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NELF, a Multisubunit Complex Containing RD, Cooperates with DSIF to Repress RNA Polymerase II Elongation

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Summary

DRB is a classic inhibitor of transcription elongation by RNA polymerase II (pol II). Since DRB generally affects class II genes, factors involved in this process must play fundamental roles in pol II elongation. Recently, two elongation factors essential for DRB action were identified, namely DSIF and P-TEFb. Here we describe the identification and purification from HeLa nuclear extract of a third protein factor required for DRB-sensitive transcription. This factor, termed *n*egative *el*ongation *f*actor (NELF), cooperates with DSIF and strongly represses pol II elongation. This repression is reversed by P-TEFb-dependent phosphorylation of the pol II C-terminal domain. NELF is composed of five polypeptides, the smallest of which is identical to RD, a putative RNA-binding protein of unknown function. This study reveals a molecular mechanism for DRB action and a regulatory network of positive and negative elongation factors.

Introduction

The elongation step of transcription is now recognized as a critical target for transcription regulation. An increasing number of elongation factors have been identified, and the regulatory mechanism of elongation seems to be as complex as that of transcription initiation (reviewed in Reines et al., 1996; Uptain et al., 1997; Shilatifard, 1998). The role played by the phosphorylation of the repetitive C-terminal domain (CTD) of pol II during elongation remains a major enigma. The CTD becomes extensively phosphorylated during the transition from transcription initiation to elongation and remains phosphorylated during elongation (Dahmus, 1996 and references therein). A nucleoside analog DRB is a specific inhibitor of pol II elongation and of certain protein kinases. Application of the drug markedly reduces the synthesis of mRNA and, at the same time, eliminates phosphorylated pol II (pol IIo) from the cells (Sehgal et al., 1976; Dubois et al., 1994), suggesting that CTD phosphorylation may be a prerequisite for the successful completion of transcription. Studies to date, however, have failed to address this issue. No difference has been observed between the efficiencies of pol IIo and hypophosphorylated pol II (pol IIa) during elongation (this study). In addition, CTD phosphorylation and even the CTD itself are dispensable for transcription in some in vitro systems (Zehring et al., 1988; Serizawa et al., 1993; Makela et al., 1995).

DRB is unique in that it shows no effect on transcription reconstituted with purified general transcription factors (GTFs) and pol II, while it potently represses transcription in more crude systems and in vivo (Yamaguchi et al., 1998 and references therein). Therefore, factors different from GTFs and pol II must be involved in DRB-sensitive transcription. Recently, we and others have identified two such elongation factors, namely DSIF and P-TEFb. DSIF (for *D*RB *s*ensitivity-*i*nducing *f*actor) represses transcription in the presence of DRB, thereby inducing DRB sensitivity (Wada et al., 1998a, 1998b; Yamaguchi et al., 1999). DSIF is composed of two subunits, which are homologs of the *Saccharomyces cerevisiae* transcription factors Spt5 and Spt4, respectively (Swanson et al., 1991; Winston, 1992; Hartzog et al., 1996). DSIF/Spt4-Spt5 genetically and physically interacts with pol II and may directly regulate pol II processivity (Hartzog et al., 1998; Wada et al., 1998a, Yamaguchi et al., 1999). On the other hand, P-TEFb (*p*ositive *t*ranscription *e*longation *f*actor *b*) stimulates elongation in a DRB-sensitive fashion, and the low level of transcription in the absence of P-TEFb is not affected by DRB (Marshall and Price, 1992, 1995; Zhu et al., 1997). P-TEFb turns out to be a DRB-sensitive protein kinase that is composed of CDK9 and cyclin T and phosphorylates the pol II CTD (Marshall et al., 1996; Zhu et al., 1997; Peng et al., 1998). Based on these data, a model has been proposed in which P-TEFb alleviates the negative effect of DSIF by phosphorylating the pol II CTD (Yamaguchi et al., 1998). We have recently obtained evidence in line with this model using a crude transcription system (Wada et al. 1998b). Nevertheless, DSIF alone had no effect on purified pol II (see below), and as such, there remain many obstacles to be overcome before we can arrive at a complete understanding of the mechanism of DRB-sensitive transcription elongation.

To further characterize this process, we have established a more defined transcription system that reconstitutes DRB sensitivity in vitro. In this system employing recombinant GTFs and DSIF plus some partially purified fractions, a requirement for an additional factor in DRB-sensitive transcription has emerged. The factor termed NELF represses pol II elongation in conjunction with DSIF but appears to be normally counteracted by P-TEFb-dependent phosphorylation of the pol II CTD. This study reveals a molecular mechanism for DRB action and a novel aspect of CTD phosphorylation. Interplay between these positive and negative elongation factors may determine the fate of pol II, deciding between processive and abortive elongation.

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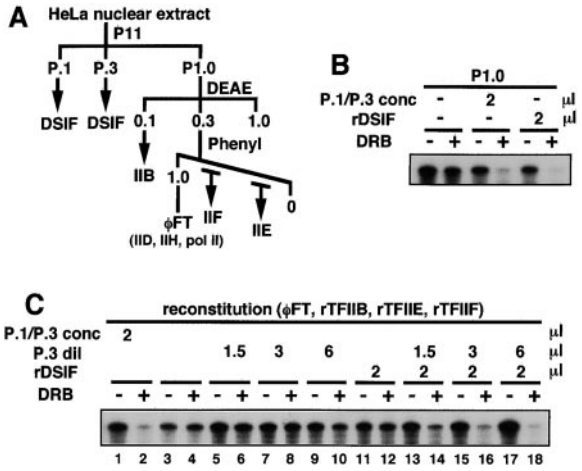

Figure 1. Identification of the Activity Required for DRB-Sensitive Transcription

(A) Diagram showing the reconstitution of DRB-sensitive transcription. HeLa nuclear extract was first applied to a phosphocellulose (P11) column, yielding three fractions, P.1, P.3, and P1.0. P1.0 was sufficient for transcription and was further fractionated on DEAE Sepharose and phenyl Superose columns.

(B) Transcription in the crude P1.0 system. Using the P1.0 fraction, the effect of crude (P.1/P.3 conc) and purified (rDSIF) preparations of DSIF was compared. Both were found to equally induce DRB sensitivity.

(C) Transcription in the reconstituted system. The effect of crude and purified DSIF preparations was compared using the reconstituted system containing the phenyl flowthrough (φFT), rTFIIB, rTFIIE, and rTFIIF. rDSIF alone did not induce DRB sensitivity in this system (lanes 11 and 12). Further addition of P.3 (P.3 dil) restored DRB sensitivity (lanes 13–18), indicating the presence of another DRB sensitivity–inducing activity in P.3.

Results

Identification of Another DRB Sensitivity–Inducing Activity

In a previous study (Wada et al., 1998a), we fractionated HeLa nuclear extract by phosphocellulose column chromatography into four fractions, which we referred to as P.1, P.3, P.5, and P.85 (the number denotes the KCl concentration of the elution buffer). Pol II and most of the GTFs were found in fractions P.5 and P.85, and a combination of these two fractions reconstituted transcription activity. However, this transcription was not sensitive to DRB, which could only be restored when the reaction was supplemented with either fractions P.1 or P.3. Using such assays, we were able to purify DSIF from P.1 and P.3 (Wada et al., 1998a).

To understand the mechanism of DRB action, a more defined transcription system that reconstitutes DRB sensitivity was required. To this end, fraction P1.0 (the 0.3–1.0 M KCl step from P11 and almost relevant to a mixture of P.5 and P.85) was passed through DEAE Sepharose and phenyl Superose columns as outlined in Figure 1A. At this phenyl Superose step, general transcription could be reconstituted with recombinant (r) TFIIB, rTFIIE, rTFIIF, and the phenyl flowthrough fraction (φFT), which contains TFIID, TFIIH, and pol II. As a template, we used a supercoiled plasmid pTF3-6C_2AT carrying a 380 bp G-free cassette under the control of the adenovirus E4 promoter (Wada et al., 1991). We utilized two different preparations of DSIF, a concentrated mixture of P.1 and P.3 achieved by ammonium sulfate precipitation (a P.1/P.3 concentration) and purified rDSIF.

Using the P1.0 fraction, both DSIF preparations converted DRB-insensitive transcription to DRB-sensitive transcription (Figure 1B), in agreement with our previous results (Wada et al., 1998a). In the reconstituted system, however, the addition of a P.1/P.3 concentration gave DRB sensitivity, but rDSIF did not (Figure 1C, compare lanes 1 and 2, and 11 and 12). This raised the possibility that an additional activity present in the crude DSIF preparation (i.e., either P.1 or P.3) was required for DRB-sensitive transcription. To test this, increasing amounts of P.1 or P.3 were added together with rDSIF to the reconstituted system. Within a range of P.3 concentrations that had little effect alone (lanes 5–10), P.3 and rDSIF together dramatically induced DRB sensitivity (lanes 13–18). P.1 did not have such an effect (data not shown). These results indicate that the P.3 fraction contains another DRB sensitivity–inducing activity. This activity may also be present in the P1.0 fraction, since purified rDSIF was sufficient when P1.0 was used in place of the reconstituted system (Figure 1B).

Purification of NELF

We termed the novel factor NELF, since it represses pol II elongation (see below). Using the reconstituted system plus rDSIF as an assay, NELF was purified from P.3 as summarized in Figure 2A. NELF was separated from DSIF at the DEAE Sepharose step (data not shown). During the course of purification, however, we noticed that the effect of DRB gradually decreased. We suspected that P-TEFb might be limiting in the reaction and supplemented the reaction with a P-TEFb active fraction prepared as described in Experimental Procedures. The addition of the P-TEFb fraction fully restored DRB sensitivity, and thus we were able to measure NELF activity up to the last purification step. In the mono S fractions, NELF activity was found between fractions 22–26 only in the presence of P-TEFb (Figure 2B, top). Finally, we established a transcription system that induces DRB sensitivity, depending on three different factors, NELF, DSIF, and P-TEFb.

SDS-PAGE and silver staining of the mono S fractions revealed five different bands of 66, 61, 59, 58, and 46 kDa associated with NELF activity (Figure 2B, bottom). On the next gel filtration column, these polypeptides were coeluted between fractions 5–9, indicating that they comprised one multimeric complex with a molecular mass of ~300 kDa (Figure 2D). NELF activity was present in these fractions, but this activity was low due to protein dilution. We refer to the five polypeptides as NELF-A to -E in accordance with their decreasing molecular weights. Since the five bands had roughly the same intensity after silver staining, they may form a heteropentamer.

We next assessed the functional relationship between NELF and P-TEFb (Figure 2C). Without P-TEFb, NELF potently repressed transcription irrespective of the presence of DRB (lanes 1–6). The addition of the P-TEFb fraction resulted in a partial reversal of the repressed

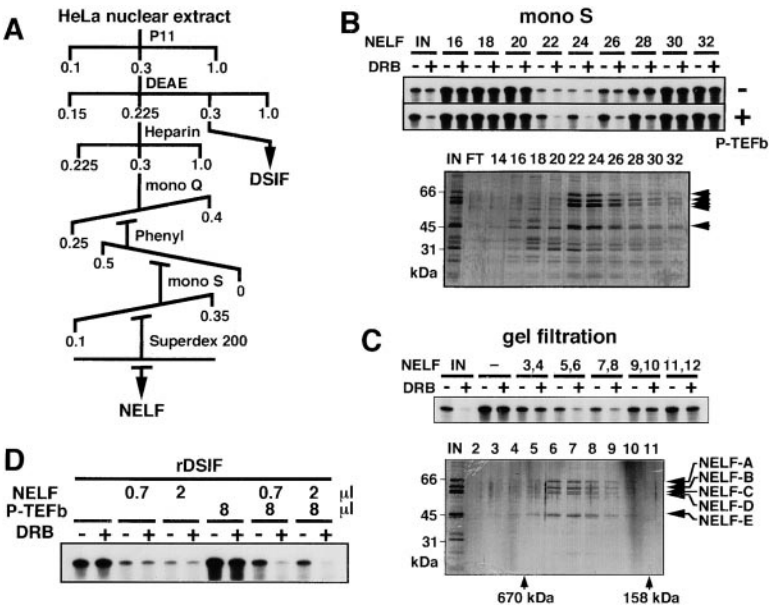

Figure 2. Purification of Negative Elongation Factor

(A) Scheme for NELF purification. NELF was purified from P.3 using the reconstituted system plus rDSIF as an assay. P-TEFb derived from P1.0 was additionally included in the reactions at later steps of purification.

(B) NELF activity on the mono S column. Mono S fractions (2 μl) were assayed for NELF activity in the presence or absence of P-TEFb. NELF activity was found in fractions 20–26 only in the presence of P-TEFb. The mono S fractions (2 μl) were also resolved by 7.5% SDS-PAGE, and the gel was stained with silver. Five different bands of 66, 61, 59, 58, and 46 kDa (indicated by arrows) were associated with the activity. IN, input; FT, flowthrough.

(C) Transcription repression by NELF. The effect of purified NELF (mono S) was examined in the presence or absence of P-TEFb as in (B). Without P-TEFb, NELF potently repressed transcription, while the addition of P-TEFb partly relieved the repression in a DRB-sensitive manner.

(D) Gel filtration of purified NELF. Fifty microliters purified of NELF (mono S) was applied to a Superdex 200 gel filtration column. An aliquot (10 μl) of each eluate fraction was assayed for NELF activity in the presence of P-TEFb as in (B), or stained with silver. The five polypeptides were coeluted with NELF activity at an apparent molecular mass of ~300 kDa and termed NELF-A to -E in accordance with their decreasing molecular weights. The positions of the native molecular weight markers are indicated at the bottom.

transcription (lanes 7–12). The restored transcription was sensitive to DRB, indicating that P-TEFb alone was responsible for the alleviation. The slight reduction in transcription seen after the addition of the P-TEFb fraction to assays containing DRB (lanes 8, 10, and 12) could not be reproduced when purified rP-TEFb was used instead of the P-TEFb fraction (data not shown). This discrepancy might be due to certain contaminating factors present in the P-TEFb fraction. Taken together, these results indicate (a) that NELF represses transcription in the absence of P-TEFb, and (b) that P-TEFb somehow counteracts this repression.

NELF Acts Cooperatively with DSIF

To elucidate the relationship between DSIF and NELF, we next tested their effect in the reconstituted system lacking P-TEFb (Figure 3A). A template pML-dC_2AT carrying a 270 bp G-free cassette under the control of the adenovirus major late promoter (Watanabe et al., 1990) was additionally used in the following assays. DSIF or NELF alone had little effect. In contrast, they repressed transcription from the E4 promoter when added together and, at the same time, resulted in the accumulation of shorter transcripts. Thus, DSIF and NELF cooperatively repress transcription. pML-dC_2AT was less sensitive and required higher doses of DSIF/NELF for repression (for example, compare lanes 2, 4, and 6 of Figure 5C). The differential effect is not due to the nature of the promoters but reflects primarily the difference in transcript length between the templates (Figure 7 and data not shown).

Since DSIF physically interacts with pol II (Wada et al., 1998a; Yamaguchi et al., 1999), DSIF and NELF might directly inhibit pol II transcription. To test this possibility, we employed an oligo dC-tailed template. This template can be efficiently transcribed by purified pol II without any GTFs and has been used for assaying general elongation factors such as TFIIF and Elongin (Kitajima et al., 1994; Aso et al., 1995). The addition of rDSIF had no effect in this system (Figure 3C, lanes 1–8), and NELF alone slightly repressed pol II transcription reproducibly (lanes 9–12). In contrast, a marked inhibition was observed when both were added together (lanes 13–16). Therefore, we conclude that DSIF and NELF exert a negative effect directly on pol II. Proteins used in this assay were analyzed by SDS-PAGE and silver staining (Figure 3B). Pol II was mostly in the IIa form, and rDSIF (2 μl) and purified NELF (2 μl) were present in excess over pol II. The amounts of pol II and P-TEFb present in the reconstituted system were roughly the same as the amounts of these factors, as estimated by immunoblotting (data not shown).

Effect of DSIF and NELF on Different Forms of Pol II

P-TEFb counteracts the repression induced by DSIF and NELF (Figure 2C). Since the pol II CTD has been suggested to be a primary target for phosphorylation by P-TEFb, it seems reasonable to propose that CTD phosphorylation modulates the negative effect of DSIF and NELF. To test this, pol IIo, pol IIa, and the CTD-less form of pol II (pol IIb) were prepared, and their susceptibility to DSIF and NELF was compared. The integrity of these polymerases was verified by silver staining (Figure 3D). In the absence of DSIF and NELF, the three forms of pol II showed similar elongation activity, indicating that neither the presence of the CTD nor its phosphorylated state affects elongation of purified pol II (Figure 3E). On the other hand, the addition of DSIF and NELF strongly repressed pol IIa but not pol IIo. To verify this further, pol IIa was converted to the

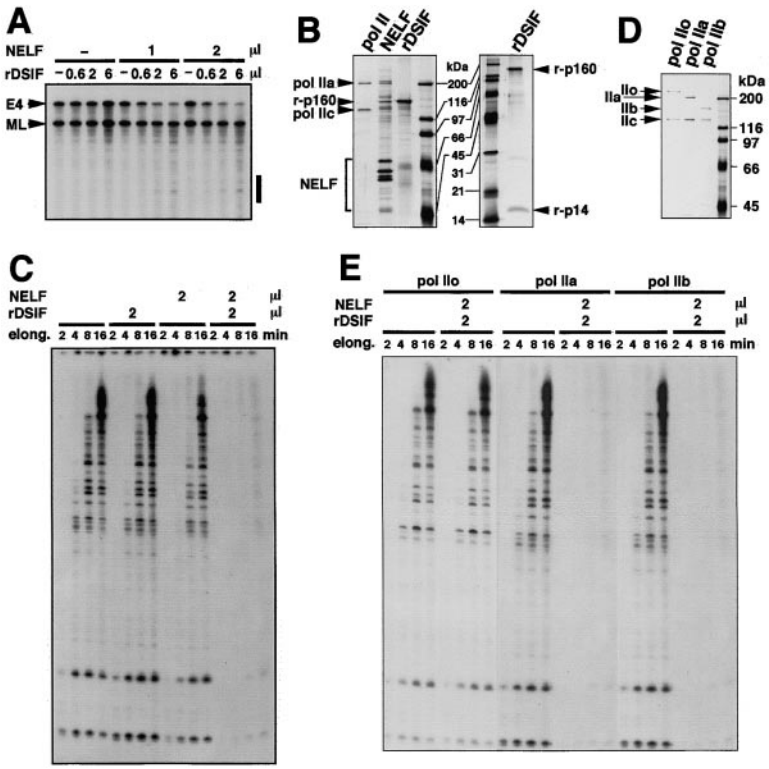

Figure 3. NELF Acts Cooperatively with DSIF to Repress Pol II Transcription

(A) Cooperative action of DSIF and NELF on promoter-specific transcription. The indicated amounts of r-DSIF and NELF (mono S) were added to the reconstituted system lacking P-TEFb. Two transcripts are generated from the adenovirus E4 and major late (ML) promoters. Transcripts from the ML promoter are less sensitive to repression. The vertical bar indicates the accumulation of short transcripts caused by DSIF and NELF.

(B) Proteins used in (C) were resolved by 7.5% (left) or 12.5% (right) SDS-PAGE and stained with silver. The amounts used are purified pol II, 0.5 μl; rDSIF, 2 μl; purified NELF, 2 μl. Pol II was mostly in the IIa form.

(C) Cooperative action of DSIF and NELF on the oligo dC-tailed template. Purified pol II and the template were preincubated together with the indicated protein factors. NTPs were added, and elongation was allowed to proceed for the indicated times.

(D) Pol IIo, pol IIa, and pol IIb were prepared as described in Experimental Procedures, and the amounts used in (E) were analyzed by 7.5% SDS-PAGE and silver staining.

(E) The different forms of pol II were assayed for elongation in the presence or absence of DSIF and NELF (2 μl each). Pol IIa and pol IIb, but not pol IIo, were susceptible to DSIF and NELF.

IIo form using P-TEFb and dATP and used in the assay. The IIo form thus prepared was also resistant to DSIF and NELF (data not shown). From these results, we conclude that CTD phosphorylation by P-TEFb (and possibly other kinases) makes pol II resistant to repression by DSIF and NELF. Since pol IIb was susceptible to DSIF and NELF (Figure 3E), the CTD is dispensable for the repression.

Molecular Cloning of NELF-E

To determine the identity of NELF complex, the NELF mono S fractions were subjected to SDS-PAGE, and the band of 46 kDa corresponding to NELF-E was excised and subjected to Lysyl-C digestion. Two peptide sequences were obtained for microsequence analysis. In a search of the databases, the two sequences were found to match part of the predicted human RD protein (Levi-Strauss et al., 1988; Cheng et al., 1993). RD is a 380 amino acid protein with a calculated molecular weight of 43.2 kDa, a size that roughly agrees with that of NELF-E. The RD protein, whose function is unknown, was named RD because it possesses the unique Arg-Asp (RD) dipeptide repeat sequence (Figure 4). RD also contains a putative RNA recognition motif (RRM) in its C terminus and a leucine zipper in its N-terminal end.

To confirm the identity of RD as NELF-E, an antibody was raised against the full-length RD protein. The antibody was highly specific, since it exclusively reacted with the ~46 kDa band of RD in the crude nuclear extract (Figure 5A, lane 1). It was then used to examine for the presence of RD in purified, as well as in crude, NELF fractions. The antibody clearly detected RD in purified NELF (lane 2). In addition, RD cofractionated with NELF activity through several purification steps (unpublished data). Thus, we conclude that NELF-E is identical to RD. Importantly, RD was found in both P.3 and P1.0, but not in P.1 (data not shown). This observation provides an answer as to why there was no requirement for NELF when the original P1.0 system was employed (Figure 1B).

Immunodepletion of NELF from HeLa Nuclear Extract

To determine whether NELF-E/RD is required for NELF activity, RD was immunodepleted from HeLa nuclear

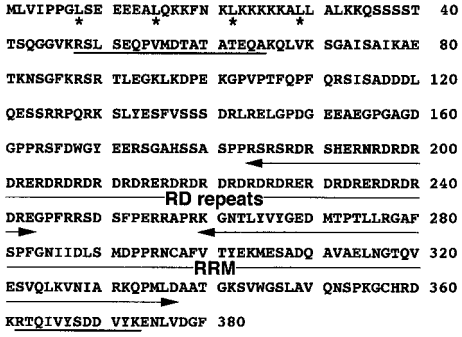

Figure 4. Identification of NELF-E as RD

The active fractions from mono S were subjected to SDS-PAGE, and the band of 46 kDa corresponding to NELF-E was subjected to microsequence analysis. Two peptide sequences (underlined) were obtained and found to match parts of the human RD protein (Cheng et al., 1993; GenBank accession No. L03411). The RD motif and the putative RRM are indicated by arrows. Asterisks denote Leu residues of the putative leucine zipper structure.

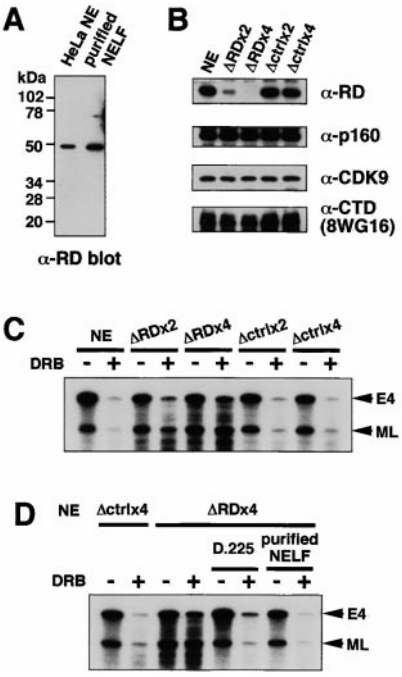

Figure 5. Immunodepletion of NELF from HeLa Nuclear Extract

(A) Specificity of anti-RD antibody. Anti-RD antibody raised against the full-length RD protein was tested for immunoblotting. The antibody detected a single band of ~46 kDa in the nuclear extract and purified NELF (mono S).

(B) Immunoblot analysis of the nuclear extracts depleted of RD. HeLa nuclear extract (200 μl) was repeatedly passed over protein G-Sepharose (20 μl) to which either anti-RD or preimmune serum was adsorbed. The supernatants (2 μl) were analyzed for the presence of RD, DSIF p160, the CDK9 subunit of P-TEFb, and the largest subunit of pol II by immunoblotting. Two or four passages over the anti-RD beads reduced or eliminated the endogenous RD protein (ΔRDx2 and ΔRDx4), while the level of other components remained constant. Depletion with the control beads had no effect.

(C) Transcription activity of the RD-depleted extract. The extracts (2 μl) were assayed using pTF3-C_2AT and pML-dC_2AT. Depletion of RD did not significantly affect transcription in the absence of DRB. In its presence, however, RD depletion alleviated transcription repression, resulting in a reduction in DRB sensitivity.

(D) Readdition of NELF. D.225 signifies the 0.225 M KCl fraction from DEAE Sepharose, which was free from DSIF. D.225 (6 μl) and purified NELF (mono S; 2 μl) were added back to the RD-depleted extract (ΔRDx4). In both cases, the addition of NELF repressed transcription in the presence of DRB and restored DRB sensitivity.

extract using the anti-RD antibody. The nuclear extract was passed through an anti-RD antibody–immobilized column or a control column twice or four times, and the presence of RD was monitored by immunoblotting (Figure 5B). After four passages, virtually all of the endogenous RD protein was removed from the extract. The procedure did not significantly affect the protein levels of DSIF, pol II, or P-TEFb (Figure 5B).

We next assessed the transcription activity of these extracts using the two templates referred to in Figure 3A (Figure 5C). The depletion of RD increased the level of transcription in the presence of DRB without affecting the basal level, which resulted in a marked reduction of DRB sensitivity. Crude (D.225, a DEAE Sepharose fraction free from DSIF) or purified NELF preparations

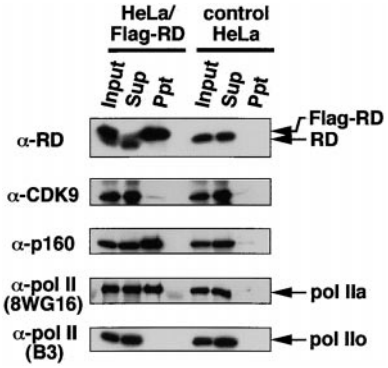

Figure 6. NELF Interacts with DSIF and Pol II

Nuclear extracts were prepared from HeLa/Flag-RD and control HeLa cells by the method of Dignam et al. (1983). The extracts (200 μl) were immunoprecipitated with anti-Flag M2 monoclonal antibody (Sigma) under transcription conditions, washed, and eluted with Flag peptide (Sigma). Each fraction was analyzed by immunoblotting with the indicated antibodies. Input and FT lanes represent 4% of the total amount.

were added back to the depleted extract (Figure 5D). In both cases, the addition of NELF repressed transcription in the presence of DRB and restored DRB sensitivity. Thus, RD is responsible, at least in part, for NELF activity. These results also fit with the model that NELF is a negative factor normally counteracted by P-TEFb. In contrast, histidine-tagged (His-) RD purified from *Escherichia coli* could not restore DRB sensitivity (unpublished data). It is therefore likely that the other NELF subunits are also essential for its function and were depleted, in addition to RD, from the extract using the anti-RD column.

NELF Interacts with DSIF and Pol II

To elucidate the molecular basis for NELF action, we investigated the physical interaction between NELF and the other components of the DRB-sensitive transcription machinery. Nuclear extracts were prepared from HeLa cells constitutively expressing Flag-tagged RD (HeLa/Flag-RD; Y. Y. and H. H., unpublished data) and control HeLa cells. Flag-RD-containing complexes were purified from the extracts by anti-Flag affinity chromatography, and the presence of various components was examined by immunoblotting. Flag-RD and the other NELF subunits were quantitatively recovered in the immunoprecipitate, while endogenous RD was found in the supernatant (Figure 6 and data not shown). Under these conditions, a fraction of DSIF p160 and pol II was coprecipitated with Flag-RD, indicating that they physically interact with NELF. P-TEFb was not detected in the immunoprecipitate.

The antibody 8WG16 recognizes both pol IIo and pol IIa (Thompson and Burgess, 1996). However, since pol II in the nuclear extract is mostly in the IIa form, it is difficult to detect pol IIo. To determine whether NELF interacts with pol IIo, another antibody B3, which specifically recognizes pol IIo, was employed (Mortillaro et al., 1996). In striking contrast to pol IIa, pol IIo was absent from the immunoprecipitate. Thus, CTD phosphorylation directly controls the interaction between NELF and pol II.

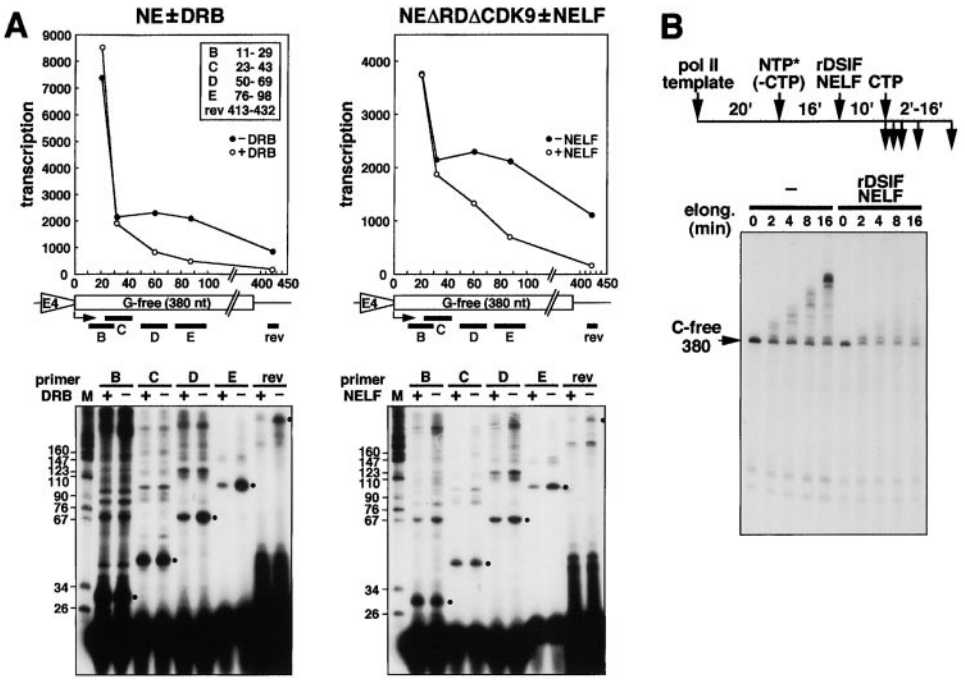

Figure 7. Kinetic Analysis of DSIF/NELF Function

(A) Primer extension analysis. Transcripts were synthesized from pTF3-6C$_2$AT using either the normal HeLa nuclear extract in the presence or absence of 50 μM DRB (left) or the extract depleted of NELF and P-TEFb with or without 1 μl NELF (right). They were analyzed using a set of oligonucleotide primers labeled to the same specific activity. M lanes show the pBR322 MspI digest. The extension products, marked with the dots, were quantified using a FLA-2000 image analyzer (Fuji), and the amounts (in arbitrary units) were plotted against the length from the transcription start site (top). Indicated in the inset are the positions of transcripts to which the primers hybridized. Longer transcripts may be underestimated due to the reduced efficiency of reverse transcription.

(B) Effect of DSIF and NELF on isolated elongation complexes. Purified pol II and the oligo dC-tailed template carrying a 380 nt C-free cassette at its dC end were incubated in the presence of NTP lacking CTP, and the paused elongation complexes were allowed to form on the template. DSIF and NELF were added where indicated, and elongation was allowed to resume for the indicated times by adding CTP.

Kinetic Analysis of DSIF/NELF Action

Which step(s) of transcription is affected by DSIF and NELF? While several lines of evidence indicate that they act on elongation, the above data showing DSIF/NELF interaction with pol IIa raise the possibility that they may also affect earlier steps of transcription such as initiation and promoter clearance. To elucidate their role in these steps, we performed primer extension assays (Figure 7A). Transcripts were synthesized from pTF3-6C$_2$AT using either the normal nuclear extract (left) or the extract depleted of NELF and P-TEFb (right), and the transcripts were analyzed using a set of oligonucleotide primers. In the former case, DRB sensitivity was examined, and in the latter case, the extent of NELF repression was directly measured in the absence of P-TEFb. The above graphs show the survival curves of transcription complexes as a function of their distance from the transcription start site. Note that the extension products reflect the amount of transcription through the primer positions as well as the amount of shorter transcripts which can anneal to the 3′-portion of the primers. This limits the resolution of the assay, which is, however, still higher than those of previous studies (Chodosh et al., 1989; Marciniak and Sharp, 1991).

We observed that a significant fraction of the initiated complexes encountered a block to elongation between positions +20 and +40. This may be due to promoter-proximal pausing, which reportedly occurs at the promoter clearance step and which is suppressed by TFIIH in an ATP-dependent manner (Dvir et al., 1997; Kumar et al. 1998). In agreement with a previous report (Chodosh et al., 1989), however, production of such short transcripts (20–40 nt) was neither sensitive to DRB (left) nor to NELF (right), indicating that DSIF and NELF do not inhibit initiation and subsequent promoter clearance steps. In contrast, elongation complexes synthesizing 50–60 nt or longer transcripts were sensitive to DRB and NELF, which were relatively processive in their absence. The qualitative change seems to occur between positions +30–40 and +50–60 in a sequence-independent manner, because the same transition was observed using a different template (data not shown). Since longer transcripts were more sensitive to DRB and NELF, it is likely that DSIF and NELF can act on elongation complexes located even further downstream.

To directly determine whether DSIF and NELF act on isolated elongation complexes, the oligo dC-tailed template was devised to carry a 380 bp C-free cassette at its dC end. Purified pol II and the template were incubated in the presence of NTP lacking CTP, and the paused elongation complexes were allowed to form on the template (Figure 7B, lane 1). Addition of CTP allowed the polymerase to resume RNA synthesis (lanes 2–5).

Addition of DSIF and NELF before CTP addition greatly reduced elongation by the paused pol II (lanes 6–15), indicating that DSIF and NELF exert a negative effect on elongation complexes that have transcribed stretches of DNA of several hundred nucleotides.

Discussion

Interplay between Positive and Negative Elongation Factors

Most of the general elongation factors known to date possess stimulatory functions. One exception is factor 2, which causes the release of pol II transcripts in an ATP-dependent manner (Xie and Price, 1996; Liu et al., 1998). In this and previous studies (Wada et al., 1998a), we have identified a novel class of elongation factors that cause pol II pausing. Since DRB-induced short transcripts can be chased by the addition of high concentrations of NTP (Wada et al., 1998a), the repression by DSIF and NELF is not due to termination, but rather to pausing by pol II. As is often seen in other biological reactions such as homeostasis, well-tuned regulation can only be achieved through the action of both positive and negative factors.

From the current data, we propose a plausible model for the interplay between DSIF, NELF, and P-TEFb. In a highly purified system (Figure 8A), transcription elongation is processive (after the promoter clearance step) and insensitive to DRB, since both the positive and negative factors are absent. In a more crude system (Figure 8B), DSIF and NELF bind pol IIa and repress elongation. However, P-TEFb normally phosphorylates the pol II CTD and thereby facilitates the release of DSIF/NELF from pol II, which alleviates the block to elongation. We have recently found that the DSIF–pol II interaction is also sensitive to CTD phosphorylation (Wada et al., 1998b). In the presence of DRB, P-TEFb action is inhibited, allowing DSIF/NELF to continue to repress elongation. Since DRB affects most of the class II genes, the repression–antirepression process likely represents a critical rate-limiting step in pol II elongation.

The CTD is dispensable for repression by DSIF and NELF, since pol IIb was susceptible to DSIF and NELF as well as pol IIa (Figure 3E). Therefore, DSIF and NELF most likely bind another region of pol II distinct from the CTD. CTD phosphorylation may induce a conformational change in pol II that could mask the interaction domain. Alternatively, phosphorylated CTD may perturb the association of DSIF and NELF with pol II ionically. It is also possible that NELF binds the nascent transcript through the RD subunit to stabilize the ternary complex (Figure 8).

Mechanism for Repression by DSIF and NELF

Primer extension analysis revealed that DSIF and NELF do not affect promoter-proximal transcription. These results as well as those of the oligo dC-tailed template assays indicate that DSIF and NELF are capable of repressing transcription at any point downstream of +50–60. The initiated polymerases pass through a structural transition at around +25, and after the synthesis of ~40 nt transcripts they form stable elongation complexes

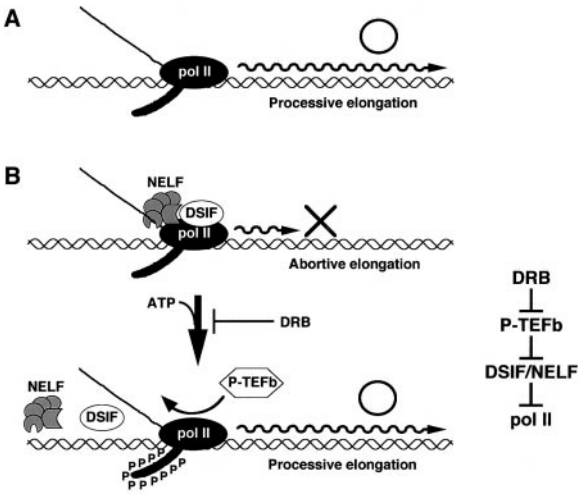

Figure 8. Interplay between DSIF, NELF, and P-TEFb

(Samkurashvili and Luse, 1998). Thus, DSIF and NELF presumably act on mature elongation complexes.

Several groups have reported that actively elongating polymerases contain the hyperphosphorylated CTD (Dahmus, 1996; Uptain et al., 1997). However, DSIF and NELF can only associate with, and inhibit, hypophosphorylated pol II. Addition of DRB or depletion of P-TEFb may create an unusual situation in which the CTD of elongating pol II remains hypophosphorylated, thereby allowing DSIF/NELF to repress elongation continuously. If so, at which step do DSIF and NELF act under physiological conditions? The most probable step is early elongation after the transcription complex becomes sensitive to DSIF/NELF. At this step, polymerases are often found in the unphosphorylated state (O'Brien et al, 1994). Kinetic analyses of P-TEFb also suggest a positive role in early elongation (Marshall and Price, 1992). As an alternative possibility, DSIF/NELF may affect elongation complexes located further downstream. Recently, a protein phosphatase specific for pol IIo was identified in humans and yeast (Chambers et al., 1995; Archambault et al., 1997, 1998). Though its action mechanism remains obscure, it seems possible that the CTD phosphatase dephosphorylates the pol II CTD during the elongation process. If this happens, DSIF and NELF may become reassociated with, and cause pausing of, this pol II.

The data described above pose another question: Why don't DSIF and NELF repress the initiation and promoter clearance steps? Since pol II recruited to promoters is in the hypophosphorylated form (Dahmus, 1996), DSIF/NELF would be expected to interact with pol II and repress these steps. However, this interpretation is not supported by the data presented above. A possible explanation is that DSIF/NELF may not be able to associate with pol II during these steps. Since pol II molecules are assembled into very large protein complexes at the promoter, this may prohibit DSIF/NELF from associating with pol II. Upon promoter clearance, the pol II complex may become "naked" and accessible to repression by DSIF and NELF. Alternatively, DSIF/NELF may be incorporated into the initiation complex

but be unable to exert a negative effect during the early steps of transcription. If this were the case, DSIF/NELF must repress some pol II function(s) specific to the elongation phase. It will be interesting to determine whether DSIF and NELF are present in the preinitiation and postinitiation complexes. Future analyses should shed more light on the mechanism of DSIF and NELF transcription repression.

Functional Roles of CTD Phosphorylation

Although the roles played by the pol II CTD and its phosphorylation have been extensively studied, their roles in processive elongation are poorly understood. Here we demonstrate that CTD phosphorylation facilitates the release of the negative elongation factors DSIF and NELF from pol II. Conversely, a novel elongation factor complex termed Elongator was recently identified in yeast. Elongator selectively binds pol IIo, and genetic evidence suggests that it plays a positive role in elongation (Otero et al. 1999). In addition, a number of proteins have been reported that interact with pol II in a CTD phosphorylation state-sensitive manner. Some GTFs and a Srb complex bind pol IIa and play roles during the initiation phase (Maxon et al., 1994; Sun et al., 1998). The 5′-capping enzyme binds pol IIo and is recruited to the elongation complex (Cho et al., 1997; McCracken et al., 1997; Yue et al., 1997). Some splicing factors also appear to bind pol IIo (Mortillaro et al., 1996; Corden and Patturajan, 1998). Thus, CTD phosphorylation functions as a molecular switch, altering the accessibility of pol II to various factors involved in transcription initiation, elongation, and mRNA processing.

There remains an important, unsolved question. Many protein kinases, such as P-TEFb, TFIIH, Srb10-Srb11, DNA-PK, MAPK, and Abl, reportedly phosphorylate the pol II CTD in vivo and/or in vitro (Dahmus, 1996). Does CTD phosphorylation by these different kinases have the same consequences? A hint as to how this question might be answered was provided recently (Hengartner et al., 1998; Sun et al., 1998). Though TFIIH and Srb10-Srb11 both belong to the cyclin-CDK family of protein kinases, they have opposing functions in general transcription. It was shown that these kinases phosphorylate different residues of the CTD in a temporarily distinct manner. Thus, as a first step, it should be determined whether DSIF and NELF respond differently to the CTDs phosphorylated by distinct kinases.

Structure of NELF

NELF is likely to be composed of RD and four additional polypeptides, NELF-A to -D. RD alone does not suffice for NELF activity, suggesting that the other subunits are essential for its function. Molecular cloning of the remaining subunits will help understand the structure and function of NELF in more detail.

RD was initially identified as a novel gene located in the major histocompatibility complex class III region of human and mouse (Levi-Strauss et al., 1988). RD possesses a putative RRM and a tract of alternating Arg-Asp residues (RD motif). Since the two motifs are also found in the splicing factor U1 70K, RD has been postulated to be involved in mRNA splicing. However, we argue against the model because (a) RD exists solely in the context of NELF (unpublished data) and (b) NELF does not interact with pol IIo, a form involved in mRNA splicing (Mortillaro et al., 1996; Corden and Patturajan, 1998). It will be important to determine whether NELF has any RNA binding activity and whether this is critical for its function. The Tat protein encoded by HIV is a well-characterized transactivator with RNA binding activity (Jones and Peterlin, 1994). Tat targets the specific RNA element downstream of the HIV long terminal repeat and stimulates elongation. Thus, NELF may also recognize RNA in a sequence-specific manner and may be recruited to some *cis*-acting elements to cause gene-specific and site-specific pol II pausing.

Experimental Procedures

Transcription Factors and Pol II

The HeLa nuclear extract and the phosphocellulose (P11) fractions (P.1, P.3, and P1.0) were prepared as described (Wada et al., 1998a). To establish a more defined transcription system that reconstitutes DRB sensitivity, P1.0 was dialyzed against HGE (20 mM HEPES [pH 7.9], 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol) containing 0.1 M KCl (HGE.1; the number following HGE denotes the molar concentration of KCl) and applied to a 60 ml DEAE Sepharose FF column (Amersham Pharmacia) equilibrated with HGE.1. The column was washed with the same buffer, and proteins were eluted with HGE.3 and HGE1.0. The 0.3 M KCl fraction was dialyzed against HGE containing 1.0 M $(NH_4)_2SO_4$ and then applied to a phenyl Superose HR5/5 column (Amersham Pharmacia; 1.0 ml). Proteins were eluted with a 15 ml linear gradient of 1.0 to 0 M $(NH_4)_2SO_4$. At this step, transcription could be reconstituted with the phenyl flowthrough fraction (ϕFT), rTFIIB, rTFIIE, and rTFIIF. ϕFT contains TFIID, TFIIH, and pol II. The recombinant GTFs were prepared as described (Wada et al., 1998a). To prepare a P.1/P.3 concentration, 12 ml each of P.1 and P.3 were mixed and precipitated with saturated $(NH_4)_2SO_4$. Following centrifugation at 13,600 $\times$ g, the pellet was resuspended in 3 ml 0.5$\times$ HGE.05 and dialyzed against the same buffer. rDSIF is a mixture of equimolar amounts of r-p160 and r-p14. For P-TEFb, P1.0 was chromatographed as above, and the presence of P-TEFb was monitored by immunoblotting and CTD kinase assays. P-TEFb activity was found in the eluate of the phenyl Superose column, and active fractions were pooled and used as the P-TEFb fraction. Pol II used in Figure 3B was prepared from HeLa nuclear pellet as described (Usuda et al., 1991). In Figure 4, pol IIa and pol IIo were separately purified to near homogeneity as described (Kim and Dahmus, 1988; Lu et al., 1991). For pol IIb, pol IIa was converted to pol IIb by mild treatment with 0.25 μg/ml chymotrypsin for 30 min at 30°C.

Promoter-Specific Transcription Assays

Supercoiled plasmids pTF3-6C_2AT and pML-dC_2AT were used as templates, which produce 380 and 270 nt G-free transcripts under the control of the adenovirus E4 and major late promoters, respectively (Watanabe et al., 1990; Wada et al., 1991). Twenty microliter reactions containing pTF3-6C_2AT (25 ng), pML-dC_2AT (225 ng), and the different protein factors were preincubated for 45 min at 30°C and processed as described (Wada et al., 1998a). In the P1.0 transcription system, 2 μl of P1.0 concentration (Wada et al., 1998a) was used. In the reconstituted system, ϕFT (5 μl), rTFIIB (30 ng), rTFIIE (α, 5 ng; β, 3 ng), and rTFIIF (RAP74, 15 ng; RAP30, 15 ng) were used. Where indicated, 6 μl of P-TEFb fraction was included. As for DSIF, either a P.1/P.3 concentration (2 μl) or rDSIF (p160 30 ng, p14 3 ng) was used. Primer extension analysis was performed essentially as described (Ausubel et al., 1995). Primers used were B, 5′-GAATAATGAGGAAAGGAGAGT-3′; C, 5′-GATGATAGATTTGG GAAATATAA-3′; D, 5′-GGAGAGTAGGGTGGTATAG-3′; E, 5′-GGAG AGTAGGGTGGTATAG-3′; and rev, 5′-AGGAAACAGCTATGACC ATG-3′. The extension products were analyzed by 8% urea-PAGE.

The expected lengths of the products were 29 nt (B), 43 nt (C), 69 nt (D), 98 nt (E), and 432 nt (rev).

Oligo dC-Tailed Template Assays

The oligo dC-tailed template dC3.8 was prepared as described (Kitajima et al., 1994). To prepare the template carrying a 380 nt C-free cassette, the plasmid p(C_2AT)19 (Sawadogo and Roeder, 1985) was digested with SmaI, and the G-free cassette was used in the reverse orientation. For the elongation assays, 20 μl reactions containing the template (100 ng), pol II (0.5–1.5 μl), and additional protein factors in 0.5× HGE.05 plus 6 mM $MgCl_2$ were preincubated for 20 min at 30°C. Elongation was started by adding 3 μl of NTP mix (50 μM ATP/CTP/GTP, 10 μM UTP, and 1 μCi [α-^{32}P] UTP). After 2 to 16 min, 5 μl aliquots were withdrawn, and the transcripts were analyzed on a 6% urea-polyacrylamide sequencing gel. In Figure 8B, elongation was started by adding the NTP mix lacking CTP. After 16 min, DSIF/NELF or buffer was added to the reactions and incubated for an additional 10 min. Elongation was resumed by adding 10 μM CTP, and the transcripts were analyzed as described above.

Purification of NELF

The P.3 fraction (~200 mg) from 120 ml (~840 mg) of HeLa nuclear extract was diluted with HGE to a KCl concentration of 0.15 M and was applied to a 60 ml DEAE Sepharose FF column equilibrated with HGE.15. The column was washed with the same buffer, and proteins were step eluted with HGE.225, HGE.3, and HGE1.0. NELF activity was eluted at the 0.225 M KCl step, while DSIF was exclusively found in the 0.3 M KCl fraction as revealed by immunoblotting. The 0.225 M KCl fraction (a 10 mg aliquot) was directly loaded onto a 5 ml HiTrap Heparin column (Amersham Pharmacia) equilibrated with HGE.225. The column was washed with the same buffer and step eluted with HGE.3 and HGE1.0. NELF activity was found in the 0.3 M KCl fraction. The 0.3 M KCl fraction (~5 mg) was diluted to a KCl concentration of 0.25 M and was loaded onto a mono Q HR5/5 column (Amersham Pharmacia; 1.0 ml). The column was eluted with a 15 ml linear gradient of 0.25 to 0.4 M KCl. The active fractions (eluting at ~0.30 M KCl) were pooled and dialyzed against HGE containing 0.75 M $(NH_4)_2SO_4$. The dialysate (~0.8 mg) was then applied to a phenyl Superose HR5/5 column, and protein was eluted with a 15 ml linear gradient of 0.5 to 0 M $(NH_4)_2SO_4$. The active fractions were pooled, dialyzed against HGE.1, and applied to a mono S PC 1.6/5 column (Amersham Pharmacia; 0.1 ml). Proteins were eluted with a 1.5 ml linear gradient from 0.1 to 0.35 M KCl. NELF activity was eluted at ~0.23 M KCl, and the fraction contained five polypeptides of 66, 61, 59, 58, and 46 kDa. The active fraction was then applied to a Superdex 200 gel filtration column (Amersham Pharmacia; 2.4 ml) equilibrated with HGE.1. The putative NELF subunits were coeluted at a molecular mass of ~300 kDa. From 840 mg of HeLa nuclear extract, 2–3 μg of each polypeptide could be obtained.

Peptide Sequencing

The mono S NELF fractions containing ~2 μg NELF-E were precipitated with acetone, and proteins were separated by 7.5% SDS-PAGE. The 46 kDa band of NELF-E was excised and digested with Lysyl-C endopeptidase (Wako) within the gel. The resulting peptides were eluted, separated by reverse-phase HPLC, and sequenced. The two sequences obtained, RSLSEQPVMDTAXAXEQA and RTQI VYSDDVYK, were found to completely match parts of the predicted human RD protein.

Preparation of Anti-RD Antisera

For the production of GST-RD fusion protein, the coding region of human RD cDNA (Cheng et al., 1993) was amplified by PCR and cloned into pGEX-5X-3 (Amersham Pharmacia). The sequence was verified by dideoxy sequencing. GST-RD was expressed and purified from *E. coli* strain DH5α using a glutathione Sepharose 4B column (Amersham Pharmacia) as instructed by the manufacturer. Purified GST-RD was mixed with complete Freund's adjuvant and injected into rats. The antisera were obtained after three rounds of immunization.

Immunodepletion of NELF

Anti-RD antiserum or preimmune serum (10 μl each) was incubated with 20 μl protein G-Sepharose CL-6B (Amersham Pharmacia) for 1 hr at 4°C, and the antibodies were immobilized on the beads. After washing three times with NETN (50 mM Tris [pH 7.9], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) and once with HGE.1, 200 μl of HeLa nuclear extract was incubated with the beads for 2 hr at 4°C. The supernatants were collected and incubated with the new antibody-immobilized beads. Nuclear extracts passed twice or four times through the beads were prepared. Depletion of P-TEFb from the nuclear extract was performed as described (Wada et al. 1998b).

Depletion of RD was monitored by immunoblotting with anti-RD antiserum. The input and the depleted extracts (2 μl each) were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The blot was sequentially incubated with anti-RD antiserum (1:200 dilution) and anti-rat IgG secondary antibody conjugated with horseradish peroxidase (1:2,000 dilution; Dako) and visualized with the ECL kit (Amersham Pharmacia).

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