Western blotting (Figure 3A). In agreement with the previous study (Zhu *et al.*, 1997), depletion of P-TEFb greatly reduced the formation of G-free transcripts (Figure 3B, lanes 1 and 3). We used partially purified human P-TEFb obtained after four chromatographic steps to restore P-TEFb activity (see Materials and methods). The human P-TEFb fraction



**Fig. 3.** DSIF and P-TEFb act antagonistically. G-free transcripts (of 380 nucleotides) were analyzed except A. (A) Depletion of the indicated factors by antibodies against DSIF p160 ( $\alpha$ -p160) (Wada *et al.*, 1998), PITALRE ( $\alpha$ -PITALRE; Santa Cruz Biotechnology) and the TFIIF Cdk7 subunit ( $\alpha$ -Cdk7; Santa Cruz Biotechnology), as revealed by Western blot analysis. Cdk7 was examined as an internal control. Lanes 1–6, 1  $\mu$ l of each nuclear extract, indicated above the gel; lane 7, 4 ng of purified-recombinant DSIF p160; lane 8, 1  $\mu$ l partially purified human P-TEFb. (B) Depletion of P-TEFb activity reduced the generation of 380 nucleotide G-free transcripts. A kinetically synchronized transcription assay was performed with nuclear extracts treated with anti-PITALRE or anti-Cdc2 antibodies in the presence (+) or absence (–) of partially purified P-TEFb (4  $\mu$ l). (C) Anti-PITALRE antibodies prevented P-TEFb from working. Reactions proceeded as described in (B), except P-TEFb (4  $\mu$ l) was preincubated for 45 min with the indicated antibodies (2  $\mu$ l aliquots) before its addition to the transcription reaction. (D) Anti-PITALRE antibodies partially inhibited P-TEFb-dependent CTD phosphorylation. The CTD kinase assay was performed as described in Material and methods using partially purified human P-TEFb (1  $\mu$ l, lanes 2–5), anti-PITALRE antibodies (0.5  $\mu$ l, lane 3), anti-Cdc2 antibodies (0.5  $\mu$ l, lane 4), DRB (final concentration 50  $\mu$ M, lane 5), and GST–CTD as substrate. The gel containing reaction products was stained with Coomassie Blue and dried for autoradiography. The results were reproduced by three independent experiments. (E) A kinetically synchronized transcription assay was performed with nuclear extracts treated with anti-PITALRE and anti-DSIF p160, or anti-Cdc2 and anti-DSIF p160 antibodies. Renatured p160 (lanes 2 and 7, 1 nM; lanes 3, 5 and 8, 4 nM) and renatured p14 (lanes 2 and 7, 1 nM; lanes 3, 4 and 8, 4 nM) were used. (F and G) A kinetically synchronized transcription assay was performed with nuclear extracts treated with anti-PITALRE and anti-DSIF p160 antibodies. Renatured p160 (4 nM) and p14 (4 nM) (lanes 2–5), and partially purified P-TEFb (lane 3, 2  $\mu$ l; lanes 4 and 5, 4  $\mu$ l) were used in (F). (H) Purified rP-TEFb was subjected to SDS–PAGE and proteins were visualized by silver staining. Numbers to the left of the gel indicate the position of protein molecular size standards. (I) A kinetically synchronized transcription assay was performed as described in (G). Renatured p160 (4 nM) and p14 (4 nM) (lanes 2–4), and purified rP-TEFb (lanes 3 and 6, 5 nM; lanes 4 and 7, 20 nM) were used.

showed DRB-sensitive CTD kinase activity (Figure 3D, lane 5). Addition of partially purified P-TEFb complemented the transcriptional defect of the P-TEFb-depleted extracts, but had little effect on the control reaction (Figure 3B). Preincubation of the P-TEFb fraction with the anti-PITALRE antibody, but not with the control antibody, inhibited restimulation of the repressed transcription, indicating that P-TEFb per se is responsible for the re-stimulation (Figure 3C). In support of this result, preincubation of the P-TEFb fraction with the anti-PITALRE antibody, but not with the control antibody, repressed the CTD kinase activity (Figure 3D).

We have now established an *in vitro* transcription system

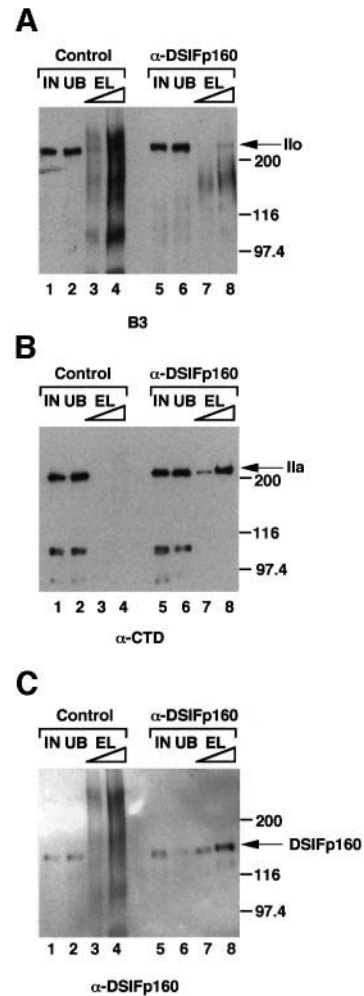
in which the level of both P-TEFb and DSIF can be controlled. To study the functional interaction between them, we immunodepleted both P-TEFb and DSIF from HeLa cell nuclear extracts (DP-dNE), and examined the effect of this immunodepletion on transcription. Surprisingly, concomitant depletion of DSIF with P-TEFb restored transcription to control levels (compare Figure 3C with E), and the addition of rDSIF repressed transcription in a dose-dependent manner (Figure 3E, lanes 1–3). Note that in the presence of P-TEFb, transcription repression by DSIF was dependent on the presence of DRB, and that DSIF had virtually no effect without DRB (Figure 2). Thus, these results indicate that the reduction of P-TEFb

activity by DRB or immunodepletion uncovers the cryptic, and negative action of DSIF on transcription, i.e. P-TEFb alleviates the inhibitory effect of DSIF. Transcription was partially repressed when p160, but not p14, was added back to DP-dNE (lanes 4 and 5). This indicates that p14 could not be fully removed from the extracts because the monoclonal antibody used for DSIF-depletion recognizes only p160 (Wada *et al.*, 1998). When purified P-TEFb was added to the reaction containing DP-dNE and rDSIF, transcription repressed by rDSIF recovered to the original level, in a DRB sensitive manner (Figure 3G). There are a number of studies showing that DRB also inhibits the kinase activity of Cdk7, a CAK component of TFIIF which is essential for transcription from some promoters (Yankulov *et al.*, 1995, 1996; Parada and Roeder, 1996; Cujec *et al.*, 1997; Garcia-Martinez *et al.*, 1997). Though DP-dNE retains Cdk7 (Figure 3A), the transcription was not sensitive to DRB (Figure 3F). This suggests that Cdk7 activity is not sensitive to DRB or is not essential for transcription under our conditions using supercoiled DNA as template.

To further confirm this conclusion, we have prepared purified-recombinant P-TEFb (rP-TEFb), which is composed of cyclin T1 and PITALRE/CDK9 proteins obtained after coexpression in Sf9 cells using the baculoviral expression system (Peng *et al.*, 1998; Figure 3H). rP-TEFb removed DSIF-dependent transcription inhibition of DP-dNE as purified P-TEFb did (Figure 3I). In contrast, the addition of rP-TEFb to DP-dNE in the absence of DSIF had no effect (Figure 3I, lanes 5–7). This suggests that P-TEFb does not have any effect on transcription in the absence of DSIF. rP-TEFb also had no effect on transcription when it was added to untreated-NE (data not shown). This suggests that the NE contained a saturating amount of P-TEFb.

DSIF interacts with RNA Pol II and may directly modulate its elongation activity (Wada *et al.*, 1998). P-TEFb strongly phosphorylates the CTD of the largest subunit of RNA Pol II and converts the hypophosphorylated Ila form to the hyperphosphorylated Ilo (Marshall *et al.*, 1996; Zhu *et al.*, 1997; Peng *et al.*, 1998). Therefore, it might be possible that CTD phosphorylation somehow alleviates the negative effect of DSIF. As a first step towards answering this question, we examined if DSIF-RNA Pol II interaction is regulated by CTD phosphorylation. p160 was immunoprecipitated from HeLa NE, and the Ila and Ilo forms in the precipitate were monitored by Western blotting. We used two different antibodies against the CTD; 8WG16 ( $\alpha$ -CTD), which recognizes both forms of RNA Pol II, and B3, which recognizes a phosphoepitope within the CTD of the largest subunit of RNA Pol II and specifically reacts with the Ilo form (Mortillaro *et al.*, 1996). As reported previously, a significant amount of RNA Pol Ila was co-precipitated with p160 (Figure 4B; Wada *et al.*, 1998). In striking contrast, the amount of RNA Pol Ilo in the precipitate was quite low and the level was the same as that in the eluate from the control resin (Figure 4A). We conclude, therefore, that the phosphorylation state of the CTD determines RNA Pol II-DSIF interaction and that CTD phosphorylation releases p160 from RNA Pol II.

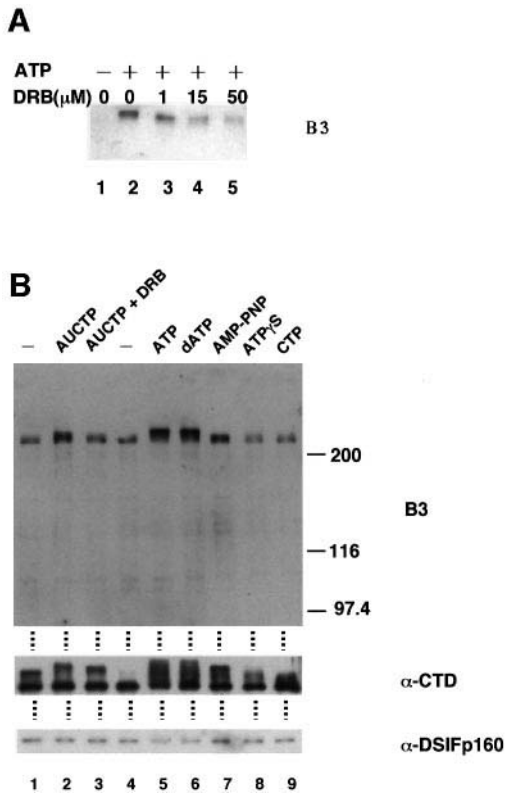
Next we tested the shift from the Ila form to the Ilo form of RNA Pol II induced by P-TEFb and the sensitivity



**Fig. 4.** Differential interaction of DSIF with RNA Pol Ila and Ilo. Western blotting of immunoprecipitates of DSIF using the B3 monoclonal antibody (A),  $\alpha$ -CTD (B), and  $\alpha$ -DSIF p160 (C). IN, nuclear extracts (2  $\mu$ l); UB, unbound fraction (2  $\mu$ l); EL, eluate (lanes 3 and 7, 5  $\mu$ l; lanes 4 and 8, 15  $\mu$ l). Numbers to the right of the gel indicate the position of protein molecular size standards. The same blot was used for the three independent assays.

of this shift to DRB (Figure 5A). Purified RNA Pol II was incubated with rP-TEFb in the absence or presence of three concentrations of DRB and reaction products were analyzed by Western blotting with the B3 antibody. In the presence of 60  $\mu$ M ATP, P-TEFb made RNA Pol Ilo efficiently (Figure 5A, lanes 1 and 2). Increasing concentrations of DRB inhibited the shift to the Ilo form (Figure 5A, lanes 2–5), suggesting that P-TEFb is involved in the transition from the Ila form to the Ilo form, a step which is sensitive to DRB. These data agree with the results reported previously (Marshall *et al.*, 1996).

We examined next the correlation between DRB sensitivity and the extent of CTD phosphorylation. Under various transcription conditions as described in Figure 1, the phosphorylated states of RNA Pol II were determined by Western blotting with B3 and 8WG16 antibodies. When the NE was incubated with the DNA template for 45 min, RNA Pol II existed mostly as the Ila form (Figure 5B, lane 2). Subsequent incubation for 10 min in the presence



**Fig. 5.** Correlation of the shift from the Ila form to the Ilo form with DRB inhibition. **(A)** Characterization of P-TEFb kinase activity. rP-TEFb (20 nM) was incubated with purified RNA Pol II (1 nM) in the absence (lane 1) or presence (lanes 2–5) of 60  $\mu$ M ATP for 10 min at 30°C in TRX buffer. Kinase reactions contained the indicated concentrations of DRB. Reaction products were analyzed by Western blotting with the B3 monoclonal antibody. **(B)** Western blotting of proteins in the kinetically synchronized transcription reactions with the B3 monoclonal antibody,  $\alpha$ -CTD, and  $\alpha$ -DSIF p160. Reactions were performed with 4  $\mu$ l of HeLa cell nuclear extracts as described in Figure 1, except that reactions were incubated for 2 min following addition of the indicated materials without [ $\alpha$ - $^{32}$ P]UTP. AUCTP (60  $\mu$ M ATP, 5  $\mu$ M UTP, 600  $\mu$ M CTP) was added to lanes 2 and 3. DRB (lane 3, 50  $\mu$ M), ATP (lane 5, 240  $\mu$ M), dATP (lane 6, 240  $\mu$ M), AMP-PNP (lane 7, 240  $\mu$ M), ATP- $\gamma$ S (lane 8, 240  $\mu$ M) and CTP (lane 9, 600  $\mu$ M) were used. Numbers to the right of the gel indicate the position of protein molecular size standards. The same blot was used for three independent assays.

of AUCTP produced the Ilo form, a transition that was reversed by 50  $\mu$ M DRB (Figure 5B, lanes 3 and 4). Notably, when the preincubation reaction was treated with either ATP or dATP for 2 min, which made transcription resistant to DRB (Figure 1), RNA Pol IIa was converted to the Ilo form (Figure 5B, lanes 6 and 7). On the other hand, treatment of NE with ATP- $\gamma$ S or CTP caused no detectable change and AMP-PNP had very little effect. We also examined the phosphorylation states of p160. p160 is highly phosphorylated by cellular kinases and shows altered mobility on SDS–polyacrylamide gels (Stachora *et al.*, 1997 and our unpublished data). However, we could not detect any mobility shift of p160 under the conditions employed here. Taken together, RNA Pol II, but not p160, was phosphorylated during transcription reactions in a manner consistent with its proposed role, suggesting that it is the functional target of phosphorylation by P-TEFb.

### DSIF represses transcription at the early phase of elongation

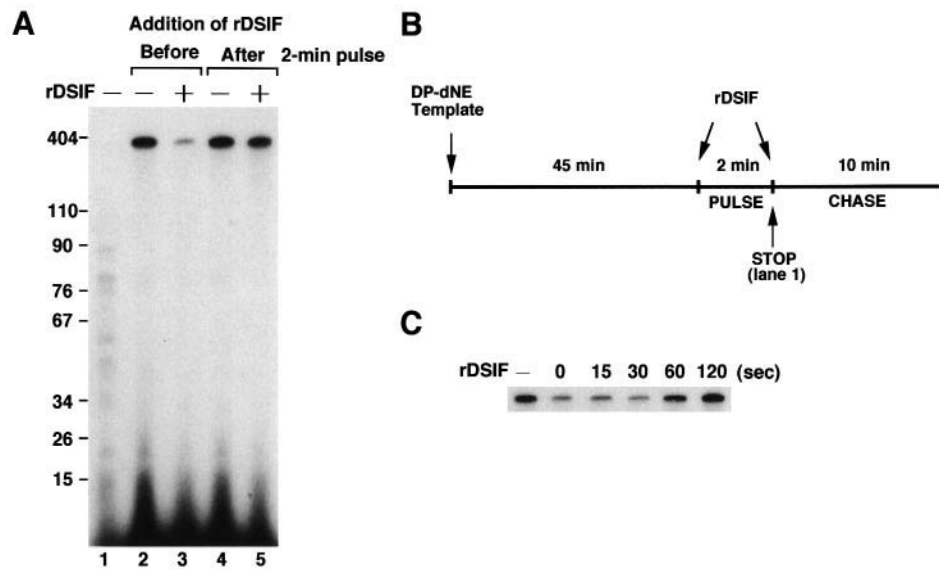
DRB inhibits the production of long transcripts, and causes the accumulation of short transcripts *in vivo* and *in vitro* (Sehgal *et al.*, 1976; Tamm and Kikuchi, 1979; Wada *et al.*, 1998). Provided that the inhibitory effect of DRB is relevant to DSIF action, DSIF should repress transcription at the early phase of elongation. Kinetic analysis demonstrated that DSIF is dispensable for its function during the preincubation period (Figure 2C). Since DRB can no longer inhibit transcription when added to the reaction 2 min after initiation (Figure 1B), it seems possible that DSIF exerts its negative effect only within the first 2 min after initiation. However, the ATP-dependent action of P-TEFb makes this difficult to interpret. To address this issue, we employed DP-dNE, and examined the negative effect of DSIF using a pulse–chase protocol (Figure 6A and B). Incubation for 2 min after the addition of ribonucleoside triphosphates was not sufficient for the generation of 380 nucleotide G-free transcripts (Figure 6A, lane 1). Addition of rDSIF during the 2-min pulse-inhibited transcription (Figure 6B). When rDSIF was added to the reaction after the 2-min pulse, no effect on transcription was observed (Figure 6A and B). This result was very similar to that of Figure 1B, suggesting that DRB and DSIF work within the same time period after initiation. In addition, DSIF has no effect on elongating RNA Pol II which had been started from the initiation site by incubation in the presence of ribonucleoside triphosphates for 2 min, and DSIF only functioned during the early phase of elongation.

### Discussion

The results presented here provide evidence that P-TEFb alleviates the negative effect of DSIF on RNA Pol II elongation. DSIF normally represses transcription only in the presence of DRB (Figure 2). However, in the absence of P-TEFb, titration of DSIF potently represses transcription without DRB (Figure 3E). Therefore, the negative effect of DSIF must be anti-repressed by P-TEFb in a DRB-sensitive fashion. These results are in agreement with our previous hypothesis (Yamaguchi *et al.*, 1998). In the presence of DSIF, P-TEFb markedly stimulated transcription (Figure 3B, lanes 1 and 3), whereas in its absence, P-TEFb did not show any detectable effect [Figure 3E (lanes 1 and 6) and I]. These results indicate that the sole function of P-TEFb is to relieve the repression by DSIF, at least in our *in vitro* conditions.

Kinetic analyses suggest that P-TEFb phosphorylates some factors other than DSIF to make transcription resistant to DRB and DSIF (Figure 1). Since a 2-min pre-treatment with ATP makes transcription resistant to DRB, transient action of P-TEFb before transcription initiation likely suffices for its function. Though P-TEFb has been reported to act on the early elongation complex (Marshall *et al.*, 1996), it is possible that the same modification by the endogenous P-TEFb could occur on the pre-initiation complex in our crude transcription system.

DSIF p160 is known to be highly phosphorylated by cellular kinases (Stachora *et al.*, 1997). We have recently found that P-TEFb extensively phosphorylates p160 *in vitro* and causes the mobility shift on SDS–polyacryl-

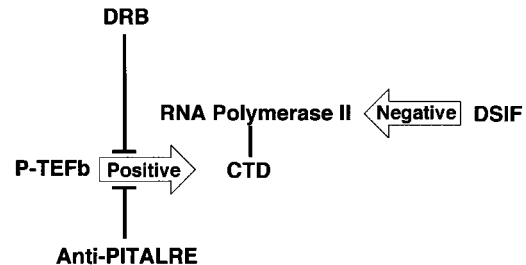


**Fig. 6.** DSIF represses transcription at the early phase of elongation. (A) A pulse–chase transcription reaction was performed with nuclear extracts treated with anti-PITALRE and anti-DSIF p160 antibodies. Renatured p160 and p14 (4 nM each) were added to the reaction as indicated. Transcription was initiated by the addition of 5  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]UTP (800 Ci/mmol), A/C/UTP mixture (final concentration: 60  $\mu$ M ATP; 600  $\mu$ M CTP; 5  $\mu$ M UTP; 80  $\mu$ M OMe-GTP) and 50 U of RNase T1 (Wada *et al.*, 1998). The pulse was continued for 2 min, after which the reaction was brought to 1.0 mM ATP, CTP and UTP. G-free transcripts were analyzed. Numbers to the left indicate the position of markers (nucleotides). (B) Reactions were performed as described in (A). (C) Renatured p160 and p14 (4 nM each) were added to the reaction at the indicated times after the addition of [ $\alpha$ - $^{32}$ P]UTP, A/C/UTP mixture and RNase T1.

amide gel as is observed *in vivo* (our unpublished data). The present study indicates that the primary target of P-TEFb is the RNA Pol II CTD, but it remains possible that the other phosphorylation event may also play a role in DRB-sensitive transcription or in P-TEFb function. Considering the functional interaction between P-TEFb and DSIF, it is natural to assume that P-TEFb regulates DSIF activity by its direct phosphorylation. Although p160 was not phosphorylated well under our transcription conditions (Figure 5B), it is still possible that p160 phosphorylation is involved in DRB-sensitivity. If P-TEFb preferentially phosphorylates promoter-bound p160, which represents only a small portion of the total p160, slight changes in its phosphorylation state might have significant impacts on its activity. We are interested in the issue and now investigating the functional relevance of p160 phosphorylation.

Even in the absence of P-TEFb, DSIF could no longer repress transcription 2 min after initiation (Figure 6). We concluded, therefore, that DSIF only affects the early elongation complex. It should be noted that the timing of DSIF action closely coincides with that of P-TEFb action (Marshall and Price, 1996). The RNA Pol II elongation complex may become resistant to DSIF after transcribing for a few minutes with a P-TEFb-independent mechanism, possibly by some structural changes in the elongation complex.

Marshall and Price (1992) have proposed that P-TEFb determines the fate of RNA Pol II (either abortive or processive elongation) at the early phase of elongation. The early elongation complex, isolated by using an immobilized DNA template, normally produces short, abortive transcripts. Addition of P-TEFb, however, causes RNA Pol II to become competent to produce full-length transcripts. In this context, they hypothesized that negative transcription elongation factors (N-TEFs) act at the early



**Fig. 7.** P-TEFb and DSIF function antagonistically to control RNA Pol II-dependent transcription elongation. P-TEFb has a positive effect on RNA Pol II activity through its CTD phosphorylation, which is specifically inhibited by either DRB (Marshall and Price, 1995, 1996; Mancebo *et al.*, 1997; Zhu *et al.*, 1997) or anti-PITALRE treatment (Figure 3), whereas DSIF represses transcription elongation of RNA Pol II and their interaction is probably important for its repression (Hartzog *et al.*, 1998; Wada *et al.*, 1998), but by an as yet unknown mechanism.

phase of elongation to induce abortive elongation in the absence of P-TEFb. The character of DSIF is quite similar to that of N-TEFs, and DSIF is probably one of the N-TEFs.

How do these two factors control elongation? Our current data, combined with previous reports, suggest the model depicted in Figure 7. DSIF and the yeast counterpart Spt4–Spt5 complex physically and genetically interact with RNA Pol II, and may directly repress RNA Pol II elongation through this interaction. Without P-TEFb, transcription would terminate prematurely. P-TEFb most probably changes the abortive elongation complex to the processive complex by phosphorylating the RNA Pol II CTD. P-TEFb-mediated CTD phosphorylation may prevent DSIF from interacting with RNA Pol II. Actually, CTD phosphorylation showed strong coincidence with the acquisition of DRB-resistance (Figure 5), and also markedly reduced the interaction between DSIF and RNA Pol II (Figure 4). Although the functional relevance of

DSIF–RNA Pol II interaction remains unclear, further investigation using a more defined transcription system will clarify this issue.

In addition to the interplay between P-TEFb and DSIF on the regulation of RNA Pol II processivity, the fact that P-TEFb is required for HIV Tat-transactivation *in vivo* and *in vitro* suggests that DSIF is possibly involved in Tat-activation. In addition, Wu-Bear *et al.*, (1998) have recently reported that the human homolog of yeast Spt5, which is thought to be identical to DSIF p160, is involved in HIV-1 Tat activation of transcription. Tat enhances the processivity of transcription complexes in a manner reminiscent of  $\lambda$  N, which forms a processive anti-terminator including host proteins and the RNA element in addition to NusG (Greenblatt *et al.*, 1993). It has been shown that DSIF has a functional similarity to NusG (Wada *et al.*, 1998), suggesting a functional association of DSIF with Tat-transactivation. The analysis of their possible interaction with other factors, such as RNA Pol II, some components of chromatin structure, and the Tat protein, will shed light on the molecular mechanism of transcription elongation, the regulation of chromatin structure, and growth of HIV.

We have performed a series of transcription assays using purified GTFs, RNA Pol II, rDSIF, and P-TEFb. In this system, DRB still does not work, and for this reason the presence of another factor required for DRB action has been hypothesized (data not shown). In accordance with this hypothesis, our preliminary *in vitro* transcription assays have identified such a factor in the phosphocellulose 0.3 M potassium chloride fraction, whose activity is necessary and sufficient for DSIF repression. The factor is not a protein kinase (Y.Yamaguchi and H.Handa, unpublished material). A complete understanding of the mechanism of DRB action requires further investigation. Nonetheless, this study has revealed a basic aspect of the transcription mechanism, i.e. the opposing functions of DSIF and P-TEFb in RNA pol II processivity (Figure 7).

## Materials and methods

### *In vitro* transcription assays

A kinetically synchronized transcription reaction containing 250 ng of supercoiled DNA template (pTF3-6C<sub>2</sub>AT) and 4  $\mu$ l of indicated nuclear extracts was performed as described previously (Wada *et al.*, 1998).

In the 2-min protocol, 12  $\mu$ l of TRX (transcription) buffer [25 mM HEPES–NaOH pH 7.9, 10% (v/v) glycerol, 50 mM KCl, 6 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol (DTT) and 0.5 mM EDTA] containing the indicated nucleotide(s) and 50 U RNase T1 (Gibco-BRL) were added immediately after the 45-min incubation, and the incubation was continued for 2 min. This was followed by the addition of 5  $\mu$ l of TRX buffer containing the missing nucleotide(s). After a further incubation of 10 min, the reaction mixtures were processed as described (Wada *et al.*, 1998). For DRB inhibition experiments, TRX buffer (12  $\mu$ l) containing nucleotides and 50 U RNase T1 was added for the 2-min incubation, followed by the addition of 5  $\mu$ l of TRX buffer containing DRB (Sigma), to give a final concentration of 50  $\mu$ M.

### Immunodepletion of human P-TEFb and DSIF

Immunodepletion of P-TEFb was performed as described previously (Zhu *et al.*, 1997). Anti-Cdc2 polyclonal antibody was used as a control material (Santa Cruz Biotechnology). Immunodepletion of DSIF was performed as described previously (Wada *et al.*, 1998).

### Partial purification of human P-TEFb

HeLa cell nuclear extracts (80 ml, 480 mg) were prepared as described (Dignam *et al.*, 1983) and fractionated on a phosphocellulose column

(Wada *et al.*, 1998). P-TEFb was monitored by Western blot analysis with the use of polyclonal antibodies against PITALRE (Santa Cruz Biotechnology) during fractionation. PITALRE was found in the 0.3–1.0 M KCl step from phosphocellulose. The step eluate was dialyzed against 0.1 M KCl and applied on a 30 ml DEAE–Sepharose fast flow column (Pharmacia) equilibrated with HGE [20 mM HEPES–NaOH pH 7.9, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT] containing 0.1 M KCl (HGE.1; the number following HGE denotes the molar concentration of KCl). After the elute was applied, the column was washed with three column volumes of HGE.1, and then step eluted with 0.3 and 1.0 M KCl. PITALRE was detected at 0.3 M KCl. The fractions were dialyzed against HGE containing 1.0 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, followed by loading onto a phenyl–Superose column (1 ml, Pharmacia). The fractions containing PITALRE were eluted at 0.3–0.1 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The pooled, active fractions were dialyzed against HGE.1 and loaded onto a Mono-S column (1 ml, Pharmacia). After loading, the column was washed with 10 column volumes of HGE.3 and eluted at 1.0 M KCl. The majority of the anti-PITALRE crossreacting material was eluted at 1.0 M KCl and used as partially purified human P-TEFb. P-TEFb activity was tested by CTD kinase assays using GST–CTD as a substrate, as described below.

### CTD kinase assay

Partially purified P-TEFb was preincubated in 8  $\mu$ l of TRX buffer (Wada *et al.*, 1998) containing 500 ng of bovine serum albumin at 30°C for 45 min with or without antibodies and then 17  $\mu$ l of TRX buffer containing 5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol), 60  $\mu$ M ATP (final concentration) and 100 ng of GST–CTD [which expression vector in *Escherichia coli* was kindly provided by R.A.Young (Whitehead Institute) (Thompson *et al.*, 1993)]. In the reactions containing DRB it was added to 50  $\mu$ M after preincubation. The reactions were incubated at 30°C for 2 min. Reaction products were analyzed on a 7% sodium dodecyl sulfate (SDS)–polyacrylamide gel and proteins were stained with Coomassie Blue, and the gel was dried for autoradiography. CTD kinase assays were repeated three times and showed the same results.

### Immunoprecipitation of DSIF

The  $\alpha$ -DSIF p160 affinity resin was prepared as described previously (Wada *et al.*, 1998). The resin (10  $\mu$ l) was incubated for 2 h at 4°C with HeLa cell nuclear extracts (0.1 ml, 8 mg/ml). An unbound fraction was separated and the resin was washed 5 $\times$  with 100  $\mu$ l of HGE.1. Then, immunoadsorbed complexes were eluted by adding 40  $\mu$ l of 4 $\times$  protein dye solution (Wada *et al.*, 1996) followed by incubation for 5 min at 98°C. The normal rat serum-conjugated protein G–Sepharose was used as the control material.

### Western blotting

The assay was performed as described previously (Wada *et al.*, 1998). Developing of the filter with the ECL system and reprobing strategies were performed according to the manufacturer's instructions (Amersham).

### Preparation of rP-TEFb

Recombinant baculoviruses were generated according to the method as described (Peng *et al.*, 1998). Plasmid pBAC–HuCDK9-T1 was kindly provided by D.Price (Iowa University, IA). rP-TEFb were prepared as described previously (Peng *et al.*, 1998). The protein concentration of purified rP-TEFb was determined by Coomassie Blue staining.

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