# Molecular characterization of *Drosophila* NELF

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## ABSTRACT

**NELF and DSIF act together to inhibit transcription elongation *in vitro*, and are implicated in causing promoter proximal pausing on the *hsp70* gene in *Drosophila*. Here, further characterization of *Drosophila* NELF is provided. *Drosophila* NELF has four subunits similar to subunits of human NELF. The amino acid sequences of NELF-B and NELF-D are highly conserved throughout their lengths, while NELF-A and NELF-E contain nonconserved regions inserted between conserved N- and C-terminal regions. Immunodepletion of NELF or DSIF from a nuclear extract desensitizes transcription *in vitro* to DRB. Immunodepletion of NELF also impairs promoter proximal pausing on the *hsp70* promoter *in vitro* without affecting initiation. Chromatin immunoprecipitation analyses detect NELF at the promoters of the *hsp70* and *β1-tubulin* genes where promoter proximal pausing has been previously detected. Heat shock induction of *hsp70* results in a marked decrease in NELF at the *hsp70* promoter. Immunofluorescence analysis of polytene chromosomes shows extensive colocalization of the NELF-B and NELF-D subunits at hundreds of interbands. Neither subunit appears to be recruited to puffs. These results provide a foundation for genetic and biochemical analysis of NELF in *Drosophila*.**

## INTRODUCTION

NELF and DSIF are two proteins that regulate transcription elongation by Pol II. They were first discovered in human cells during an investigation aimed at understanding how the nucleoside analog, DRB, inhibits transcription elongation (1–3).

Biochemical analyses indicated that NELF and DSIF act together to inhibit elongation by Pol II (3). Hyperphosphorylation of Pol II by the kinase, P-TEFb, appears to alleviate this inhibition (2,4). Since DRB is a potent inhibitor of P-TEFb, it has been proposed that DRB blocks phosphorylation of Pol II, thus allowing DSIF and NELF to inhibit elongation (5).

The opposing actions of P-TEFb and the combination of NELF and DSIF could serve as an important mechanism for transcriptional control. P-TEFb associates with several genes during activation, and direct interaction between P-TEFb and transcriptional activators has been observed (6–9). A fusion protein composed of P-TEFb and the Gal4 DNA-binding domain was observed to stimulate transcription of a test promoter containing binding sites for Gal4 (6). We recently showed that NELF and DSIF cause Pol II to pause in the promoter proximal region of the *hsp70* gene in *Drosophila* under normal growth conditions (10). Heat shock induction results in rapid association of P-TEFb with *hsp70* (11). NELF but not DSIF appears to dissociate from the elongation complex during heat shock induction (10). In contrast to *hsp70*, NELF was found to be recruited to estrogen-stimulated genes where it attenuates the level of induction achieved by the addition of estrogen (12). Thus, studies so far indicate that NELF may play different roles on different genes.

Much remains to be learned about the roles of P-TEFb, NELF and DSIF. Although early studies indicated that hyperphosphorylation of the CTD of Pol II might be sufficient to overcome inhibition by NELF and DSIF (3), more recent results indicate that phosphorylation of NELF and DSIF may also be involved (13–15). Flavopiridol, a potent inhibitor of P-TEFb, did not significantly diminish the level of Pol II associated with the *hsp70* gene after heat shock induction as would be expected if phosphorylation by P-TEFb was responsible for releasing paused Pol II (16). Biochemical data indicated that NELF and DSIF might provide a checkpoint during early elongation that ensures proper capping of nascent transcripts (17). The broad and overlapping distributions of NELF

and DSIF observed on polytene chromosomes are consistent with these proteins affecting transcription of many genes (10).

Although DSIF and P-TEFb have homologs in eukaryotes ranging from yeast to human, no homologs of the four subunits of NELF identified in humans are evident in model organisms such as yeast or *Caenorhabditis elegans* (18). Thus, the regulatory potential provided by NELF could be restricted to a subset of eukaryotes. Our previous work focused on NELF-D and NELF-E from *Drosophila* and its role in promoter proximal pausing on the *hsp70* gene (10). Here, we report on the characterization of the entire NELF complex from *Drosophila*, and provide a foundation for further study of this protein in *Drosophila*.

## MATERIALS AND METHODS

### Preparation of NELF-A and NELF-B antibodies

NELF-A and NELF-B subunits were identified through BLAST searches of the *Drosophila* genome using the sequences of human NELF subunits. dNELF-A has the gene identification CG5874 and dNELF-B has the gene identification CG32721. Two EST cDNA clones, SD09448 (NELF-A) and GH10333 (NELF-B) were obtained from the Berkeley *Drosophila* Genome Project.

NELF-A is predicted to encode a 1248 amino acid polypeptide. The region of cDNA clone SD09448 encoding amino acids 1150–1248 was amplified with the following primers: 5′-CGCGGATCCCGTGGACTCTCTCTATCGAA and 5′-CCGGAATTCGCGTATGACCCTTGTGGA. The resulting DNA fragment was digested with NheI and HindIII and subcloned into HindIII/NheI cut pET28a(+)vector (Novagen). His-tagged NELF-A protein was expressed in BL21(DE3) cells and purified with a HIS-Select™ Cartridge (Sigma) in the presence of urea. The isolated protein was dialyzed into 15 mM Tris–Cl, pH 8.0 and used to raise antibodies in guinea pigs (Pocono Rabbit Farm & Laboratory).

NELF-B is predicted to encode a 594 amino acid polypeptide. A primer set, NELF-B (5′)-5′-CGCAATCCAT-ATGATAATGAGCACACCGGCGAAA and NELF-B (3′-1)-5′-TCGAAGCTTCTATTGGACATTATAGTGC, was used to amplify the region of GH10333 encoding full-length NELF-B. The amplified DNA fragment was digested with NdeI and HindIII and subcloned into HindIII/NdeI cut pET28a(+)vector. His-tagged NELF-B protein was expressed in BL21(DE3) cells and purified in the presence of urea with nickel-NTA agarose (Qiagen). Protein was dialyzed against 100 mM KCl/HEMG and used to produce antisera in guinea pigs. HEMG is 25 mM HEPES, pH 7.6, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol and 1 mM DTT.

### Isolation of FLAG-NELF-E complexes

NELF-E cDNA was obtained by RT–PCR from total RNA of S2 cells. A DNA fragment encoding full-length NELF-E and encoding FLAG peptide (DYKDDDDK) at its N-terminus was inserted into the plasmid pA5CΔP, which contains the *Drosophila* actin 5C promoter and polyadenylation signals (19). The resulting plasmid was called pA5CΔP-FLAG-NELF-E. To generate pA5CΔP-neo, the neomycin phosphotransferase gene cassette was excised from the plasmid pKO SelectNeo (Lexicon Genetics) and inserted into pA5CΔP.

*Drosophila* S2 cells were grown at 25°C in *Drosophila* S2 medium supplemented with 10% fetal calf serum (Gibco BRL). Two micrograms of pA5CΔP–FLAG–NELF–E and 0.5 μg of pA5CΔP-neo were cotransfected into 1 ml of cells ($5 \times 10^5$) using SuperFect reagent (Qiagen). Cells transfected with pA5CΔP-neo alone served as a negative control. Cells were cultured for 2 days in the absence of gentamicin and then cultured with regular passages for 1 month in media containing gentamicin (500 μg/ml; Invitrogen) to establish stably transformed cell lines.

To purify NELF complexes from transformed cells, nine 150 cm² T-flasks, each containing 40 ml of Flag-NELF-E expressing cells were cultured in the presence of gentamicin (100 μg/ml) for 3 days. Cells were harvested by centrifugation at 1000 g at 4°C, and washed once with cold phosphate-buffered saline. The cell pellet was rapidly frozen in liquid nitrogen and stored at −80°C. All subsequent steps were carried out at 4°C. Cell pellets were resuspended in 15 ml of modified TBS buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 10% glycerol; 1 mM DTT; and 1× complete proteinase inhibitor) and sonicated four times for 8 s intervals. Supernatant was collected by centrifugation at 8000 r.p.m. for 15 min in a Sorvall SS34 rotor, and incubated with 1.5 ml of anti-FLAG M2 beads (Sigma) for 1 h. The beads were poured into a column and washed with 15 ml of modified TBS buffer. NELF was eluted with FLAG peptide (100 μg/ml) in TBS.

### Preparation of antibody resins for immunodepletions

Antibody resins were generated by incubating 500 μl of serum with 250 μl of protein A-sepharose followed by covalent coupling with dimethyl pimelimidate as described by Harlow and Lane (20). Immunoaffinity sepharose was prepared that contained antibody against NELF-B (described above), a mixture of antibodies against both NELF-D and NELF-E (10), or antibody against the largest subunit of DSIF (21). Control antibody-sepharose was prepared from preimmune antibody. To reduce nonspecific binding during depletions, the antibody-sepharose was first incubated with 500 μl of nuclear extract for 4 h. Antibody-sepharose was washed extensively with 0.15 M KCl/HEMG buffer, followed by two washes with 0.1 M glycine, pH 2.5, and extensive washing with 0.15 M KCl/HEMG buffer. Antibody-sepharose was stored at 4°C.

### Immunodepletion of NELF and DSIF from nuclear extracts and evaluation of DRB sensitive transcription

Nuclear extracts were prepared from *Drosophila* embryos as described by Biggin and Tjian (22), but dialyzed at the final step to a conductivity that matched 0.15 M KCl/HEMG. To deplete DSIF or NELF, 500 μl of nuclear extract was incubated for 1.5 h with 100 μl of the appropriate antibody-sepharose. This step and all subsequent steps for the depletion were done at 4°C. Supernatants were collected through a Handee Spin Cup (Pierce) and then incubated with a fresh 100 μl portion of antibody-sepharose. While the second cycle of depletion was occurring, the first portion of antibody-sepharose was washed three times with 0.15 M KCl/HEMG, two times with 0.1 M glycine, pH 2.5 and then three times with 0.15 M KCl/HEMG. Two more rounds of depletion were performed by alternating between regenerated batches of antibody-sepharose. Depleted extracts were stored at −80°C.

The DNA template used for *in vitro* transcription consisted of the *Drosophila hsp70* promoter region spanning from –194 to +1 fused upstream of a 410 bp G-less cassette from p(C2AT) (23). Transcription reactions were performed by first preparing a 20 µl solution of 7.5 ng/µl supercoiled G-less DNA template, 27.5 mM HEPES, pH 7.6, 0.125 U/µl RNase T1, 45 mM KCl, 30 µM Na₂EDTA, 3.75 mM MgCl₂, 3% glycerol, 0.5 mM DTT and 6 µg/µl nuclear extract protein. The mixture was incubated at 25°C for 25 min to allow formation of preinitiation complexes. Transcription was started by adding a 10 µl solution of 1 mM ATP, 1 mM UTP, 0.1 mM CTP, 0.5 µCi/µl [$^{32}$P]CTP (6000 Ci/mmol), 0.02 mM 3′ *O*-methyl GTP, 22.5 mM HEPES, pH 7.6, 75 mM KCl, 1.25 mM MgCl₂, 0.5 mM DTT and 1% glycerol. When added, DRB was present in the 10 µl solution at 150 µM so that the final concentration in the 30 µl transcription reaction was 50 µM. Transcription was allowed to occur at 25°C for 20 min. Transcription was stopped by adding 120 µl of 20 mM EDTA, pH 8, 0.2 M NaCl, 1% SDS and 0.1 µg/µl yeast RNA. Samples were digested for 30 min with proteinase K followed by phenol/chloroform extraction. RNA was ethanol precipitated and analyzed on a 6% polyacrylamide gel containing 7 M urea.

### Evaluation of promoter proximal pausing *in vitro*

Transcription reactions were performed with 10 µl of NELF-depleted or mock-depleted extracts in 40 µl reaction as described previously (24). The permanganate footprinting analysis was performed as described previously (24,25).

### Chromatin immunoprecipitation (ChIP) analysis

ChIP analysis was performed as described by Park *et al*. (26). Cross-linked chromatin from 10$^7$ *Drosophila* S2 cells was immunoprecipitated with the following amounts of sera: 7 µl of NELF-B preimmune serum or antiserum, 7 µl of NELF-E preimmune serum or antiserum, 4 µl of DSIF preimmune serum or antiserum, 4 µl of GAGA factor preimmune serum or antiserum. DNA recovered from immunoprecipitates and from 10, 1 and 0.1% input DNA was analyzed by PCR as described previously (10). The following primers were used to amplify 5′ and 3′ regions of each gene: *hsp70* −48F (AAAAGAGCGCCGGAGTATAAATAGA) and *hsp70* +250R (GCAGGCATTGTGTGTGAGTTCT); *hsp70* +1357F (CTGTGCAGGCCGCTATCC) and *hsp70* +1730R (GGCCTTGCCCGTACTCATCTC); *β1-tubulin* −40F (ACTGCGGCCATCGTATAAAAG) and *β1-tubulin* +222R (TGGCAACTGTACTCTGGATGG); *β1-tubulin* +3890F (CCACCTTCATCGGCAACTC) and *β1-tubulin* +4104R (GAACTCGGCGTCCTCGTC).

## RESULTS

### Identification of the *Drosophila* NELF complex

BLAST analyses identified *Drosophila* candidates for each of the four subunits of NELF. Antibodies against *Drosophila* NELF-D and NELF-E were previously shown to recognize proteins of the expected sizes in *Drosophila* nuclear extracts, and co-immunoprecipitation analyses showed that NELF-D and NELF-E associated with each other (10). cDNA clones putatively encoding *Drosophila* NELF-A and NELF-B were obtained from the *Drosophila* Genome Resource Center and portions encoding each protein were subcloned into bacterial expression vectors. Antibodies were raised against portions of NELF-A and NELF-B isolated from *Escherichia coli*. These antibodies were observed to react with proteins of sizes
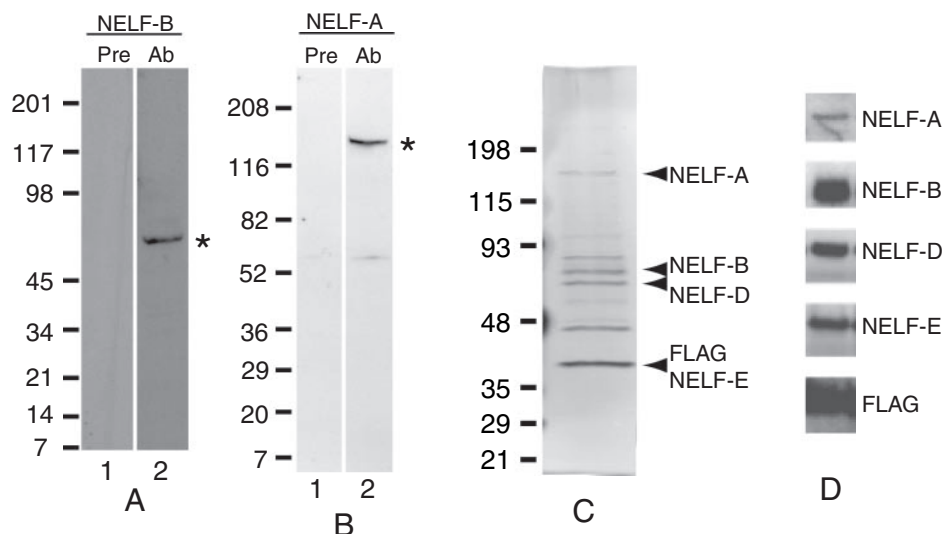


**Figure 1.** Identification of subunits of the NELF complex. (**A**) Immunoblot analysis of *Drosophila* nuclear extract with preimmune antiserum (lane 1) and anti-NELF-B antiserum (lane 2). The polypeptide marked by an asterisk matches the predicted size of NELF-B. (**B**) Immunoblot analysis of *Drosophila* nuclear extract with preimmune antiserum (lane 1) and anti-NELF-A antiserum (lane 2). The polypeptide marked by an asterisk matches the predicted size of NELF-A. (**C**) SDS–PAGE analysis of immunoaffinity purified FLAG-NELF-E complex. Polypeptides were separated on a 4–15% polyacrylamide gradient gel and visualized by silver staining. NELF subunits designated on the right were identified based on co-migration with polypeptides detected on immunoblots with respective antibodies. (**D**) Immunoblot analysis of immunoaffinity purified NELF. Separate blots were probed with antiserum against different subunits of NELF or antibody against the FLAG-tag. Only the portion of each blot showing signals is presented. The variation in the molecular weight markers designated by the numbers flanking (A, B and C) are due to batch to batch variation in the calibration of pre-stained markers (BioRad).

matching those predicted by the *Drosophila* genome (Figure 1A and B, asterisks). The size of *Drosophila* NELF-A is notable as the protein is approximately twice the size of the human ortholog. Nevertheless, this size is consistent with the current annotation of the *Drosophila* genome.

To test if the four putative NELF subunits formed a complex, we generated a *Drosophila* cell line that expressed a FLAG-tagged version of NELF-E. Analysis of the immunoaffinity purified sample on SDS–PAGE stained with silver revealed prominent polypeptides corresponding to the predicted sizes of the four subunits of NELF (Figure 1C). The prominent polypeptide migrating at ∼48 kDa is also recovered from *Drosophila* cells that do not express FLAG-tagged NELF-E so it appears to be unrelated to NELF (data not shown). The identities of the remaining polypeptides have not been determined. Immunoblotting with antibodies against each subunit of NELF verified that the silver stained polypeptides comigrated with NELF subunits (Figure 1D).
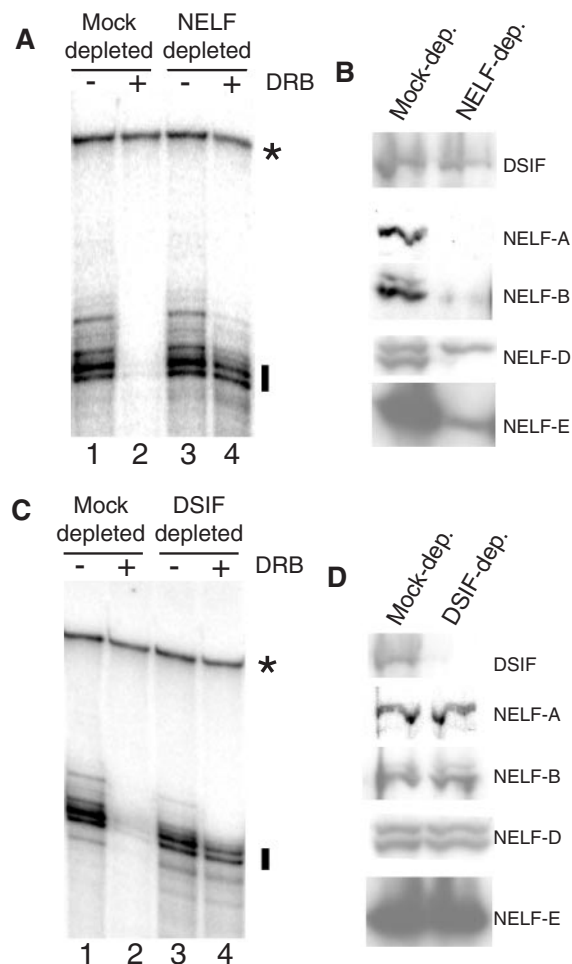
## Immunodepletion of NELF or DSIF diminishes the sensitivity of transcription *in vitro* to DRB

NELF and DSIF were originally isolated from human cell extracts because they were involved in rendering transcription reactions sensitive to DRB (1,3). To determine if *Drosophila* NELF and DSIF functioned like their human counterparts, we assessed whether depletion of either protein from a *Drosophila* nuclear extract would affect the sensitivity of transcription towards DRB. Transcription was performed with nuclear extracts from nonheat shocked embryos on a plasmid that contained the *hsp70* promoter fused at +1 to a 410 bp G-less cassette.

As shown in Figure 2A, immunodepletion of NELF from the *Drosophila* nuclear extract desensitized the transcription reaction to DRB. Fifty micromolar DRB markedly inhibited the level of transcription that occurred in extract subjected to a mock depletion with preimmune antibody (Figure 2A, compare lanes 1 and 2). In contrast, extract subjected to depletion by a mixture of antibodies against NELF-E and NELF-D was significantly less sensitive to DRB inhibition (Figure 2A, compare lanes 3 and 4). Immunoblots confirmed the specificity of the immunodepletion: the levels of all four subunits of NELF were significantly less in the NELF-depleted extract than the mock-depleted extract (Figure 2B). In contrast, comparable amounts of DSIF were present in both extracts.

Interestingly, two polypeptides were detected on the western blot with antibody against NELF-D, but only the more rapidly migrating polypeptide was depleted with NELF antibodies (Figure 2B, NELF-D). Both polypeptides were detected with crude NELF-D antiserum or with affinity-purified NELF-D antibody (data not shown). More work is required to assess whether the polypeptide that is refractory to depletion in Figure 2B is a form of NELF-D that is independent of the rest of NELF or a cross-reacting polypeptide that is detected on the immunoblots. NELF-B also appeared as a doublet. In this case, both polypeptides were depleted suggesting that both are NELF-B and associated with the other subunits of NELF.

Immunodepletion of DSIF from the *Drosophila* nuclear extract also desensitized the transcription reaction to DRB (Figure 2C). Fifty micromolar DRB markedly inhibited the



**Figure 2.** Immunodepletion of NELF or DSIF desensitizes transcription *in vitro* to DRB. (**A**) Mock-depleted (lanes 1 and 2) or NELF-depleted (lanes 3 and 4) extracts were tested for transcription *in vitro* in the presence or absence of DRB. NELF was depleted with a mixture of antibodies against NELF-D and NELF-E. Transcription was measured using an *hsp70*-G-less cassette substrate. The radiolabeled G-less transcription products are marked by a vertical line. The identity of the band marked with an asterisk is not known, but this band is evident even when transcription reactions are done in the presence of alpha amanitin (data not shown). It serves as a convenient control for RNA recovery. (**B**) Immunoblot analysis of proteins in mock-depleted and NELF-depleted extracts. (**C**) Mock-depleted (lanes 1 and 2) or DSIF-depleted (lanes 3 and 4) extracts were tested for transcription *in vitro* in the presence or absence of DRB. Antibody against the largest subunit of DSIF was used to deplete DSIF. (**D**) Immunoblot analysis of proteins in mock-depleted and DSIF-depleted extracts.

level of transcription that occurred in extract subjected to a mock depletion with preimmune antibody (Figure 2C, compare lanes 1 and 2). In contrast, extract subjected to depletion by DSIF antibody was significantly less sensitive to DRB inhibition (Figure 2C, compare lanes 3 and 4). Immunoblot analysis confirmed that the DSIF depletion had indeed removed DSIF but not the four subunits of NELF (Figure 2D).

## Immunodepletion of NELF from a *Drosophila* nuclear extract impairs promoter proximal pausing

Previously, we determined that reducing the level of NELF-E using RNA interference impaired promoter proximal pausing

on the *hsp70* gene in salivary glands (10). It remained possible, however, that the depletion of NELF-E was affecting initiation rather than pausing. To address this possibility, we evaluated the impact of depleting NELF from the nuclear extract on promoter proximal pausing *in vitro*. Previously, we showed that promoter proximal pausing can be reconstituted in *Drosophila* nuclear extracts and that depletion of DSIF from the extract with antibody inhibited pausing but not initiation (10,25).

Depletion of NELF was achieved with a mixture of NELF-D and NELF-E antibodies or with NELF-B antibody alone. Antibodies from respective preimmune sera served as negative controls. After four successive incubations of extract with antibody conjugated to sepharose, immunoblots showed that NELF subunits were substantially depleted from the extract while DSIF was mostly unaffected (Figure 3C). Approximately half the protein detected with the NELF-D antibody on the immunoblot was refractory to depletion. This is in accord with the result in Figure 2B showing that there is a second protein that migrates closely to NELF-D but is not depleted by NELF antibodies.

Transcription reactions were performed in the presence of all four nucleotides with the NELF-depleted and mock-depleted extracts. After 30 min, the presence of Pol II on the promoters was monitored with permanganate (25). Permanganate reacts preferentially with thymines in single-stranded regions, and promoter proximal pausing on *hsp70* causes thymines at +22, +30 and +34 on the nontranscribed strand to be hyper-reactive (25,27,28). Extracts depleted of NELF exhibited less permanganate reactivity at these positions than the mock-depleted extracts (Figure 3A, compare lanes 3 to 6 and lanes 9 to 12). Quantification of band intensities at +22 and +30 after subtracting background intensities in lanes 1, 4, 7 and 10 are provided in Figure 3B. The intensities of the +22 and +30 bands for the NELF-depleted samples were ∼4-fold less than the preimmune controls. Importantly, similar results were obtained when the depletions were done with two different NELF affinity resins—one composed of a mixture of NELF-D and NELF-E antibodies and the other composed of NELF-B antibodies.

To test if depletion of NELF impaired initiation, we did transcription reactions with 3′ *O*-methyl GTP instead of GTP. 3′ *O*-methyl GTP causes the Pol II to arrest at +15 where the first guanylate nucleotide is incorporated, and this arrest results in permanganate hyper-reactivity at +8 and +22 (10). Since initiation must occur for Pol II to reach +15, the intensities of the bands at +8 and +22 reflect the level of initiation. As shown in Figure 3A (lanes 2, 5, 8 and 11), permanganate analysis detects little difference in the reactivity of thymines at +8 and +22. Thus, depletion of NELF is affecting promoter proximal pausing but not initiation.

Unfortunately, we were unable to assess the fate of the Pol II that failed to pause as a consequence of depleting NELF. It may continue to elongate on the template or it might disengage. The efficiency of pausing in our extract is never complete. Consequently, read-through products are always generated even in the presence of NELF, so these obscure detection of read-through transcripts that might be generated as a consequence of NELF depletion.

## ChIP analyses of *hsp70* in nonheat shocked and heat shocked cells

Previously, we observed that NELF-D and NELF-E cross-linked to the promoter region but not the 3′ region of the *hsp70* gene in nonheat shocked cells (10). We were interested in determining if any changes in the association of NELF would be apparent during heat shock. Results in Figure 4 indicate that there was ∼5-fold less NELF-B and NELF-E cross-linking to the *hsp70* promoter region in heat shocked cells than in nonheat shocked cells. The level of each NELF subunit cross-linking to the 3′ region of *hsp70* was low and essentially the same for heat shocked and nonheat shocked cells. The residual cross-linking of NELF subunits to the promoter region in heat shocked cells appears to be specific because the amount of DNA recovered in NELF precipitates from the 5′ region exceeded the amount from the 3′ region or the amounts of either 5′ or 3′ regions recovered in the pre-immune precipitates. Some evidence indicates that promoter proximal pausing persists transiently during heat shock induction (29), so this could account for the NELF detected on the *hsp70* promoter region during heat shock.

We also analyzed DSIF and GAGA factor as both have been implicated in promoter proximal pausing (10,35). As seen before, both proteins cross-link to the *hsp70* promoter region but not the 3′ region prior to heat shock induction. The level of DSIF cross-linking to the promoter region decreased ∼2-fold following heat shock. In contrast, the level of DSIF cross-linking to the 3′ region of *hsp70* increased ∼2-fold following heat shock. These results are in good agreement with rapid cross-linking results reported by Saunders *et al*. (30). The level of GAGA factor cross-linking to the promoter region also diminished following heat shock, but the level of cross-linking to the 3′ region remained low and similar to the level seen in nonheat shocked cells. The apparent absence of GAGA factor from the downstream region of *hsp70* in heat shocked cells is not in agreement with previously reported UV cross-linking experiments (31) but is in agreement with recent immunofluorescence data showing that GAGA factor does not colocalize with elongation complexes on polytene chromosomes (16). The heat shock-dependent reduction in GAGA factor cross-linking to the promoter region could be due to displacement by HSF, whose binding sites are interdigitated and overlapping with GAGA factor binding sites in the promoter region (32).

## ChIP analysis of the *β1-tubulin* gene

Previous analyses detected paused Pol II on the promoter proximal region of the *β1-tubulin* gene in *Drosophila* cells (27,33), so we tested whether NELF and DSIF were present at this gene. ChIP analysis revealed that both NELF and DSIF were present in the promoter region but not the 3′ region (Figure 5). Interestingly, significantly less GAGA factor was detected on the *β1-tubulin* promoter than on the *hsp70* promoter. This is consistent with visual inspection of the sequences constituting these two promoters (data not shown)—multiple GAGA elements are evident in *hsp70* but only two short elements are evident in the region from +1 to −250 of *β1-tubulin*. Nevertheless, the signal for GAGA factor on the *β1-tubulin* promoter region is above the background shown by the preimmune control. GAGA factor might be
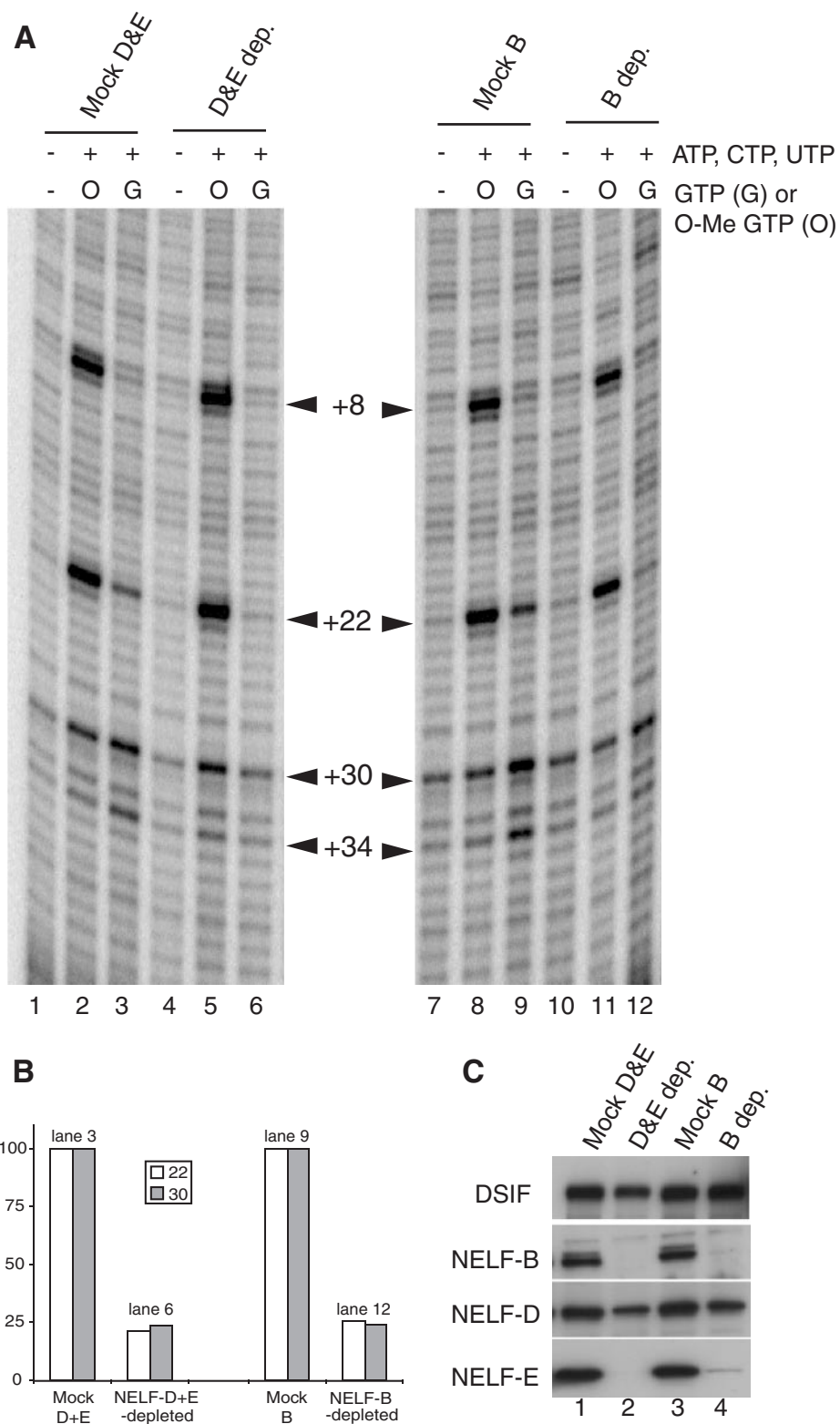
**Figure 3.** Immunodepletion of NELF impairs promoter proximal pausing *in vitro*. (**A**) Permanganate footprinting analysis of mock-depleted and NELF-depleted extracts. Transcription reactions were done in the absence of nucleotides (lanes 1, 4, 7 and 10), the presence of ATP, CTP, UTP and 3′ *O*-methyl GTP (lanes 2, 5, 8 and 11), or the presence of ATP, CTP, UTP and GTP (lanes 3, 6, 9, and 12). At the end of 30 min, Pol II was detected on the DNA by permanganate footprinting. Permanganate reactivity at +8 and +22 in lanes 2, 5, 8 and 11 was due to Pol II arresting at +15 following incorporation of the 3′ *O*-methyl GTP. Permanganate reactivity at +22, +30 and +34 in lanes 3 and 9 was due to Pol II stably paused in the promoter proximal region of *hsp70*. (**B**) Quantification of band intensities detected at +22 and +30 in lanes 3, 6, 9 and 12. Background intensities detected in lanes 1, 4, 7 and 10 have been subtracted. (**C**) Immunoblot analysis showing the specificity of the NELF depletions. Depleted extracts were run-on a 4–15% gradient gel and blotted to nitrocellulose. The largest subunit of DSIF and the indicated subunits of NELF were detected with antibodies.

**A**

hsp70

5′
(-48 to +250)

3′
(+1357 to +1730)

**B**



**C**
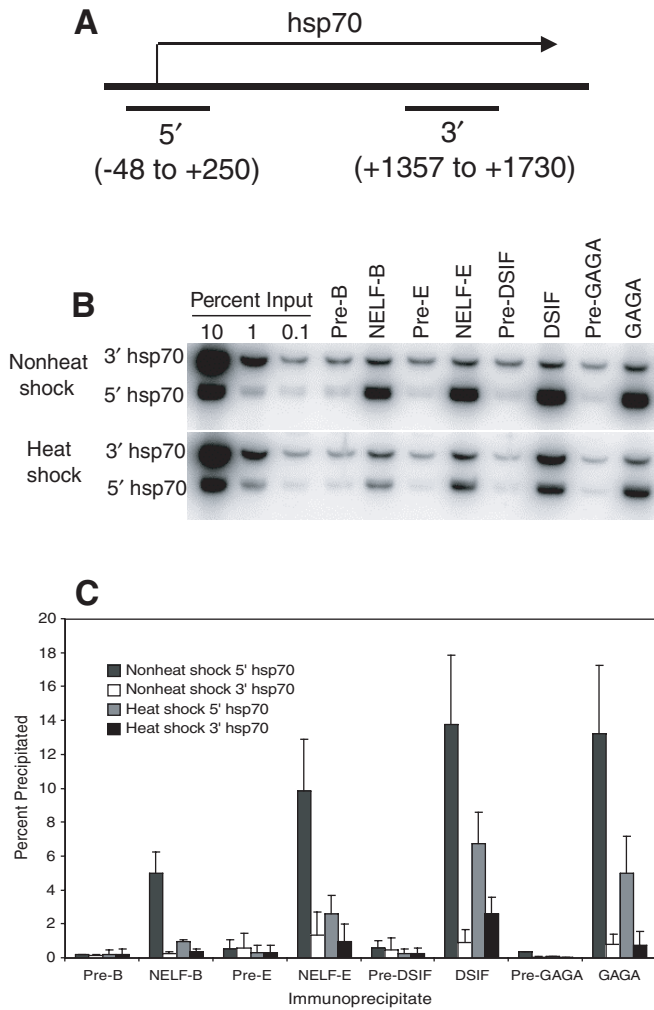


**Figure 4.** ChIP analysis of proteins associated with the *hsp70* gene before and after heat shock. Cross-linked chromatin was prepared from nonheat shocked cells and cells that had been heat shocked for 15 min. The cross-linked chromatin was subjected to immunoprecipitation with antisera against NELF-B, NELF-E, DSIF or GAGA factor, or with corresponding preimmune sera. The immunoprecipitated DNA and the indicated amounts of input DNA were analyzed by PCR to determine the amount of DNA from the promoter region and the 3′ region cross-linked to each protein. (**A**) Schematic of the *hsp70* gene showing regions that were amplified by PCR (not drawn to scale). (**B**) Representative results obtained from a ChIP analysis. (**C**) Quantification of results obtained from three independent ChIP analyses. Error bars represent standard deviations.

involved in setting up the paused Pol II on *β1-tubulin*, since GAGA factor is involved in setting up paused Pol II on *hsp70* (34,35).

### Immunofluorescence analysis of NELF-B and NELF-D on polytene chromosomes

Immunofluorescence microscopy was used to compare the distributions of NELF-B and NELF-D on polytene chromosomes. Previously, we observed that antibody against NELF-D stained hundreds of loci on polytene chromosomes (10). Figure 6A–C show that antibody against NELF-B stains hundreds of loci and that there is extensive overlap in the distributions of NELF-B and NELF-D on polytene chromosomes. Both proteins are concentrated in regions known as

**A**

β1-tubulin

5′
(-40 to +222)

3′
(+3890 to +4104)

**B**



**C**
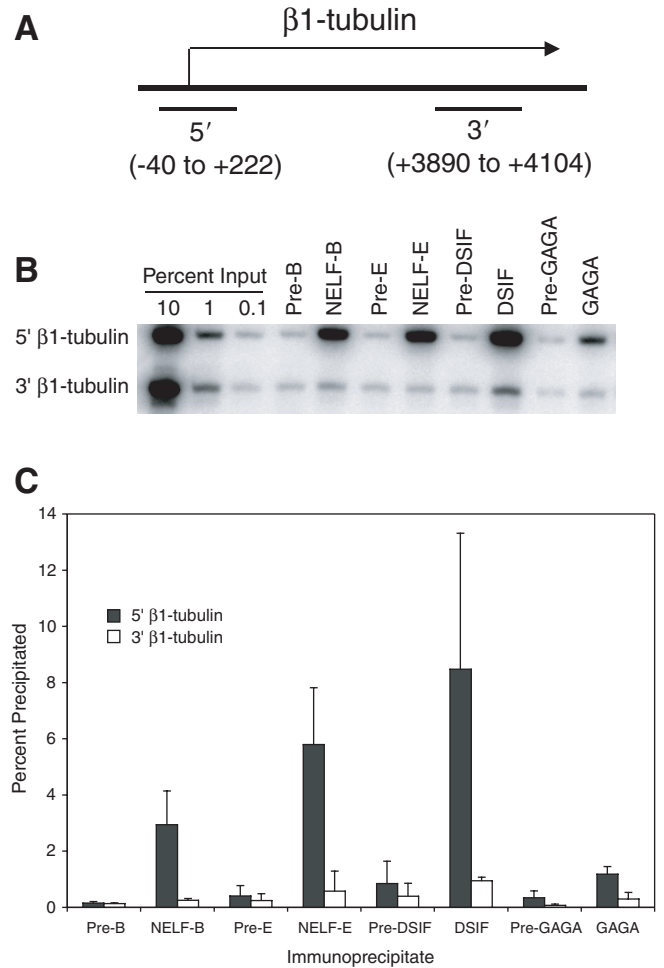


**Figure 5.** ChIP analysis of proteins associated with the *β1-tubulin* gene in nonheat shocked cells. Cross-linked chromatin from nonheat shocked cells was subjected to immunoprecipitation with antisera against NELF-B, NELF-E, DSIF or GAGA factor and with corresponding preimmune sera. (**A**) Schematic of *β1-tubulin* gene showing regions that were amplified by PCR (not drawn to scale). (**B**) Representative results obtained from a ChIP analysis. (**C**) Quantification of results obtained from three independent ChIP analyses.

interbands, which are regions of decondensed chromatin. Control experiments showed that the preimmune serum for each antibody did not stain the chromosomes. Moreover, preincubation of either antibody with its respective antigen but not with an unrelated protein blocked staining of the chromosomes.

We were particularly interested to see staining at 67B, 87A, 87C and 87E because all of these locations are known to harbor Pol II paused in the promoter proximal regions of particular genes (27,36). 67B contains several small heat shock genes, and 87A and 87C contain copies of the *hsp70* heat shock gene. 87E contains an *hsp70* transgenic promoter previously shown to interact with NELF-D and to have paused Pol II (10). A fly line lacking the transgene at 87E showed reduced staining of 87E with antibodies against NELF-B or NELF-D (data not shown).

The NELF-D antiserum detected two polypeptides in *Drosophila* nuclear extracts raising concern that immunofluorescence could be due to a polypeptide that was not part of the NELF complex (Figure 2C). This is unlikely for two reasons.
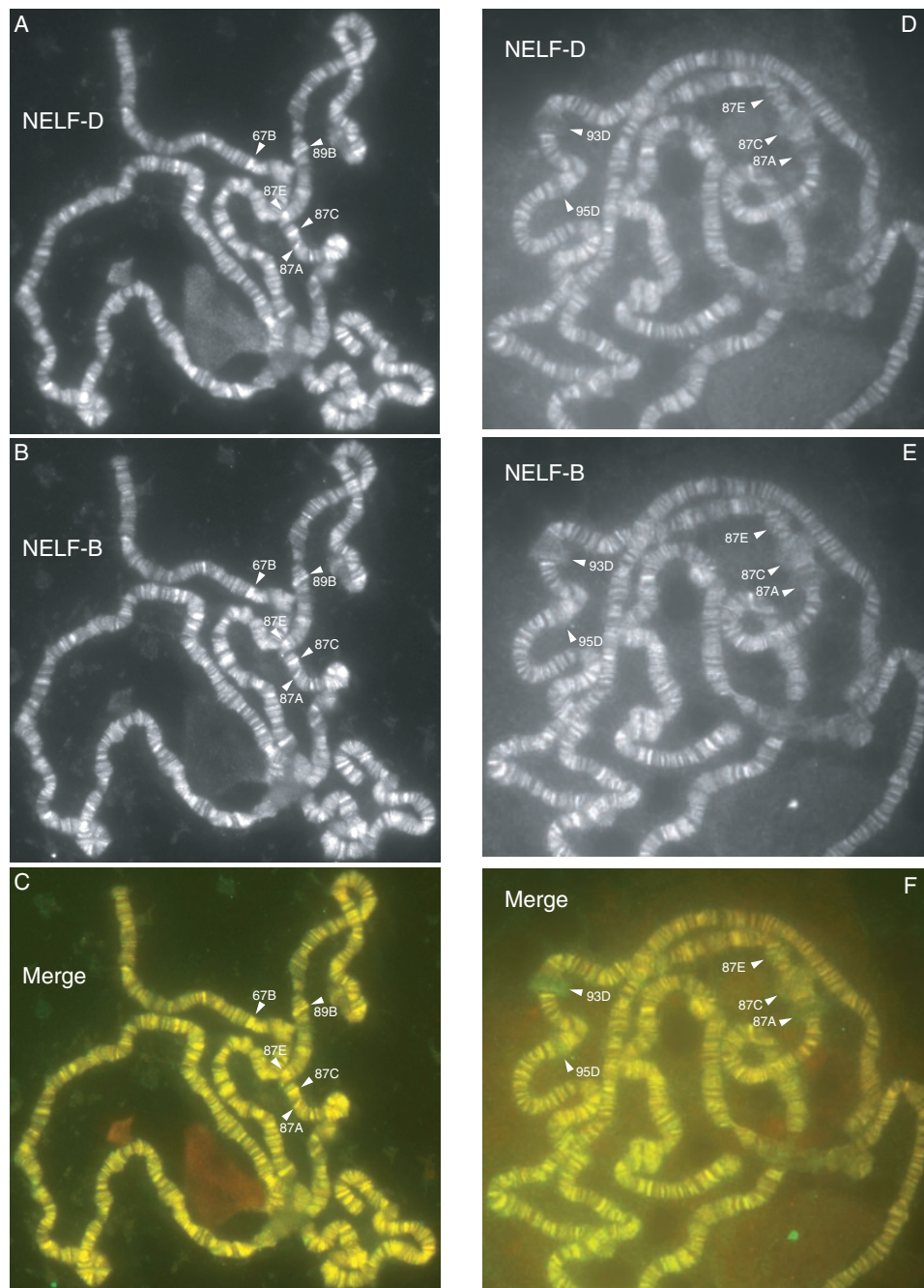
**Figure 6.** Immunofluorescence analyses of NELF-D and NELF-B on polytene chromosomes derived from nonheat shocked and heat shocked larvae. Images in (A, B and C) are of chromosomes from nonheat shocked larvae. Images in (D, E and F) are of chromosomes from heat shocked larvae. NELF-D was detected with affinity-purified antibody made in rabbit. NELF-B was detected with antiserum made in guinea pig. NELF-D antibody on the chromosomes was detected with Alexa 568-tagged secondary, giving red fluorescence [converted to grayscale in (A and D)]. NELF-B antibody bound to the chromosomes was detected with Alexa 488-tagged secondary, giving green fluorescence (converted to grayscale in panels B and E). Merged images for the two antibodies are shown in (C and F).

First, the immunodepletion results in Figure 2B are consistent with the majority of NELF-B being associated with NELF-D. Second, the slowly migrating polypeptide that appears to be independent of NELF seems to be unable to associate with the NELF-D antibody in its native state, since it fails to be immuno-depleted with NELF-D antibody (Figure 2B).

Figure 6D–F show the distributions of NELF-B and NELF-D on polytene chromosomes from heat shocked larvae.

As was the case for the nonheat shocked samples, there is extensive overlap in the distributions of NELF-B and NELF-D. In accord with our previous observation (10), stain-ing of the puffs with antibody against NELF-D was low. Heat shock induced genes that cause puffs in the specimen shown in Figure 6 reside at 87A, 87C, 87E, 93D and 95D. Although staining of puffs with antibody against NELF-B was slightly elevated relative to NELF-D, the level of staining was

markedly less than the level of staining observed for DSIF at puffs (10) (data not shown).

We have also stained polytene chromosomes with antibodies against NELF-A and NELF-E (data not shown). There was considerable overlap in the staining patterns with NELF-B and NELF-D at interbands. As was the case with NELF-B antibody, the staining of puffs with NELF-A and NELF-E antibodies were variable and much less intense than the staining observed with DSIF antibody. More analysis is required to determine the significance of the weak staining at puffs as this staining could be due to nonspecific binding by the NELF antibodies.

## DISCUSSION

### Comparison of human and *Drosophila* NELF

Using immunoaffinity techniques, we have determined that *Drosophila* NELF contains four polypeptides with similarity to the four subunits of human NELF. All four polypeptides copurify with a FLAG-tagged version of NELF-E expressed in *Drosophila* cells. Moreover, immunodepletions done with antibodies against subsets of NELF subunits result in the removal of other subunits. The amino acid sequences of the four *Drosophila* polypeptides have significant similarity to their human counterparts indicating that they might have similar structures. In addition, *Drosophila* and human

NELF appear to be functionally similar because depletion of NELF from human (1,3) and *Drosophila* nuclear extracts desensitizes transcription to DRB.

We aligned the amino acid sequences of the various NELF subunits to learn more about NELF (Figure 7). The sequences of NELF-B and NELF-D orthologs exhibit several regions with >50% identity distributed over their entire lengths. Analysis of subunit interactions lead Narita *et al.* (18) to propose that NELF-B and NELF-D form a central core that brings together NELF-A and NELF-E. NELF-A associates with NELF-D while NELF-E associates with NELF-B. The high degree of homology between NELF-B and NELF-D orthologs is consistent with constraints that might be dictated by the requirements that these two proteins interact with each other and with other subunits of NELF. The extensive colocalization of NELF-B and NELF-D on polytene chromosomes is also consistent with these two subunits forming the core of the NELF complex.

When NELF-A was initially identified through a BLAST search, we were concerned about the genome annotation because dNELF-A was over twice the size of human NELF-A. Our immunoblot analysis verifies that dNELF-A is approximately twice the size of hNELF-A. The alignment in Figure 7 shows that the size difference is due to a large nonconserved region located approximately between amino acids 300 and 1100. Inspection of the amino acid sequence in this region of *Drosophila* NELF-A reveals numerous tracts
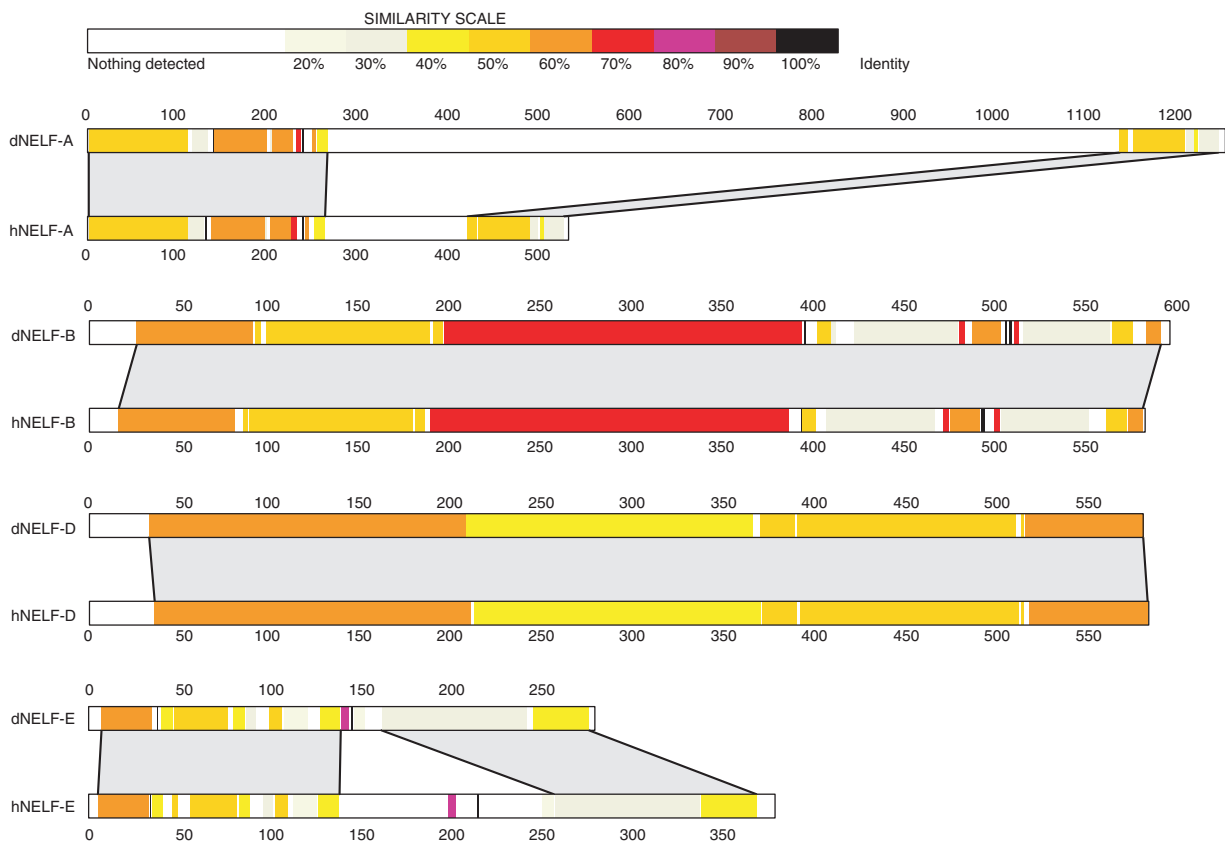


**Figure 7.** Schematic representation of amino acid sequence alignments between human and *Drosophila* subunits of NELF. Regions of homology between *Drosophila* and human NELF subunits were determined using LFasta and illustrated using LalnView. The nonhomologous region of ∼800 amino acids in dNELF-A spans from approximately amino acids 300 to 1100. The RNA recognition motif in dNELF-E corresponds to amino acids 167–232.

of poly-glutamine, poly-asparigine, poly-threonine and poly-alanine that are absent from hNELF-A. This lack of conservation raises the possibility that this region serves as a linker between two distinct functional domains defined by the regions of homology spanning the first 250 amino acids and the last 100 amino acids of NELF-A. The N-terminal region of homology in hNELF-A has been shown to interact with Pol II and NELF-D (37). Moreover, this region is required for NELF-mediated repression *in vitro* (18). Deletion of amino acids 431–528 of hNELF-A, which corresponds to the last 100 amino acids of dNELF-A, does not impair the ability of the NELF complex to inhibit transcription *in vitro* (18). Future analysis using genetic and molecular genetic approaches in *Drosophila* may help elucidate the function of the large nonconserved region and the C-terminal domain.

hNELF-E binds RNA, and the region responsible for this activity exhibits 30% identity to amino acids 167–232 in dNELF-E. This RNA recognition motif has been shown to be required for NELF inhibition in human extracts (18). Two features found in hNELF-E are notably absent in dNELF-E. A 46 amino acid stretch spanning from 196 to 242 in hNELF-E that is composed primarily of alternating arginine and aspartic acid residues is absent—the function of this region in hNELF-E is not known. Also absent from dNELF-E are serines that correspond to amino acids 181, 185, 187 and 191 in hNELF-E. These serines in hNELF-E are phosphorylated *in vitro* by P-TEFb, and these modifications reduce the binding between hNELF-E and RNA (15). It will be interesting to learn if phosphorylation of dNELF-E regulates its RNA binding activity.

### NELF is involved in promoter proximal pausing

Previously, we provided two results that implicated NELF in promoter proximal pausing on the *hsp70* heat shock gene (10). Depleting salivary glands of NELF using RNA interference diminished the level of promoter proximal pausing occurring on *hsp70* in salivary glands. Also, NELF was found to cross-link to the *hsp70* promoter region prior to heat shock induction. Here, we provide additional data strengthening the conclusion that NELF is involved in promoter proximal pausing.

Immunodepletion of NELF from nuclear extracts impaired formation of the paused Pol II. Importantly, the cell-free transcription reactions with 3′ O-methyl GTP indicated that NELF does not contribute to steps in the transcription process encompassing initiation and elongation to +15. We have also determined that NELF is associated with the *β1-tubulin* promoter. Genomic footprinting with permanganate and nuclear run-on analysis indicate that promoter proximal pausing occurs on the *β1-tubulin* gene (27,36).

### Changes in NELF and DSIF associations at *hsp70* during heat shock induction

Based on strong immunofluorescence staining detected for DSIF and the weak staining for NELF at heat shock puffs, we proposed that NELF dissociates from elongation complexes during activation but DSIF remains associated with the elongation complexes (10). Our observation that the level of NELF cross-linking to the *hsp70* promoter region after heat shock is ∼5-fold less than before heat shock is consistent with this model. However, we also observed a 2-fold decrease in the level DSIF cross-linking to the *hsp70* promoter region after heat shock, in agreement with a recent report from Saunders *et al.* (30). The decrease in DSIF cross-linking near the promoter was unexpected since heat shock puffs on polytene chromosomes stain very strongly with DSIF antibody. However, much of the signal detected on the polytene chromosomes probably originates from DSIF associated with elongation complexes in the body of the gene. In addition, the interaction of DSIF with Pol II in the promoter proximal region might be transient or not occur at all on some elongation complexes in the promoter proximal region. The rate of reinitiation at the *hsp70* promoter is exceptionally high, once every few seconds, so a significant portion of the Pol II molecules might escape into the body of the gene before associating with DSIF.

### NELF and its role in gene expression

The broad distribution of NELF-B and NELF-D on polytene chromosomes could mean that hundreds of genes harbor paused Pol II similar to that found on *hsp70*. Alternatively, NELF could have additional functions. mRNA capping is coupled to transcription (38), and recent results lead to the hypothesis that pausing induced by NELF might be important for coupling transcription and mRNA capping (17). A subset of genes like *hsp70* and *β1-tubulin* might use cofactors such as GAGA factor to generate a stably paused state.

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