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Evidence that Negative Elongation Factor Represses Transcription Elongation through Binding to a DRB Sensitivity-Inducing Factor/RNA Polymerase II Complex and RNA

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Negative elongation factor (NELF) is a human transcription factor complex that cooperates with DRB sensitivity-inducing factor (DSIF)/hSpt4-hSpt5 to repress elongation by RNA polymerase II (RNAPII). NELF activity is associated with five polypeptides, including NELF-A, a candidate gene product for Wolf-Hirschhorn syndrome, and NELF-E, a putative RNA-binding protein with arginine-aspartic acid (RD) dipeptide repeats. Here we report several important findings regarding the DSIF/NELF-dependent elongation control. First, we have established an effective method for purifying the active NELF complex using an epitope-tagging technique. Second, the five polypeptides each are important and together are sufficient for its function in vitro. Third, NELF does not bind to either DSIF or RNAPII alone but does bind to the preformed DSIF/RNAPII complex. Fourth, NELF-E has a functional RNA-binding domain, whose mutations impair transcription repression without affecting known protein-protein interactions. Taken together, we propose that NELF causes RNAPII pausing through binding to the DSIF/RNAPII complex and to nascent transcripts. These results also have implications for how DSIF and NELF are regulated in a gene-specific manner in vivo.

Transcription elongation by RNA polymerase II (RNAPII) is controlled by a number of *trans*-acting factors called transcription elongation factors as well as by *cis*-acting elements (2, 26). Transcription elongation factors such as transcription factor IIF (TFIIF), elongin, and TFIIS interact with elongating RNAPII to prevent its pausing or to reactivate it from an arrested configuration. *cis*-acting elements are mainly located on nascent transcripts. Some RNA elements cause RNAPII to pause or arrest without the aid of protein factors by forming structures that destabilize RNAPII-DNA-RNA complexes (19, 26). Other types of RNA elements include the one called TAR, which exists at the 5′ end of human immunodeficiency virus (HIV) transcripts and serves as a binding site for the viral activator Tat and cellular cofactors (9); together these strongly stimulate RNAPII elongation. The functions of many other RNA elements are largely unknown.

The recent discovery of a new class of positive and negative elongation factors, including DRB sensitivity-inducing factor (DSIF), negative elongation factor (NELF), and positive transcription elongation factor b (P-TEFb), has shed new light on the control of RNAPII elongation (21, 27, 31–33, 35). Biochemical studies have established that DSIF and NELF cooperatively repress RNAPII elongation, whereas P-TEFb alleviates the repression in a manner sensitive to the kinase inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (28, 29, 31). DSIF and NELF coimmunoprecipitate with the unphosphorylated form of RNAPII (IIa), but not with the hyperphosphorylated form (IIo) (28, 31). In addition, P-TEFb strongly phosphorylates the C-terminal domain (CTD) of RNAPII and a subunit of DSIF in a DRB-sensitive manner (8, 12, 35). From these data, we have proposed that transcription repression is caused by the physical association of DSIF, NELF, and RNAPIIa and is reversed by P-TEFb-dependent phosphorylation of the CTD. Such phosphorylation-dependent control allows the transcription elongation step to play a critical role in gene expression in response to various extracellular stimuli and during development (1, 5, 10, 13, 15, 16, 33). Furthermore, recent findings suggest that other cellular and viral proteins, such as TFIIF (22) and HDAg (33) (also see below), may also control DSIF/NELF action in a phosphorylation-independent manner.

DSIF is composed of 14- and 160-kDa subunits, which are human homologues of *Saccharomyces cerevisiae* Spt4 and Spt5 (7, 27). Previous studies have shown that DSIF not only represses but also activates RNAPII elongation under limiting concentrations of nucleoside triphosphate (27), in a Tat/TAR-dependent transcription system (13), or when a DNA template that produces long transcripts is used (5). The positive role for DSIF/Spt4-Spt5 is also supported by genetic evidence in *S. cerevisiae* (7) and by cytogenetic studies with *Drosophila melanogaster* (1, 10). Considering the selective binding of DSIF to RNAPIIa, DSIF may affect RNAPIIo indirectly through an as-yet-unknown mechanism. Recently, Parada and Roeder (20) have identified a large protein complex containing hSpt5, P-TEFb, and Tat-stimulatory factor 1 (SF1) that is capable of activating RNAPII elongation. This complex may be the molecular entity for the stimulatory function of DSIF.

NELF activity is associated with five polypeptides—A (66 kDa), B (61 kDa), C (59 kDa), D (58 kDa), and E (46 kDa)—of which only NELF-A and NELF-E have been sequenced and cloned (31, 33). The structure of NELF-E is characterized by an N-terminal leucine zipper motif, a central domain rich in Arg-Asp dipeptide repeats (the RD motif), and a C-terminal RNA recognition motif (RRM). RRMs, which

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typically encompass 80 to 90 amino acids with two highly conserved elements called RNP1 and RNP2, often bind to RNA in a sequence- or structure-specific manner (14, 17). It is not known whether the NELF-E RRM, which is slightly divergent from the consensus, binds to RNA and is required for NELF function. NELF-A is encoded by *WHSC2*, a candidate gene for Wolf-Hirschhorn syndrome (30, 33). Interestingly, its N-terminal segment shows sequence similarity to HDAg, the hepatitis delta virus (HDV) protein that binds to RNAPII and activates transcription elongation (32). These proteins thus may have functional and evolutionary relationships. The identity and significance of the other polypeptides associated with NELF activity are yet to be determined.

To obtain more insight into the mechanism underlying DSIF/NELF/P-TEFb-dependent elongation control, we have established a simple method for purifying the multisubunit factor NELF. This method allows us to characterize the biochemical properties of NELF, including its subunit structure, protein-protein interactions, and protein-RNA interactions. We provide evidence that NELF causes RNAPII pausing through binding to the DSIF/RNAPII complex and nascent transcripts.

MATERIALS AND METHODS

In vitro transcription assays. Promoter-specific transcription assays were performed as described (27). Briefly, 25-μl reaction mixtures containing the supercoiled plasmid templates pTF3-6C_2AT (25 ng) and pML-dC_2AT (100 ng), HeLa nuclear extracts (NE) (2 μl), 60 μM ATP, 600 μM CTP, 5 μM UTP, 5 μCi of [α-^{32}P]UTP, 80 μM 3′-*O*-methyl-GTP (Amersham Pharmacia), and 50 U of RNase T_1 (Invitrogen) were incubated with or without 50 μM DRB (Sigma) for 10 min at 30°C. Preparation of the dC-tailed template and transcription assays using this template were performed as described (31). Briefly, 25-μl reaction mixtures containing the dC-tailed template (100 ng), purified RNAPII (30 ng), recombinant p160/hSpt5 (30 ng), recombinant p14/hSpt4 (3 ng), indicated amounts of Flag-NELF, 50 μM ATP, 50 μM GTP, 50 μM CTP, 10 μM UTP, and 5 μCi of [α-^{32}P]UTP were incubated for the indicated times at 30°C. Synthesized RNAs were purified by phenol-chloroform extraction and ethanol precipitation and analyzed by electrophoresis on 8 or 6% polyacrylamide gels containing 7 M urea.

Construction of NELF-E derivatives. NELF-E PM carries Arg-to-Glu, Asn-to-Gln, Cys-to-Met, and Phe-to-Thr substitutions at positions 295, 296, 297, and 299, respectively, all of which lie within the RNP1 motif. Amino acid substitutions were introduced so as not to change the predicted secondary structure of this region. NELF-E Del has a deletion of amino acids 260 to 342 spanning the entire RRM. NELF-E Swap has the CstF-64 RRM (amino acids 14 to 99) instead of the natural RRM (amino acids 260 to 342). These alterations were made by a combination of PCR and standard recombinant DNA techniques. An oligonucleotide encoding the Flag epitope (DYKDDDDK) was attached to the 5′ end of the NELF-E open reading frame by PCR. Tagged cDNAs for wild-type and mutant NELF-E were inserted into the mammalian expression vector pCAGGS (18). Partial cDNAs encoding the region from amino acid 247 to the C-terminal ends were inserted into pGEX-5X-3 (Amersham Pharmacia). The C-terminal segments of NELF-E were expressed as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli* and purified by glutathione-Sepharose chromatography as recommended by the manufacturer (Amersham Pharmacia).

Purification of Flag-NELF. HeLa S3 cells were maintained in minimal essential medium supplemented with 10% fetal calf serum and 0.03% L-glutamate. pCMV-Flag-E (10 μg) and pSV2-neo (0.5 μg) were transfected into 2×10^6 HeLa cells using the standard calcium phosphate method, and the cells were cultured for 2 weeks in the presence of Geneticin (500 μg/ml; Invitrogen). Drug-resistant clones were isolated, and cell lines expressing Flag-NELF-E (HeLa/Flag-E) were selected by immunoblotting with anti-Flag M2 (Sigma). Clone 15, which expressed Flag-E in a fewfold excess of endogenous E, was expanded further and adapted for cultivation in a large spinner apparatus in the presence of Geneticin (100 μg/ml). The whole-cell extracts were prepared from a 30-liter culture (~2×10^{10} cells). Cells were harvested by centrifugation at 2,000 × *g* at 4°C and washed twice with phosphate-buffered saline. All the subsequent steps were carried out at 4°C. Cell pellets were resuspended in 250 ml of high-salt buffer (50 mM Tris-HCl [pH 7.9], 500 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride) and homogenized with a Dounce homogenizer. After a brief sonication, the cell extracts were cleared by centrifugation at 13,600 × *g* twice for 20 min each and filtration through a 0.45-μm-pore-size filter. A 40-ml aliquot of the extracts was loaded onto a 1-ml anti-Flag M2 affinity column (Sigma) at a flow rate of 0.4 ml/h. Flowthrough fraction 1 (FT 1) was reapplied to the column, and FT 2 was saved. The column was washed twice with 10 ml of high-salt buffer (washes 1 and 2) and once with 5 ml of HGE.1 (20 mM HEPES [pH 7.9], 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 100 mM KCl) (wash 3). The flow was stopped, the resin was resuspended with 1 ml of HGE.1 containing 100 μg of Flag peptide (Sigma), and a 5-min incubation was followed by collection of the eluate (eluate 1). This was repeated four more times (eluates 2 to 5).

Protein samples, either purified Flag-NELF (2 ml) or crude HeLa NE (100 μl), were applied to a Mono Q PC1.6/5 column (Amersham Pharmacia) equilibrated with HGE.1. The column was washed with the same buffer, and bound proteins were eluted with a 1.0-ml linear gradient from 100 to 500 mM KCl.

Immunological techniques. The following antibodies were used for carrying out the immunological techniques depicted in Fig. 2, 3, and 6: a rat monoclonal antibody against DSIF p160 (27), a rat polyclonal antibody against NELF-E (31), a mouse monoclonal antibody against NELF-A (33), and a mouse monoclonal antibody against the RNAPII CTD (clone 8WG16; Babco). For the technique depicted in Fig. 5, mouse monoclonal antibodies against NELF-E were used. These antibodies, directed against full-length NELF-E protein produced in *E. coli*, were prepared essentially as described (6). The antibodies were highly reactive to NELF-E but differed in their cross-reactivities to other proteins in HeLa NE, suggesting that they recognize different parts of NELF-E. Immunodepletion of NELF, DSIF, and RNAPII from HeLa NE was performed essentially as described (31). Immunodepletion was repeated a few times until the proteins of interest decreased substantially. Immunoprecipitation and immunoblotting were performed as described (27, 31). For immunoprecipitation with anti-Flag and anti-RNAPII CTD, proteins bound to antibody-immobilized matrices were eluted with Flag peptide at a concentration of 0.1 mg/ml or by boiling in the presence of 1% sodium dodecyl sulfate (SDS), respectively.

Electrophoretic mobility shift assays (EMSA). The RNA probes used in this study are summarized below (see Fig. 4D). For the CstF-64-binding site (25) and the SF2/ASF-binding site (B1) probes (24), synthetic oligonucleotides were annealed to make partially single-stranded templates containing a T7 promoter sequence. For the G-free cassette and the HIV TAR probes, transcription templates were generated by PCR using p(C_2AT)19 (23) or pHIV-LTR (13) as a template and appropriate pairs of primers, one of which contained a T7 promoter sequence. Probes were synthesized from these templates using T7 RNA polymerase and [α-^{32}P]UTP. The HDV RNA probe was synthesized from *Eco*RI-linearized pHS103 (3) using T3 RNA polymerase and [α-^{32}P]UTP. Transcription reactions were incubated further in folding buffer [50 mM 3-(cyclohexylamino)-2-hydroxyl-1-propanesulfonic acid (pH 8.6), 0.5 M NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA] for 2 h at 60°C and processed as described (3). Indicated amounts of protein factors and probes (~0.1 pmol) were incubated in 10 μl of buffer containing 12 mM HEPES (pH 7.9), 12% glycerol, 0.12 mM EDTA, 0.3 mM dithiothreitol, 60 mM KCl, 1 μg of yeast tRNA, and 5 μg of bovine serum albumin for 30 min at 30°C. Reactions were analyzed by electrophoresis on 4% native polyacrylamide gels using 0.5× Tris-borate-EDTA as a running buffer at 4°C.

RESULTS

Immunoaffinity purification of epitope-tagged NELF. Previously, NELF was purified from HeLa NE by a series of column chromatography procedures (31). To obtain a sufficient quantity of NELF for further biochemical study, we sought to purify the multiprotein complex by immunoaffinity chromatography using epitope-tagged NELF-E (Flag-E). HeLa derivative cell lines expressing Flag-E were established, and whole-cell extracts were prepared from three Flag-E-expressing cell lines (clones 3, 15, and 17) and a Flag-E-negative cell line. The extracts were incubated with anti-Flag Sepharose, and after extensive washing with a buffer containing 500 mM KCl and 1% NP-40, bound proteins were eluted with the Flag peptide (Fig. 1A). Eluate fractions contained an ~48-kDa polypeptide

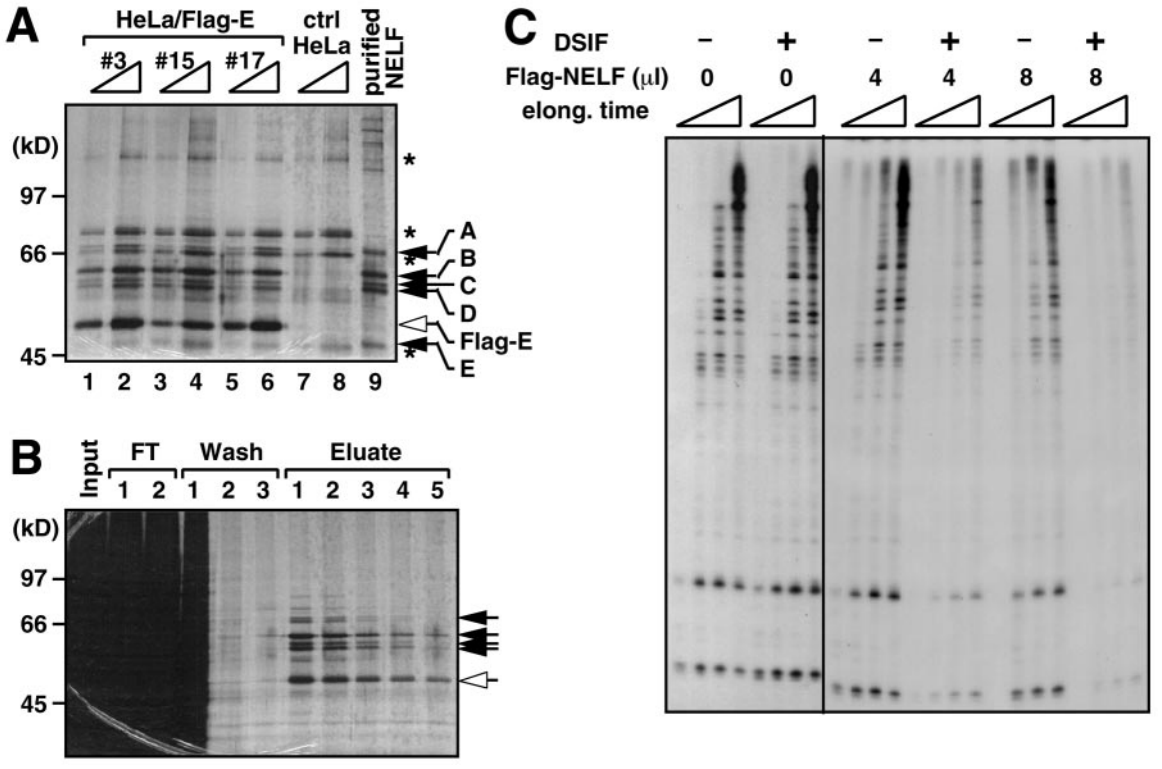

FIG. 1. Immunoaffinity purification of Flag-NELF. (A) Whole-cell extracts were prepared from HeLa-derivative cell lines expressing Flag-E (3, 15, and 17) or not (ctrl) and subjected to batchwise purification using anti-Flag Sepharose. Eluate fractions (5 and 20 μl) were analyzed by SDS-PAGE and silver staining. Lane 9 shows 2 μl of native NELF on a Mono Q column (31). Filled arrows indicate the positions of the NELF subunits, whereas the open arrow indicates the position of Flag-E. Asterisks denote the positions of nonspecific bands that appeared in the control eluate fraction. (B) Large-scale purification of Flag-NELF. Whole-cell extracts prepared from a 30-liter culture of clone 15 were subjected to immunoaffinity chromatography as described in Materials and Methods. Each fraction (4 μl) was analyzed by SDS-PAGE and silver staining. (C) NELF activity of Flag-NELF. Purified RNAPII and dC-tailed template were preincubated with or without DSIF (a mixture of recombinant p160/hSpt5 and p14/hSpt4) and Flag-NELF (eluate 1). Nucleoside triphosphates were added, and elongation (elong.) was allowed to proceed for 2, 4, 8, and 16 min. Two bands close to the bottom of the gel correspond to the transcripts of ~50 and ~70 nt.

corresponding to Flag-E and four additional polypeptides with higher molecular masses. The four polypeptides showed mobility indistinguishable from those of NELF-A, -B, -C, and -D (Fig. 1A, lane 9), and the largest one was indeed recognized by an anti-NELF-A antibody (data not shown). Therefore, Flag-E appears to form a stable NELF-like complex within the cells.

This complex, termed Flag-NELF, was examined for NELF activity using a dC-tailed template. This template is efficiently transcribed by pure RNAPII without additional factors and is useful for assaying the activity of transcription elongation factors. To do this assay, Flag-NELF was purified on a large scale as described in Materials and Methods. As a result, some of the nonspecific bands observed in Fig. 1A were eliminated, and almost-pure preparations of Flag-NELF were obtained (Fig. 1B). Simultaneous addition of DSIF and Flag-NELF to reaction mixtures containing RNAPII and the dC-tailed template strongly repressed RNAPII elongation (Fig. 1C). Flag-NELF alone weakly repressed RNAPII elongation reproducibly, whereas DSIF alone had little effect on the reactions. These results are similar to those obtained previously using native NELF (31), indicating that Flag-NELF is active in vitro.

Identification of a NELF subcomplex. As shown in Fig. 1A, NELF-B and Flag-E were stained by silver more intensely than NELF-A, -C, and -D. This prompted us to ask whether the fractions contained subcomplexes of Flag-NELF. Flag-NELF proteins were allowed to bind to a Mono Q column and were then eluted with a linear gradient from 100 to 500 mM KCl (Fig. 2A). This resulted in the resolution of the Flag-NELF complexes: Fractions peaking at ~180 mM KCl contained only NELF-B and Flag-E (peak 1), whereas fractions peaking at ~340 mM KCl contained all the polypeptides (peak 2). In addition, we noted a small difference in the elution profiles of NELF-C and NELF-D at peak 2. Preliminary microsequencing data indicate that NELF-C and NELF-D are closely related proteins (Y. Yamaguchi and H. Handa, unpublished data). It is therefore possible that holo-NELF is a four-subunit complex composed of A-B-C-E or A-B-D-E, with peak 2 being a mixture of these complexes. However, because it is difficult to resolve the putative related complexes, we do not pursue this issue in the present work.

The activities of these fractions were then compared using a crude transcription system. HeLa NE immunodepletion was performed using an anti-NELF-E antibody, and this procedure eliminated NELF-A as well as NELF-E from the extracts (Fig. 2B). In normal HeLa NE, 50 μM DRB strongly inhibits the synthesis of 380- and 270-nucleotide

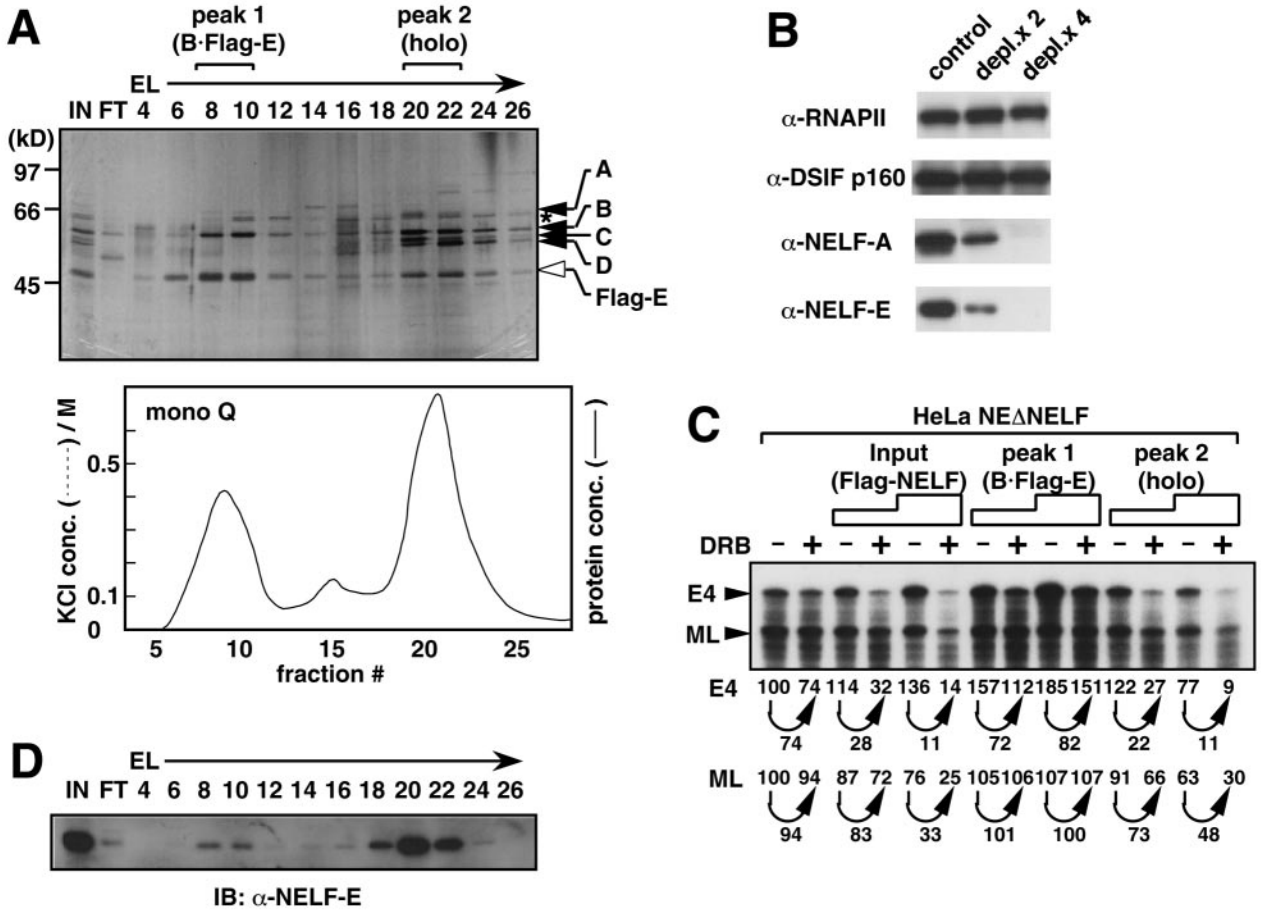

FIG. 2. Identification of the B-E subcomplex of NELF. (A) Chromatography of Flag-NELF on a Mono Q column revealed the presence of the B-E subcomplex. Each fraction (2 μl) on the Mono Q column was analyzed by SDS-PAGE and silver staining. The fractions at ~180 mM KCl (peak 1) contained only NELF-B and Flag-E, whereas the fractions at ~340 mM KCl (peak 2) contained all five polypeptides. (B) Immunodepletion of NELF from HeLa NE. HeLa NE were repeatedly passed over anti-NELF-E immobilized protein G-Sepharose as described (31). The protein composition of the flowthrough was analyzed by immunoblotting with the indicated antibodies. (C) NELF activity of the B-E subcomplex. Mono Q input, peak 1, and peak 2 fractions (0.5 and 2 μl) were assayed for NELF activity using NEΔNELF. The first two lanes represent the activity of NEΔNELF with no NELF complex added back in. Transcription reactions were performed with or without 50 μM DRB. Arrows indicate 380- and 270-nt G-free transcripts synthesized from the adenovirus E4 and ML promoters. Digital images were collected by scanning the film, and band intensities were measured using NIH Image software. The intensities in lane 1 are arbitrarily expressed as 100. (D) Chromatography of HeLa NE on the Mono Q column revealed the presence of the B-E subcomplex in the crude extracts. Each fraction (2 μl) was analyzed by immunoblotting (IB) with anti-NELF-E (α-NELF-E). Abbreviations: EL, eluate fraction; IN, input.

(nt) G-free transcripts from the adenovirus E4 and major-late (ML) promoters (27). DRB exerts its effect by inhibiting endogenous P-TEFb that would otherwise reverse the negative effect of DSIF/NELF on transcription. Consistent with the previous report (31), DRB did not substantially affect transcription in NE immunodepleted of NELF (NEΔNELF) (Fig. 2C, lanes 1 and 2). Add-back of purified NELF results in the recovery of DRB inhibition (31). Similarly, addition of Flag-NELF or the peak 2 fraction to NEΔNELF strongly repressed transcription in the presence of DRB but not in its absence, thereby restoring DRB sensitivity (Fig. 2C). In contrast, the peak 1 fraction did not restore DRB sensitivity but slightly increased overall transcription. The weak activation by the B-E subcomplex may result from its dominant-negative effect on the trace amount of endogenous NELF that remained in NEΔNELF. Taken together, peak 2 has transcription repression activity similar to that of the input fraction, whereas peak 1 has no or little activity, suggesting that not only NELF-B and NELF-E but also NELF-A and NELF-C/D are required for transcription repression. Transcription from the ML promoter was less sensitive and required higher doses of NELF for repression (Fig. 3C). The differential effect is not due to the nature of the promoters but reflects primarily the difference in transcript length between the templates.

The B-E subcomplex may exist in normal cells or, alternatively, may arise from Flag-E overexpression. To distinguish these possibilities, normal NE were directly subjected to Mono Q column chromatography, and eluate fractions were analyzed for the presence of NELF-E by immunoblotting (Fig. 2D). A small amount of NELF-E was found in fractions 8 to 10 and was thus thought to be associated with NELF-B alone. Therefore, the B-E subcomplex exists in HeLa cells, although its functional significance, if any, is unclear.

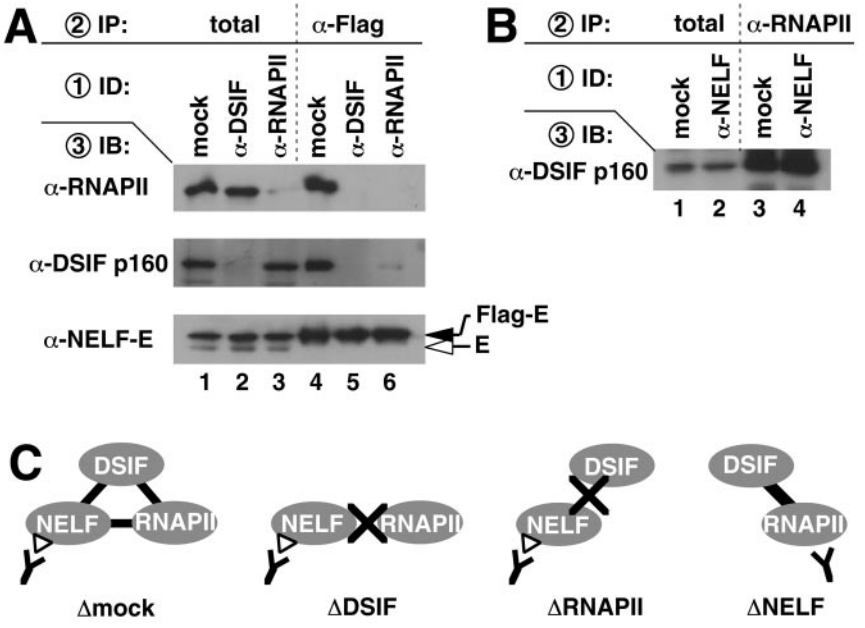

FIG. 3. NELF binds to the preformed DSIF/RNAPII complex. NE prepared from HeLa/Flag-E cells (A) or normal HeLa cells (B) were subjected to immunodepletion (ID), immunoprecipitation (IP), and immunoblotting (IB) with the indicated antibodies. See text for details. Lanes below the label "total" represent 2% of the untreated or immunodepleted NE used. (C) Schematic representation of results in panels A and B. Thick bars and Xs indicate the presence or absence of interactions. Ys and triangles indicate antibodies and Flag epitopes.

NELF binds to the preformed DSIF/RNAPII complex. NELF is thought to interact with DSIF and RNAPII, based on the observation that, under mild conditions (100 mM KCl and 0.1% NP-40), Flag-NELF coimmunoprecipitates with DSIF and RNAPII from HeLa cells expressing Flag-E (31) (Fig. 3A, lanes 1 and 4). However, the structural basis for the complex formation remains elusive. The coimmunoprecipitation does not necessarily indicate the formation of a ternary complex, because distinct fractions of NELF may be responsible for DSIF and RNAPII binding. Alternatively, NELF may bind only to DSIF or RNAPII directly, with the other being associated indirectly through the DSIF-RNAPII interaction.

To distinguish these possibilities, we took the following strategy. DSIF and RNAPII were first immunodepleted from HeLa NE expressing Flag-E. These procedures removed most of the DSIF or RNAPII proteins from the NE without substantially affecting the levels of the other proteins examined (Fig. 3A, lanes 1 to 3). Flag-NELF was then immunoprecipitated from the depleted extracts under mild conditions, and the presence of DSIF and RNAPII in the precipitates was examined by immunoblotting. To our surprise, prior removal of RNAPII markedly reduced the interaction between NELF and DSIF, and prior removal of DSIF similarly reduced the interaction between NELF and RNAPII (Fig. 3A, lanes 4 to 6). These results indicate that NELF binds to the preformed DSIF/RNAPII complex but not to either DSIF or RNAPII alone. NELF-DSIF and NELF-RNAPII interactions may be weak individually but stable together. Alternatively, the DSIF-RNAPII interaction may allosterically induce the incorporation of NELF.

We also examined a possible effect of NELF on the DSIF-RNAPII interaction (Fig. 3B). RNAPII was immunoprecipitated from either mock- or NELF-depleted extracts, and the presence of DSIF in the precipitates was examined. Prior removal of NELF did not substantially affect the amounts of DSIF associated with RNAPII. Therefore, the DSIF-RNAPII interaction does not appear to be regulated by NELF. Note that the immunoprecipitation data do not conflict with the immunodepletion data. Only a few to 10% of these proteins are associated with each other in the extracts as estimated from the immunoprecipitation data. Therefore, immunodepletion of one protein does not cause coimmunodepletion of the other proteins but leaves significant fractions of the other proteins in the supernatant.

NELF-E has RNA-binding activity distinct from that of CstF-64. We were next interested in the putative RRM encoded by NELF-E. To characterize this motif, we generated three RRM mutants: a mutant lacking the entire RRM (Del), a mutant carrying four point mutations within the conserved RNP1 motif (PM), and a mutant having an RRM derived from a 64-kDa subunit of the cleavage stimulation factor (CstF-64) in place of the natural RRM (Swap). The C-terminal fragments of wild-type (WT) and mutant NELF-E were expressed as GST fusion proteins [GST-E(C)] (Fig. 4A), and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 4B). PM showed a lower mobility than that of WT. The RNA-binding activity of the purified proteins was then examined by EMSA (Fig. 4C). Five RNA species were tested as a first step (Fig. 4D): a binding site for the CstF-64 RRM (25), a binding site for RRMs encoded by the splicing factor ASF/SF2 (24), an HDV antigenomic fragment with a rod-like structure that serves as a template for RNAPII transcription (3), an initially transcribed sequence of the G-free cassette (23), and an initially transcribed sequence of HIV or the TAR (9). GST-E(C)

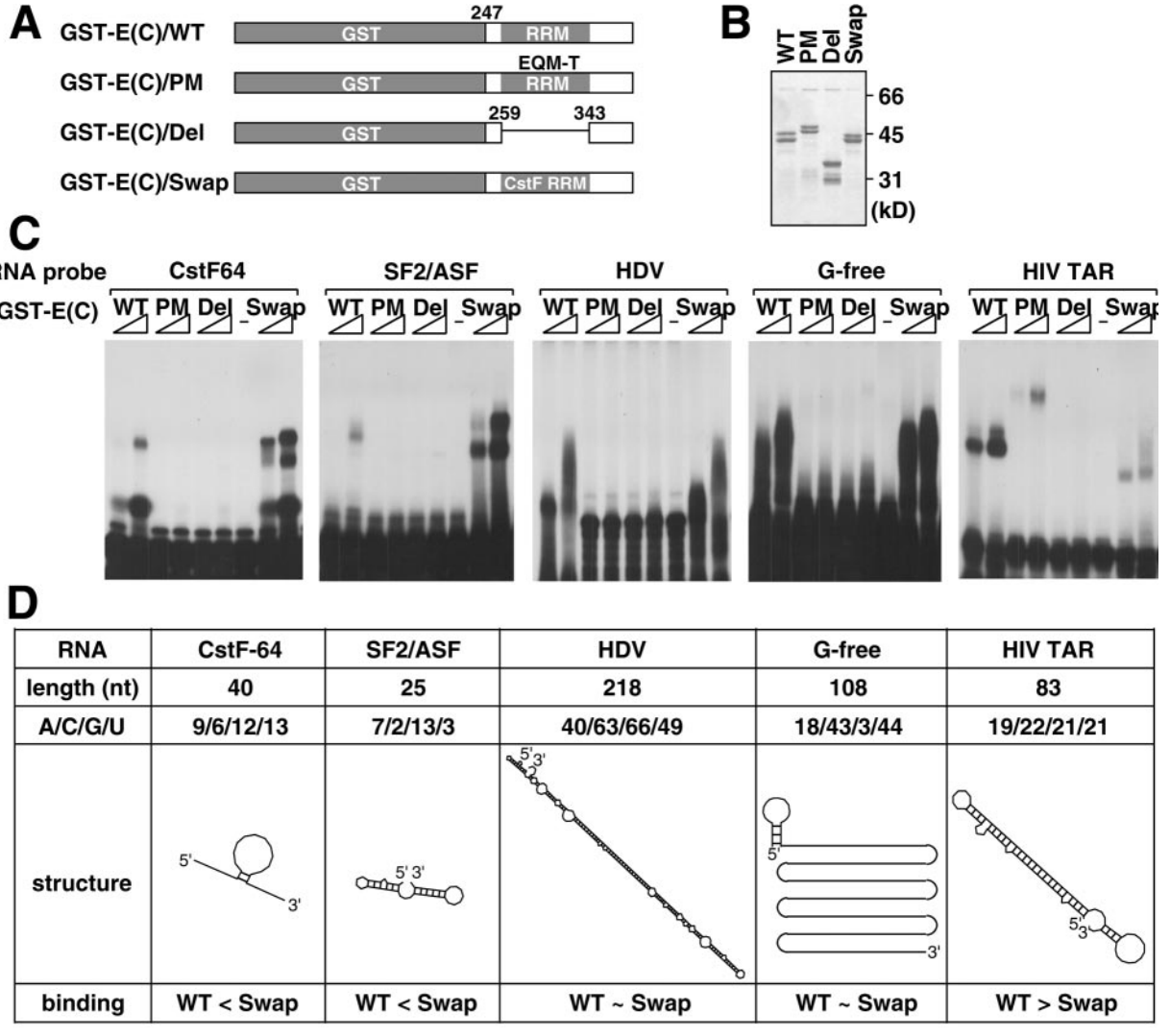

FIG. 4. NELF-E binds to various RNAs through the RRM. (A) Schematic structure of GST fusion proteins used for EMSA. The C-terminal segments of WT and mutant NELF-E were expressed as GST fusion proteins. (B) Purified GST-E(C) proteins (~300 ng) analyzed by SDS-PAGE and Coomassie blue staining. (C) GST-E(C) proteins (100 and 300 ng) were incubated with various labeled RNAs (~0.1 pmol) for 30 min at 30°C. Reactions were analyzed by electrophoresis on 4% native polyacrylamide gels at 4°C. (D) Sequence and structure of RNA probes used in panel C. Secondary structures were predicted using the program *mfold* with default settings (37).

PM and Del had little or no RNA-binding activity, whereas WT and Swap showed various degrees of RNA binding (Fig. 4C). Swap bound to the CstF-64- and the SF2/ASF-binding sites more strongly than WT. While these proteins bound equally to the HDV and the G-free probes, WT bound to the HIV TAR more strongly than Swap. Swap showed a pattern of RNA binding indistinguishable from that of the CstF-64 RRM fused to GST (data not shown). Taken together, the C-terminal fragment of NELF-E bound to various RNAs in a manner sensitive to mutations within the putative RRM, suggesting that it constitutes a bona fide RNA-binding domain. In addition, the RRMs of NELF-E and CstF-64 appear to have distinct RNA-binding specificity.

Some RRMs are known to bind to single-stranded DNA. To determine if NELF-E has such an activity, EMSA were performed using single- or double-stranded DNAs containing either the CstF-64-binding site or the TAR sequence as probes (data not shown). The Swap mutant strongly bound to the single-stranded CstF-64-binding site probe, indicating that the CstF-64 RRM does not discriminate DNA and RNA well. In contrast, the other proteins, including WT, did not substantially bind to any of these probes. Therefore, the NELF-E RRM seems to have a strong preference for RNA over DNA.

NELF complex binds to RNA. We then asked whether NELF binds to RNA as a complex. Due to the low protein concentration of Flag-NELF, we only assayed 6 μl of Flag-NELF containing 30 ng of Flag-E and compared it with various amounts of GST-E(C) WT (10 to 1,000 ng) (Fig. 5A). The amount of these proteins was verified by SDS-PAGE and Coomassie blue staining (Fig. 5B). When the HIV TAR probe was used, Flag-NELF produced a slower-migrating complex, with an apparent affinity similar to that of the recombinant RRM

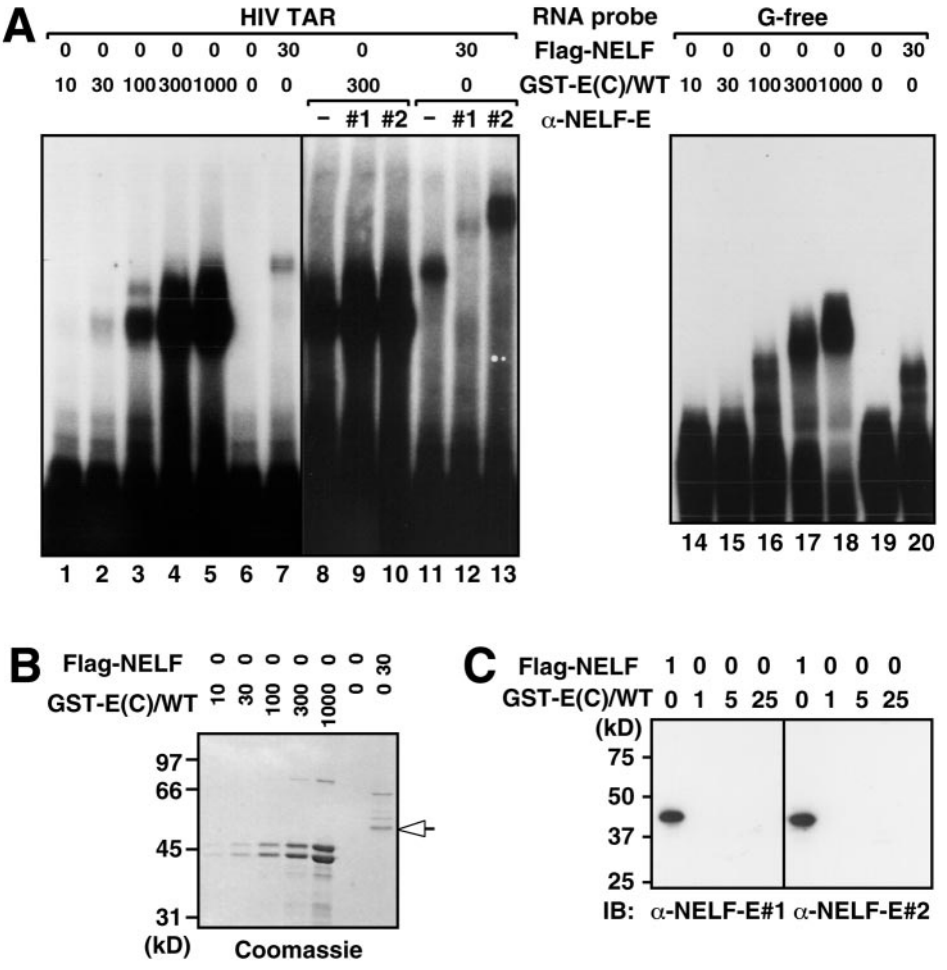

FIG. 5. NELF complex binds to RNA. (A) The indicated amounts (in nanograms) of GST-E(C) WT or 6 μl of Flag-NELF containing 30 ng of Flag-E were incubated with either the HIV TAR or the G-free RNA probe (~0.1 pmol) with or without one of two different anti-NELF-E monoclonal antibodies (1 μg) for 30 min at 30°C. Reactions were analyzed by electrophoresis on 4% native polyacrylamide gels at 4°C. In lanes 8 to 13, the film was exposed for a longer time. (B and C) The same protein samples containing indicated amounts (in nanograms) of GST-E(C) WT or Flag-E were analyzed by SDS-PAGE and either Coomassie blue staining (B) or immunoblotting (C) with the anti-NELF-E antibodies used in panel A.

(Fig. 5A, lanes 1 to 7). To confirm the identity of the novel complex, two monoclonal antibodies against NELF-E were included in the reaction mixtures. These antibodies, both of which recognized full-length NELF-E but not the C-terminal fragment (Fig. 5C), selectively caused supershifts of the slower-migrating band (Fig. 5A, lanes 8 to 13). On the other hand, when the G-free probe was used, Flag-NELF as well as the low doses of GST-E(C) produced only a smear (Fig. 5A, lanes 14 to 20), suggesting that these protein-RNA complexes tend to be dissociated during electrophoresis. Taking these results together, it is likely that the NELF complex binds to RNA with an affinity and specificity similar to those of NELF-E.

A role for the NELF-E RRM in transcription repression. To investigate the functional significance of the NELF-E RRM, we sought to isolate and characterize NELF complexes containing mutant NELF-E. HeLa-derivative cell lines expressing various Flag-E mutants (Fig. 6A) were established, and whole-cell extracts were prepared and subjected to immunoaffinity chromatography under stringent conditions. As shown in the silver-stained gel (Fig. 6B), the eluate fractions contained mutant Flag-E of various sizes and, in addition, the four polypeptides NELF-A to -D with similar stoichiometry. When NE were prepared instead and subjected to immunoaffinity chromatography under mild conditions, the Flag-NELF mutants as well as WT were found to be associated with DSIF and RNAPII (Fig. 6C). On the basis of these results, the NELF-E RRM is likely dispensable for NELF complex formation and for its interactions with DSIF and RNAPII.

We then asked whether the NELF-E RRM is involved in transcription repression. Add-back of Flag-NELF mutants to NELF-depleted NE reproducibly caused diverse effects on transcription in the presence of DRB (Fig. 6D). Specifically, Del had no detectable effect on transcription, whereas PM repressed transcription weakly. Swap showed repression activity slightly higher than that of PM but substantially lower than that of WT, suggesting that the CstF-64 RRM partially complemented the functional defects associated with those RRM mutations. In the absence of DRB, WT and mutant Flag-

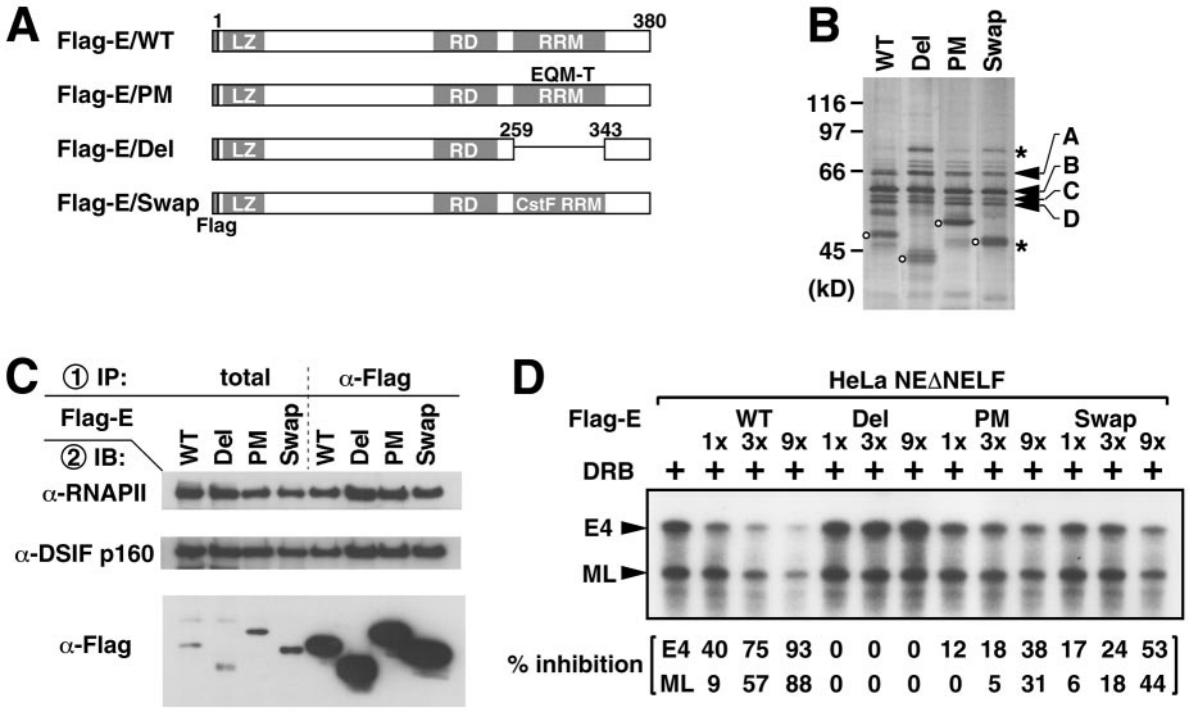

FIG. 6. Isolation and characterization of NELF complexes containing RRM mutant NELF-E. (A) Schematic structure of WT and mutant Flag-E. LZ, leucine zipper motif. (B) Flag-NELF containing mutant Flag-E. The whole-cell extracts were prepared from HeLa-derivative cell lines expressing mutant Flag-E and subjected to immunoaffinity purification under stringent conditions. Aliquots of eluate fractions (2 to 8 μl) were analyzed by SDS-PAGE and silver staining. (C) Interactions of Flag-NELF mutants with DSIF and RNAPII. NE were prepared from the HeLa-derivative cell lines and subjected to immunoaffinity purification (IP) under mild conditions. Eluate fractions were then analyzed by immunoblotting (IB) with the indicated antibodies. Lanes below the label "total" represent 2% of the NE used. (D) Purified Flag-NELF mutants were assayed for NELF activity using HeLa NE immunodepleted of NELF in the presence of 50 μM DRB. 1×, 3×, and 9× correspond to a ninth, a third, and one part of the amounts shown in panel B. The intensities of the bands were quantified using the STORM image analyzer (Molecular Dynamics).

NELF had little effect on transcription (data not shown). These results essentially conform to the idea that RNA binding by NELF is important for its repressive function.

DISCUSSION

To overcome the difficulty associated with purification of the multisubunit factor NELF by conventional column chromatography, we have developed an effective method using an epitope-tagging technique. Using single-step chromatography, we were able to obtain a large quantity of Flag-NELF that contained all the previously identified polypeptides and was capable of repressing RNAPII elongation in conjunction with DSIF. Using this approach, we were also able to prepare and assay mutant NELF complexes for structure-function analysis. Thus, the method developed here has proven useful for elucidating the mechanism of action of NELF, and will be used until all the NELF subunits are cloned, expressed, and assembled in an active form.

NELF is a structure that is composed of five polypeptides. As mentioned above, NELF-C and NELF-D are related proteins, and NELF is likely a heterotetramer composed of subunits A, B, either C or D, and E. We propose that these polypeptides exist largely in the context of a NELF complex within cells, based on two complementary observations. First, under stringent conditions, Flag-E coimmunoprecipitated exclusively with near stoichiometric amounts of NELF-A, -B, and -C or -D (Fig. 1). This makes it unlikely that NELF-E exists in the context of other protein complexes. Second, immunodepletion with anti-NELF-E eliminated not only NELF-E but also NELF-A from HeLa NE (Fig. 2). This indicates that most molecules of endogenous NELF-A are stably associated with NELF-E.

The B-E subcomplex is an unexpected finding. This inactive complex may regulate NELF activity by acting in a dominant-negative fashion or, alternatively, may be an intermediary form during NELF complex assembly. Regardless of its physiological role, the present data demonstrate that NELF-B interacts directly with NELF-E, and that not only NELF-B and NELF-E but also NELF-A and NELF-C/D are required for NELF function. Sequence similarity between NELF-A and HDAg, the HDV protein that binds RNAPII and activates transcription elongation (33), suggests the intriguing idea that NELF-A may mediate the interaction between NELF and RNAPII, thereby being required for its function.

The results of Fig. 3 demonstrate that the interaction between DSIF and RNAPII plays a critical role during the assembly of the DSIF/NELF/RNAPII complex. In other words, DSIF creates a scaffold for NELF by binding to RNAPII. This predicts that NELF localizes to chromosomal regions occupied

by both DSIF and RNAPIIa. Immunostaining of polytene chromosomes in *Drosophila* shows results that are essentially consistent with this prediction (C.-H. Wu, Y. Yamaguchi, H. Tang, H. Handa, and D. S. Gilmour, unpublished data), suggesting that the stepwise assembly of the DSIF/NELF/RNAPII complex also occurs in vivo.

We have shown that the putative RRM encoded by NELF-E is functional and capable of binding to various RNAs. RNA is not a structural component of the NELF complex, because purified Flag-NELF did not contain distinct RNA species and because the integrity of NELF was not affected by an RNase treatment (data not shown). Likely physiological targets for NELF are nascent transcripts synthesized by RNAPII. Consistent with this, the three RNA species that were shown here to bind to NELF-E are all transcribed by RNAPII in vivo and subject to NELF repression in vitro. It remains elusive what elements or structures are recognized by the NELF-E RRM. NELF-E strongly bound to the HDV and the HIV TAR probes, both of which are predicted to form long stems with bulges and loops, whereas it also bound to the G-free probe, which does not form a stable higher-order structure (Fig. 4D). Probably, NELF-E recognizes the 2′-OH groups of RNA, because the RRM discriminated RNA from DNA.

We propose that the NELF-E binding to RNA is important for transcription repression by NELF. This is based on the observation that the RRM mutations of Del and PM, which abolished RNA-binding activity, strongly impaired transcription repression without affecting known protein-protein interactions (Fig. 6). A question then arises from the results of Fig. 6D: why didn't the CstF-64 RRM fully complement the functional defects associated with those RRM mutations? Three alternative explanations are possible. (i) The NELF-E RRM, but not the CstF-64 RRM, encodes an important function other than RNA binding. (ii) Sequence-specific RNA binding by NELF is important for transcription repression, and substitution did not work because RNA-binding specificity is different between NELF-E and CstF-64. (iii) Substitution did not work simply because some structural problem prevented the chimeric protein from working in the context of the NELF complex. Among these possibilities, the second seems less likely, because WT and Swap, which bound to the G-free RNA with similar affinity, showed different abilities in repressing transcription of the G-free cassette (Fig. 4 and 6). We speculate that transcription repression by NELF in vitro involves rather indiscriminate binding to nascent transcripts because NELF can strongly repress transcription of several templates encoding various sequences. To study this point further, perhaps one may need to examine the effect of nascent transcripts on NELF-dependent transcription repression or, in other words, to quantify the level of transcription with or without growing transcripts.

Increasing evidence suggests that the regulatory system involving DSIF, NELF, and P-TEFb plays a critical role in the control of RNAPII elongation in response to various extracellular stimuli and during development. Examples include Tat-dependent transcription of HIV (13, 35; reviewed in references 4 and 11), HDAg-dependent replication and transcription of HDV (33), heat shock-dependent transcription of *Drosophila hsp* genes (1, 10, 15), and neuronal development of zebra fish embryos (5; reviewed in references 34 and 36). In some cases, P-TEFb is recruited to the gene loci by known and unknown mechanisms, resulting in transcription activation in response to the corresponding signals. In contrast, it is largely unknown whether, and how, DSIF and NELF are controlled. Regulation of DSIF and NELF may be achieved by their association with *trans*-acting factors or *cis*-acting RNA elements. In particular, sequence-specific interactions between the NELF-E RRM and nascent transcripts may facilitate the formation of DSIF/NELF/RNAPII complexes in a gene-specific manner. Strong NELF-E binding to the HIV TAR and the HDV RNA is potentially interesting and may be relevant to the growth regulation of these pathogenic viruses. Consistent with this idea, our preliminary data suggest that NELF is associated with HDV genomic RNA in HDV-positive human cells (Yamaguchi and Handa, unpublished data).

In this work, we have described (i) the establishment of an effective method for the purification of NELF, (ii) analysis for the composition of NELF subunits, (iii) analysis for interactions among DSIF, NELF, and RNAPII, and (iv) identification and initial characterization of RNA binding by NELF. The new findings greatly advance our understanding of the control mechanisms for RNAPII elongation. On the other hand, it remains to be determined what elements or structures of RNA are recognized by the NELF-E RRM and whether specific RNA binding is involved in NELF-dependent transcription repression. These are interesting questions that will need to be addressed in the future.

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