Human Negative Elongation Factor Activates Transcription and Regulates Alternative Transcription Initiation

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The human negative elongation factor (NELF) is a four-subunit protein complex that inhibits the movement of RNA polymerase II (RNAPII) at an early elongation stage in vitro. NELF-mediated stalling of RNAPII also attenuates transcription of a number of inducible genes in human cells. To obtain a genome-wide understanding of human NELF-mediated transcriptional regulation in vivo, we carried out an exon array study in T47D breast cancer cells with transient small interfering RNA knockdown of individual NELF subunits. Upon depletion of NELF-A, -C, or -E, the vast majority of NELF-regulated genes were downregulated. Many of the down-regulated genes encode proteins that play key roles in cell cycle progression. Consequently, NELF knockdown resulted in significant reduction in DNA synthesis and cell proliferation. Chromatin immunoprecipitation showed that NELF knockdown led to dissociation of RNAPII from the promoter-proximal region of the cell cycle-regulating genes. This was accompanied by decreased histone modifications associated with active transcription initiation (H3K9Ac) and elongation (H3K36Me3), as well as reduced recruitment of the general transcription factor TFIIIB and increased overall histone occupancy at a subset of the down-regulated promoters. Lastly, our study indicates that NELF regulates alternative transcription initiation of BSG (Basigin) and elongation (H3K36Me3), as well as reduced recruitment of the general transcription factor TFIIIB and increased overall histone occupancy at a subset of the down-regulated promoters. Taken together, our data suggest a diverse transcriptional consequence of NELF-mediated RNAPII pausing in the human genome.

Historically, mechanistic studies of eukaryotic gene regulation have predominantly been focused on the control of transcription initiation (1). Until recently, the prevailing view was that assembly of the preinitiation complex (PIC) that contains RNA polymerase II (RNAPII) at the promoter region was the most critical step in transcription of the vast majority of eukaryotic genes. However, recent genome-wide examination of the physical distribution of RNAPII along the chromosomes in mammals and Drosophila has led to the unexpected finding that RNAPII is enriched at the promoter-proximal regions of a large portion of both transcriptionally active and inactive genes (2–5). For example, the majority of silenced genes in embryonic stem cells are associated with RNAPII and initiation-specific histone modifications (2). Clearly RNAPII pausing is a widespread phenomenon in eukaryotic genomes. It has been suggested that paused RNAPII keeps the silenced genes in a transcriptionally “poised” state, which can be rapidly activated in response to various environmental and developmental cues (6). However, a comprehensive understanding of the impact of RNAPII pausing on mammalian gene expression is still lacking.

NELF was biochemically characterized as a regulatory complex for RNAPII movement during transcription elongation (7). Together with 5,6-dichloro-1-β-D-ribobenzimidazole sensitivity-inducing factor, NELF interacts with the hypophosphorylated form of RNAPII (IIa) and stalls it at an early stage of elongation in an in vitro transcription system (7, 8). The inhibitory effect of NELF on RNAPII elongation is alleviated by the positive transcription elongation factor-b (7), which phosphorylates serine 2 of the C-terminal domain of RNAPII. Phosphorylation of 5,6-dichloro-1-β-D-ribobenzimidazole sensitivity-inducing factor and NELF by the positive transcription elongation factor-b is also thought to contribute to the release of RNAPII from the stalled mode (9–12).

Consistent with the biochemical findings, NELF induces temporary pausing of RNAPII at promoter-proximal regions and represses the transcription of a number of inducible genes, including Drosophila Hsp70 (13), human immunodeficiency virus (10, 15), estrogen-responsive genes in breast cancer cells (16, 17), and inflammatory response genes in macrophages (18). NELF can modulate basal and/or induced transcription of these genes, depending on the gene and cell context. Genome-wide investigation of Drosophila NELF indicates that NELF is recruited to a large number of Drosophila genes (19), and consistently, a separate study demonstrates that ~60% of RNAPII stalling events are mediated by NELF (4). Interestingly, almost half of the most highly expressed genes in Drosophila cells are associated with NELF (19), and most of the NELF-regulated polymerase pausing events are associated with gene activation rather than repression in the Drosophila genome (20), indicating that the in vivo function of NELF may not be limited to inhibition of tran-

**References**

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2. The abbreviations used are: PIC, preinitiation complex; NELF, negative elongation factor; RNAPII, RNA polymerase II; siRNA, small interfering RNA; RT, reverse transcription; ChIP, chromatin immunoprecipitation; FACS, fluorescence-activated cell sorting; BrdUrd, bromodeoxyuridine; PBS, phosphate-buffered saline.

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4. The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and Figs. S1–S6.

5. Primary array data sets can be accessed through the NCBI Gene Expression Omnibus under GEO accession number GSE19940.

6. The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and Figs. S1–S6.
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scription elongation. Consistent with this notion, human NELF has also been implicated in several pre-mRNA processing events, which include 5' capping (21, 22), 3' processing of replication-dependent histone mRNA (23), and alternative splicing (24). Thus NELF may have a broader impact on multiple aspects of gene regulation than indicated by the initial biochemical studies in vitro.

In the current study, we used an exon array to examine the functional impact of NELF on the abundance of mRNA transcripts at the levels of individual exons in the human genome. Our data uncovered an important role for NELF in sustaining transcription of a large number of cell cycle-regulating genes. The whole genome study also suggests that NELF regulates the usage of alternative promoters in the human genome.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Human breast cancer cell line T47D was obtained from ATCC and cultured in Dulbecco's modified minimum essential medium supplemented with 10% fetal bovine serum. The following antibodies used in chromatin immunoprecipitation assay were commercially available: anti-RNAPII (H224, sc-9001x; Santa Cruz), anti-RNAPII C-terminal domain phospho-S2 (ab5095; Abcam), anti-RNAPII C-terminal domain phospho S5 (ab5131; Abcam), anti-TFIIF (sc-225x; Santa Cruz), anti-histone H3 trimethyl K4 (ab8580; Abcam), anti-histone H3 trimethyl K9 (ab4414; Abcam), anti-histone H3 terminal K4 (ab8580; Abcam), anti-histone H3 trimethyl K36 (ab9050; Abcam), and anti-α-tubulin (CP06; CalBiochem). Anti-NELF-A, -B, -C, and -E antibodies used in the Western blot analysis and ChIP assay have been previously described (17, 25). Primers for RT-PCR and ChIP assays were designed by Primer Express of Affymetrix and synthesized by Invitrogen. Primer sequences and their positions in the corresponding genes are listed in supplemental Table S1.

siRNA Transfection and mRNA/Protein Expression Analysis—siRNAs targeting the individual NELF subunits and luciferase control have been previously described (25). Transfection of the siRNA duplex into T47D cells was carried out by using RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 1.5 × 10⁶ cells in a 10-cm culture dish were transfected with 10 nM of siRNA duplex into T47D cells was carried out by using the RNAiMAX reagent. Growth medium was replenished 48 h after transfection, and the cells were allowed to grow for additional 48 h before harvest. The procedures for extraction of total RNA and protein, RT-PCR analysis, and Western blot analysis have been previously described (17).

Array Hybridization and Processing—Human GeneChip Exon 1.0 ST Array was purchased from Affymetrix. The service for RNA processing and labeling, array hybridization, and scanning was provided by Expression Analysis Inc. (Durham, NC), using whole transcript sense target labeling and control reagents (900652; Affymetrix) according to the manufacturer's instruction. The RNA samples were prepared from T47D cells 96 h after transfection with siRNA oligonucleotides for the control and individual NELF subunits. Triplicates were made for each condition. 1 μg of total RNA from each sample was used to generate labeled probes for array hybridization. The quality of isolated RNA was first measured by Bioanalyzer QC assay (Agilent). Ribosomal RNA was then removed with the RiboMinus Kit (Invitrogen), and the reduction efficiency was verified by capillary electrophoresis. cDNA was synthesized from the resultant RNA followed by amplification, fragmentation, and terminal labeling. The array was hybridized with the labeled targets, washed, and scanned for data collection.

Data Analysis—The exon array data were analyzed using ArrayAssist (Stratagene) and GeneSpring GX 10 (Agilent) with default settings. The CEL files were imported, and the triplicates from the same siRNA treatment were assigned into the same group. Probe sets at the core level were summarized by using the ExonPLIER algorithm. Quantile normalization was performed, and antigeneomic probes were used as background probes. For variance stabilization, the number of 16 was added to probe set intensity values before transformation to a log₂ scale. Once the summarized dataset was obtained, a series of analysis were applied to assess the quality of the samples, hybridization procedures, and actual data. In brief, the poly(A) controls view was used to monitor the target labeling process. The hybridization controls view depicts the hybridization quality. The principal component analysis allows viewing of separations between groups of replicates. The correlation coefficient for each pair of arrays was calculated for all of the samples. Through these analyses, one of the siNELF-B samples was found to be an outlier, and data from the three arrays of the siNELF-B group were therefore excluded from subsequent analyses.

Probe sets below the noise level were filtered out by the detection above background analysis. A transcript was retained if one of its probe sets had p value of <0.05 in at least six of the remaining 12 arrays. Gene level expression was estimated with the same algorithm from all of the probe sets in each transcript. A splicing index was then calculated for all of the probe sets using siControl samples as the reference group. Pairwise analysis was conducted to compare all groups with the siControl reference group using unpaired t test. Transcripts that were commonly changed in siNELF-A, -C, and -E groups with a fold change of >1.5 and a p value of <0.05 were selected as the differentially expressed genes, and probe sets with an absolute value of differential splicing index of >1 and a p value of <0.05 were selected as the alternatively spliced probe sets. The list of probe sets with significant splicing index was further filtered to retain those with at least two nonoverlapping probes and unique hybridization pattern. Hierarchical clustering analysis on differentially expressed genes was performed with GeneSpring GX. Gene ontology analysis was performed using the DAVID Bioinformatics Resources and confirmed with ArrayAssist and GeneSpring GX.

Cell Proliferation and FACS Analysis—Cell proliferation of the control and NELF knockdown cells was measured by cell counting with a hemocytometer. Briefly, the same number of T47D cells were plated for siRNA transfection, and triplicate samples were prepared for each condition. Cell number was counted on the second, third, and fourth day after siRNA transfection, and data were presented as the averages of the triplicate samples ± standard deviation. For FACS analysis, the cells were collected at the fourth day after siRNA transfection, fixed overnight with 90% ethanol at 4 °C, and stained with 50 μg/ml of
propidium iodide at 37 °C for 30 min. Cell cycle distribution was analyzed with FACS. For bromodeoxyuridine (BrdUrd) analysis, asynchronously growing cells 3 days after siRNA transfection were pulse-labeled with 50 μM BrdUrd for 1 h, washed twice with PBS, and replenished with growth medium for various periods of time. Cells were trypsinized, collected in growth medium, and centrifuged at 1,500 rpm for 5 min. Cell pellet was then washed once with PBS-SN (PBS with 1% fetal bovine serum and 1% NaNO3) and fixed overnight with 90% ice-cold ethanol at 4 °C. The fixed cells were washed again with PBS-SN, denatured with 2 M HCl by incubation at room temperature for 20 min, and neutralized with 0.1 M boric acid, pH 8.5, for 5 min. After wash with PBS-TB (PBS with 0.5% Tween 20 and 0.05% bovine serum albumin), the cells were resuspended with PBS-TB, stained with propidium iodide for 30 min, and analyzed on a FACScalibur flow cytometer (Becton Dickinson).

Chromatin Immunoprecipitation Assay—ChIP protocol was adapted from published work (26) with several modifications. The cells were cross-linked with 1% formaldehyde for 10 min followed by termination with 125 mM glycine for 5 min. The cells were collected and washed twice with ice-cold PBS and once with lysis buffer I (5 mM HEPES, pH 8.0, 85 mM KCl, 0.5% Triton X-100, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 mM Na3VO4, 10 mM Na4P2O7, 10 mM NaF). After centrifugation, the cells were resuspended and incubated in lysis buffer II (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1% SDS, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 mM Na3VO4, 10 mM Na4P2O7, 10 mM NaF) on ice. After centrifugation, the cells were resuspended and incubated in lysis buffer II (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1% SDS, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 mM Na3VO4, 10 mM Na4P2O7, 10 mM NaF) for 10 min on ice. The cells were then sonicated with Bioruptor (Diagenode sa, Belgium), and insoluble debris was removed by centrifugation at 14,000 rpm for 10 min. Chromatin extract was diluted by 10-fold with dilution buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1 mg/ml sonicated single-strand DNA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 mM Na3VO4, 10 mM Na4P2O7, 10 mM NaF) and precleared with a 50% slurry of protein A-agarose preblocked with sonicated single-strand DNA. Extract from ~2 × 106 cells was used for each immunoprecipitation reaction by incubating with 2 μg of antibody at 4 °C overnight. 5% of input extract was saved prior to immunoprecipitation as input control for normalization. Immune complexes were captured with preblocked protein A-agarose for rabbit polyclonal antibodies or protein G-agarose for monoclonal antibodies for 2 h. Precipitated protein-DNA complexes were washed, and DNA was eluted, reverse cross-linked, and purified as previously described (26). DNA from the input samples was recovered with a MinElute reaction clean-up kit (Qiagen) after reverse cross-linking at 65 °C overnight. Input and immunoprecipitated DNA was analyzed by real time PCR.

RESULTS

NELF-A, -C, and -E Knockdown Results in Reduced Expression of a Common Set of Genes—To assess the impact of acute NELF depletion on global gene expression, human T47D cells were transfected with minimal amounts of siRNA duplex that were required for efficient and rapid knockdown of individual NELF subunits. As shown in Fig. 1A, the NELF-specific siRNA duplexes readily reduced the protein levels of the corresponding targets. Consistent with published finding of co-dependence of protein stability among the NELF subunits (23, 25), knockdown of one NELF subunit also reduced the protein (Fig. 1A) but not the mRNA levels (supplemental Fig. S1) of the other three NELF subunits. Among all four knockdowns, the NELF-E siRNA duplex was most effective in co-depleting the entire NELF complex (Fig. 1A).

Using mRNA from the control and individual NELF knockdown cells, we carried out a gene expression profiling analysis with the exon array from Affymetrix. The exon array assesses the abundance of all exons from both empirically curated mRNA sequences and computationally predicted transcripts. In addition to gene level changes, the exon array can also reveal variations in individual exons that may result from alternative splicing, promoter utilization, termination, and polyadenylation (27–29). Principle component analysis indicates that the data points for NELF-A, -C, and -E knockdown cells were all clustered with each other and well separated from those of the control knockdown cells (supplemental Fig. S2). However, the three data points from NELF-B knockdown cells were scattered on the plot (supplemental Fig. S2) and yielded low correlation coefficient (supplemental Fig. S3), suggesting poor reproducibility among the triplicates. Curiously, none of the three data points for NELF-B knockdown cells were in close proximity to any of the other NELF knockdown samples in the principle component analysis plot (supplemental Fig. S2). Although the biological significance of this NELF-B-specific phenomenon remains unclear and is subject to future investigation, we choose to focus on the common gene expression profiles shared by NELF-A, -C, and -E knockdown cells in the current study.

A total of 543 genes were differentially expressed at the gene level by at least 1.5-fold with a p value of <0.05 between the control and all three NELF knockdown cell populations (supplemental Table S2). A visual inspection of the hierarchical clustering revealed three groups of genes with regard to the effects of NELF knockdown (Fig. 1B). Group A consisted of genes that were commonly down-regulated by knockdown of different NELF subunits. The vast majority of NELF-affected genes fell into this category. Of note, ~28% of the genes in this group were down-regulated more than 3-fold by NELF-E knockdown, as compared with 8% that were affected to the same extent by NELF-A or -C knockdown. This correlated with the more complete depletion of the NELF complex by the NELF-E siRNA oligonucleotides. Group b contained genes whose expression was increased in all NELF-depleted samples. This included JUNB, an immediate early response gene that was previously shown to be negatively regulated by NELF (14). Unlike the first two groups, genes in group c were affected in opposite directions by knockdown of different NELF subunits.
This could be due to possible actions of individual NELF subunits that were independent of the NELF complex. Alternatively, it might result from potential off-target effects of siRNA oligonucleotides. Taken together, the gene expression profiling analysis suggests that the human NELF complex predominantly functions to sustain active expression of its target genes.

**Direct Effect of NELF on Transcription of Cell Cycle-related Genes**—Gene Ontology analysis indicated that the NELF-regulated genes were significantly enriched with those involved in various aspects of cell cycle progression (Fig. 1C). These gene products, which are all reduced by NELF knockdown (group a), are important for DNA replication (e.g. CDT1, MCM5, POLA1, TK1, and TOP2A), DNA recombination (e.g. RECQL and SMC6), DNA packaging (e.g. histone genes), spindle organization and biogenesis (e.g. AURKA, NDC80, and UBE2C), mitotic chromosome condensation (e.g. CDC6, CDCA5, POLS, and SMC2), and regulation of progression through cell cycle (e.g. CCND1, CCNE2, and MYBL2). The microarray results for representatives of these cell cycle genes were verified by RT-PCR, using mRNA samples from independent siRNA transfection experiments. As shown in Fig. 2A, mRNA levels of CCNB2, TOP2A, DLG7, CCNE2, and MYBL2 were all reduced by NELF-A, -C, and -E knockdown. Consistent with published data (14), JUNB expression was elevated in all three knockdown cell populations. When active transcription was blocked by actinomycin D, mRNA levels of the potential NELF target genes decreased with similar kinetics in control and NELF-E knockdown cells (Fig. 2B). This suggests that the changes in steady-state mRNA levels upon NELF depletion are more likely due to the impact of NELF on transcription rates than on mRNA half-lives.

To determine whether the siNELF-affected genes were direct targets of NELF, we used ChIP to examine the occupancy of NELF-C at these gene loci. NELF-C was chosen for the ChIP analysis because of the high efficiency of the corresponding antibody in immunoprecipitation (data not shown). As shown in Fig. 3A, the NELF-C signal peaked at the promoter-proximal region (position b) of both NELF-activated and -repressed genes tested. Low or no NELF-C signals were detected in regions between 300 and 500 bp upstream (position a) or 1.5–2 kb downstream (position c) of the transcription initiation sites (Fig. 3A). Importantly, the NELF-C signals at promoter-proximal regions were significantly dampened in the NELF-C knockdown cells (Fig. 3B), demonstrating the specificity of the ChIP signals. These data indicate that the cell cycle-related genes identified by the exon array are most likely direct targets of NELF.

**NELF Enhances Occupancy of RNAPII and TFIIB at the NELF Target Genes**—The positive effect of human NELF on transcription of a large number of cell cycle-related genes is apparently distinct from its well documented activity in inhibition of transcription elongation. To elucidate the underlying mecha-
nism(s) of a positive role of NELF in transcriptional regulation, we first examined distribution of RNAPII at the NELF target genes in control and NELF-E knockdown cells. Using an antibody that recognizes the N terminus of the largest subunit of RNAPII, we found by ChIP that the total RNAPII levels were enriched at the promoter-proximal regions of these genes in the control cells (compare positions a and b in Fig. 4A). Consistent with its effect on gene expression, NELF depletion significantly reduced the total RNAPII levels at the 5’/H11032 end of the NELF-activated genes (CCNB2, CCNE2, DGL7, MYBL2, and TOP2A), without corresponding increases of RNAPII density in the downstream regions of the same genes. In contrast, NELF depletion resulted in increases in the RNAPII level at JUNB, a known NELF-repressed gene (Fig. 4A). As a negative control, NELF depletion did not affect the RNAPII density at RPLPO, whose expression was not subjected to NELF-mediated regulation (Fig. 4A and data not shown). Using the antibodies that specifically recognize unphosphorylated RNAPII and those phosphorylated at the serine 5 or serine 2 positions, we also examined the effect of NELF-E knockdown on the density of these subpopulations of RNAPII at different transcriptional states. When normalized with the density of total RNAPII, no differences in unphosphorylated or phosphorylated RNAPII at the NELF-regulated genes were found between the control and NELF-C knockdown cells. T47D cells were transfected with control or NELF-C siRNA oligonucleotides for 96 h and then harvested for ChIP. Position b of the target genes was analyzed for NELF-C occupancy. After being normalized with input, the level of NELF-C in knockdown cells was expressed as fold change against that in the control cells for each gene tested. The results shown in this figure were the averages ± S.D. from two independent experiments. In this and the following figures, the statistical significance was analyzed by Student’s t test, and asterisks indicate p < 0.05.

FIGURE 2. NELF is directly involved in transcription of a number of cell cycle-associated genes. A, real time PCR analysis of gene expression in control and NELF knockdown cells. T47D cells were transfected with siRNA oligonucleotides for 4 days. Total RNA was then isolated and treated with DNase before cDNA synthesis. Real time PCR analysis was performed, and the value was normalized against that of 18s rRNA. B, mRNA half-life analysis of the NELF target genes. T47D cells were transfected with control or NELF-E siRNA oligonucleotides for 84 h. Actinomycin D (5 μg/ml) was added to culture medium, and the cells were incubated for 0, 1, 2, 4, 8, and 12 h before collected for RNA isolation. mRNA level at the 0 h time point is set at 100.

FIGURE 3. In vivo association of NELF-C with NELF-regulated genes. A, recruitment of NELF-C to the promoter-proximal regions of NELF targets. Sonicated chromatin extract from T47D cells was immunoprecipitated with IgG or NELF-C antiserum. Association of NELF-C was analyzed at three positions of NELF-regulated genes: a, between 300 and 500 bp upstream of initiation site; b, between 15 and 70 bp downstream of initiation site; and c, between 1.5 and 2 kb downstream of initiation site. NELF-C levels at all positions were first normalized against input, and the levels at positions a and c were expressed as fold change against that at position b for each gene. Non-specific association was evaluated by ChIP with IgG, and the levels were shown as fold change against the NELF-C level at position b. B, NELF-C recruitment in the control and NELF-C knockdown cells. T47D cells were transfected with control or NELF-C siRNA oligonucleotides for 96 h and then harvested for ChIP. Position b of the target genes was analyzed for NELF-C occupancy. After being normalized with input, the level of NELF-C in knockdown cells was expressed as fold change against that in the control cells for each gene tested. The results shown in this figure were the averages ± S.D. from two independent experiments. In this and the following figures, the statistical significance was analyzed by Student’s t test, and asterisks indicate p < 0.05.
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**A**

**Figure 4.** NELF depletion decreases RNAPII and TFII B densities at the NELF target genes. **A**, chromosomal association of RNAPII was examined in control and NELF-E knockdown cells at two positions of each NELF-regulated gene: a, between 10 and 70 bp downstream of initiation site; and b, between 300- and 700 bp downstream of initiation site. Antibody (H224) that recognized the N terminus of the largest subunit of RNAPII was used for measuring the level of total RNAPII associated with a specific genomic site. The levels of RNAPII at position b in control cells and at positions a and b in NELF-E knockdown cells are expressed as fold change against that at position a in control cells. B, association of TFII B at position a of NELF regulated genes was examined in control and NELF-E knockdown cells. Level of TFII B in NELF-E knockdown cells was expressed as fold change against that in control cells. The results shown here are the averages ± S.D. from at least three independent experiments.

**B**

regions of all NELF-activated genes, the increase was statistically significant only for two of the five NELF-activated genes examined (CCNB2 and TOP2A; Fig. 5A). In comparison, the histone H3 acetyl lysine 9 (H3K9Ac) signal was substantially diminished by NELF depletion for all of the NELF-activated genes examined, after normalization with the total histone H3 density at the same chromosomal location (Fig. 5B). Histone H3 trimethyl lysine 4 (H3K4Me3), another histone modification marker associated with active transcription initiation (31), was reduced by NELF knockdown to a much smaller extent, with a statistically significant difference detected only at one NELF-activated gene (MYBL2; Fig. 5C). In addition, we also measured the level of histone H3 trimethyl lysine 36 (H3K36Me3), a histone modification marker associated with active transcription elongation (31). As shown in Fig. 5D, the H3K36Me3 signal in the body of all NELF-activated genes was markedly decreased by NELF-E depletion. The same histone modification signals at the NELF-repressed JUNB locus were either unaffected by NELF knockdown (H3K9Ac and H3K4Me3) or altered in the opposite direction as the NELF-activated genes (H3K36Me3). Taken together, these results suggest a role for NELF in rendering the local chromatin configuration of its activated genes more accessible to the transcription machinery. However, it remains to be determined whether NELF does so directly by recruiting histone-modifying enzymes or indirectly by maintaining the association of RNAPII at the same regions.

**NELF Influences Alternative Transcription Initiation of the BSG Gene**—In addition to the effect of NELF knockdown on gene expression, the exon array also revealed 205 exons that were differentially expressed in control and NELF knockdown cells (supplemental Table S3). Interestingly, the majority of the exons influenced by NELF knockdown are not known to be alternatively utilized, raising the possibility that NELF may have a potential role in ensuring the proper splicing of constant exons. Approximately 20% of the NELF-affected exons have been previously reported to be alternatively utilized via alternative promoter utilization, termination, or splicing (supplemental Fig. S5). For example, the exon array indicates that expression of exon 1, but not the other exons of the BSG (Basigin) gene, is significantly reduced in NELF knockdown cells (supplemental Fig. S6). As illustrated in the diagram in Fig. 6A, the BSG gene produces four transcripts via alternative transcription initiation and alternative splicing of internal exons. Transcription of variants I (NM_001728.2) and II (NM_198589.1) starts from exon 2, whereas those of III (NM_198590.1) and IV (NM_198591.1) are initiated from exon 1. The differential effect of NELF knockdown on the levels of exon 1 and 2 was confirmed by exon-specific RT-PCR (Fig. 6B). Furthermore, ChIP indicates that NELF-C is physically associated with both exons, with a stronger signal detected at exon 2 (Fig. 6C). Consistent with its effect on exon 1 expression, NELF-E depletion led to an increase in total histone H3 signal at exon 1 (Fig. 6E) and substantial reduction of total RNAPII (Fig. 6D), H3K9Ac (Fig. 6F), and H3K4Me3 (Fig. 6G) signals. In contrast, no or fewer robust changes in total RNAPII and histone markers were observed at exon 2. Thus NELF can selectively influence utilization of two neighboring transcription initiation sites.
NELF Is Important for Cell Cycle Progression—Consistent with a role for NELF in sustaining expression of multiple cell cycle-regulating genes, knockdown of NELF-A, -C, and -E in T47D cells significantly reduced the cell proliferation rate (Fig. 7A). Curiously, knockdown of NELF-B did not reduce cell growth to the same extent as depletion of the other three NELF subunits (Fig. 7A). Analysis by FACS indicates that NELF knockdown results in fewer cells in the S phase and more in the G1 phase of the cell cycle (Fig. 7B). To specifically examine the impact of NELF knockdown on DNA synthesis and cell cycle progression, control and NELF-E knockdown cells were pulse-labeled with BrdUrd for 1 h, and the BrdUrd-positive cells were chased for 6 h by FACS (Fig. 7C). As quantitated in Fig. 7D, the NELF-E knockdown cell population had significantly fewer BrdUrd-positive cells than the control during the pulse-labeling, which is consistent with the lower percentage of the S phase fraction in the NELF-E knockdown cells (5.6% versus 17.8% in control; Fig. 7B). Furthermore, the BrdUrd-positive fraction in the NELF knockdown cells also progressed to the next G1 phase at a lower rate than its counterpart in the control population (Fig. 7E). For example, the number of BrdUrd-positive control cells in G1 increased by 3-fold between 2 and 4 h of the chasing, whereas no substantial increase in BrdUrd-positive cells in the G1 phase was observed until 6 h into chasing. Together these results strongly suggest that NELF-mediated gene expression is crucial for S phase replication and cell cycle progression.

DISCUSSION

In the current study, we examined the impact of the NELF complex on gene expression in breast cancer cells at the level of individual exons. Compared with the conventional microarrays for gene level expression profiling, exon arrays have the advantage of interrogating exon level variations as a result of regulation of both transcription and RNA processing. Our data indicate that NELF is required for maintaining the gene level expression of a large number of key cell cycle regulators, thus ensuring proper rates of cell proliferation and cell cycle progression. This positive effect of NELF on transcription is likely due to its ability to sustain chromatin accessibility and promote PIC assembly at the promoters of the NELF-activated genes. In

FIGURE 5. NELF depletion increases total histone density and decreases activation-associated histone modifications at NELF target genes. T47D cells transfected with control and NELF-E siRNA oligonucleotides were used for ChIP with antibodies recognizing total histone H3 (A), histone H3 acetyl K9 (H3K9Ac) (B), histone H3 trimethyl K4 (H3K4Me3) (C), and histone H3 trimethyl K36 (H3K36Me3) (D). The levels of histone modifications for H3K9Ac and H3K4Me3 at position a (between 10 and 70 bp downstream of initiation site) and H3K36Me3 at position b (between 1.5 and 2 Kb downstream of initiation site) were normalized against the total histone H3 levels at the corresponding loci. The level of total histone H3 and its modifications in knockdown cells were expressed as fold change against those in control cells. The results shown here were the averages ± S.D. from at least three independent experiments.

FIGURE 6. Differential effects of NELF depletion on the alternative transcription initiation of BSG. A, Diagram indicating four known variants of BSG transcripts, with I and II initiated from exon 2 and III and IV initiated from exon 1. B, real time PCR analysis of mRNA expression initiated from alternative transcription initiation sites of BSG in control and NELF knockdown cells. C, recruitment of NELF-C to the BSG locus. Chromatin extracts from T47D cells were immunoprecipitated with IgG or NELF-C antiserum. Exon 1, exon 2, and exon 8 were examined for NELF-C occupancy. D, RNAPII distribution on BSG locus in control and NELF-E knockdown cells. The level of total RNAPII at exon 1 and exon 2 of BSG in control and NELF-E knockdown cells was determined by ChIP with antibodies recognizing total RNAPII. Exon 1, exon 2, and exon 8 were examined for RNAPII occupancy. E, recruitment of NELF-C to the BSG locus. Chromatin extracts from T47D cells were immunoprecipitated with IgG or NELF-C antiserum. Exon 1, exon 2, and exon 8 were examined for NELF-C occupancy. F, RNAPII distribution on BSG locus in control and NELF-E knockdown cells. The level of total RNAPII at exon 1 and exon 2 of BSG in control and NELF-E knockdown cells was determined by ChIP with antibodies recognizing total RNAPII. Exon 1, exon 2, and exon 8 were examined for RNAPII occupancy. G, total histone H3 density at BSG locus in control and NELF-E knockdown cells. H, H3K9Ac signals at the two BSG locations. I, H3K4Me3 signals at the BSG gene. The ChIP signals shown for H3K9Ac and H3K4Me3 were normalized against total histone H3 levels of the corresponding loci. The level of total histone H3 and its modifications in knockdown cells were expressed as fold change against those in control cells. The results shown in this figure were the averages ± S.D. from two independent experiments.
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![Graph A](image)

**FIGURE 7. NELF depletion delays cell cycle progression.** A, effect of NELF knockdown on cell proliferation. T47D cells were transfected with siRNA oligonucleotides against the individual NELF subunits, and the cell number was counted on days 2, 3, and 4 after siRNA transfection. B, effect of NELF knockdown on cell cycle distribution. T47D cells were transfected with the siRNA oligonucleotides for 4 days, and the cells were then collected, fixed, and stained with propidium iodide for FACS analysis. C, BrdUrd pulse-chase analysis of control and NELF-E siRNA transfected cells. T47D cells were transfected with control or NELF-E siRNA for 3 days. The cells were then labeled with 50 μM BrdUrd for 1 h and incubated in BrdUrd-free medium for 2, 4, and 6 h. The fixed cells were stained with an Alexa Fluor 488-conjugated anti-BrdUrd antibody and PI and analyzed by flow cytometry. Dot plots of a representative experiment from two repeats were shown. D, percentage of BrdUrd-positive cells at 0 time point in Fig. 7C. The data shown are the averages ± S.D. from two repeats. E, percentage of G1, BrdUrd-positive cells at different time points after incubation with BrdUrd-free growth medium. The percentage of G1, BrdUrd-positive cells were calculated by dividing the percentage of BrdUrd-positive cells with 2n DNA content (top left panel in C) with the percentage of total BrdUrd-positive cells (top left and top right panels). The data shown are averages ± S.D. from two repeats.

addition, the exon array data also suggest a role for NELF in influencing the utilization of individual exons in the human genome. This is exemplified by a differential impact of NELF knockdown on alternative transcription initiation sites of the same gene.

Several recent gene level expression profiling studies in HeLa cells and Drosophila S2 cells have uncovered a number of interesting in vivo functions of NELF that might not have been predicted based on its in vitro biochemical properties (20, 23, 32). For example, Handa and co-workers (23) showed that human NELF mediated the 3′ end processing of replication-dependent histone genes, the transcripts of which normally end with a stem-loop rather than polyadenylated tail. NELF depletion resulted in a reduction in the stem-loop form and a concurrent increase in the polyadenylated species (23). The same phenomenon was observed in Drosophila cells (20).

The use of random primer rather than the oligo(dT) method for labeling array probes in the current study precluded us from directly assessing the effect of NELF on the choice of 3′ termination. However, consistent with the published work, we did observe significantly reduced expression of many replication-dependent histone mRNAs in NELF-depleted cells (supplemental Table S2).

A recent genome-wide study of Drosophila NELF indicates that NELF is responsible for a large proportion of RNAPII pausing events in the fly genome (4), many of which facilitate active transcription of genes involved in response to various stimuli (20). Although our data reveal that a distinct group of genes are affected by NELF in human cells, the mechanistic study suggests that the positive effect of NELF on transcription is likely evolutionarily conserved. It will be of interest to determine whether Drosophila NELF is also involved in the regulation of the abundance of individual exons.

Promotion of RNAPII pausing, the best characterized biochemical activity of NELF, most likely accounts for its inhibition of transcription elongation in vitro and transcription repression of multiple NELF-target genes in vivo. However, as demonstrated in the current study, alleviation of NELF-mediated RNAPII pausing in the promoter-proximal region of NELF-activated genes in vivo does not result in a concurrent increase in RNAPII density in downstream regions, and curiously, we did not observe any obvious reduction of RNAII occupancy at these loci either, despite the significantly reduced levels of the corresponding transcripts. Although the ChIP condition used in our current study might not be sensitive and quantitative enough to detect the difference at regions with relatively low RNAPII density, a similar effect of NELF depletion on RNAPII association in Drosophila cells was also observed with ChIP-on-chip analysis (4) or permanganate footprinting assays (20). Collectively, these data suggest that the major in vivo function of NELF at these gene loci is likely to promote the stable association of RNAPII with the actively transcribing genes rather than prevent the polymerase from engaging in active transcription elongation.

How NELF-mediated RNAPII pausing facilitates transcription in vivo remains to be elucidated. Studies of Drosophila NELF-mediated gene activation suggest that the high RNAPII density mediated by NELF may prevent nucleosomal encroachment into the promoter-proximal region (20). Furthermore, NELF-mediated RNAPII pausing may further facilitate chromatin accessibility by helping preserve histone modification markers associated with active transcription (e.g. H3K4Me3) (20). ChIP analysis of human NELF in cell cycle-regulating genes indicates that H3K9Ac is universally reduced by NELF knockdown at the promoter-proximal regions of all NELF-activated genes examined. Interestingly, changes in H3K4Me3 modification and overall histone density upon NELF depletion in T47D cells were only observed in a subset of these genes and less robust than that of H3K9Ac. Therefore, the exact impact of NELF on nucleosome occupancy and specific histone modification markers may depend on species, cell type, and chromosomal location. Nevertheless, our study supports the notion that the presence of NELF at the promoter-proximal region helps maintain a favorable epigenetic status for active transcription. This may be carried out indirectly by NELF through stabilization of RNAPII-containing tran-
scriptional machinery, which in turn recruits the histone-modifying enzymes. Alternatively, it is possible that NELF may interact with the histone-modifying enzymes and thus have a more direct impact on the epigenetic status of its transcriptional target genes.

In addition to its impact on nucleosome density and histone modifications, NELF knockdown also reduced the promoter association of TFIIB at a subset of NELF-activated genes in T47D cells, suggesting a role for NELF in the assembly of PIC. The less efficient transcription initiation and/or reinitiation in NELF-depleted cells may reflect reduced chromatin accessibility for PIC at the promoter-proximal regions. Alternatively, one could envision a more direct role for NELF in promoting the recruitment of PIC components through putative protein-protein interactions.

Consistent with a previous report (14), we found that transcription of JUNB was repressed by NELF. Our study further indicates that RNAPII density at both promoter-proximal and downstream regions of JUNB is increased in NELF-depleted T47D cells. This is accompanied by elevation of the activation-associated histone markers at the JUNB locus. Thus our comparative study of JUNB and those NELF-activated, cell cycle-regulating genes clearly demonstrates the gene-specific effects of NELF in transcriptional regulation, which could be determined by the chromatin context, promoter strength and configuration of a given NELF-regulated gene. The previous work on NELF-mediated regulation of JUNB showed that RNAPII stalling at the promoter-proximal regions of JUNB was reduced upon NELF-E knockdown (14), a finding that is different from our observation. The earlier work focused on interleukin 6-induced JUNB expression in HeLa cells growing under a serum-free condition, where the overall transcription level of the gene was relatively low. On the other hand, the T47D cells used in our current study were continuously cultured in serum-containing medium. The relative impact of NELF on initiation versus post-initiation stages could be influenced by the availability of additional growth factors in serum and the overall transcriptional potency of its target gene.

The fact that a large number of cell cycle-associated genes are affected by NELF-A, -C, and -E knockdown is consistent with the presumed functional commonality of these proteins as integral components of the NELF complex. However, it is somewhat surprising that none of the three NELF-B data points of the exon array shares significant overlap with those from the other NELF knockdown samples. This could be partially because of the technical inferiority of some of the NELF-B data points. However, using gene-specific RT-PCR with separate NELF-B-depleted samples, we did not observe any significant effects of NELF-B knockdown on the same cell cycle-regulating genes either (data not shown). Consistently, NELF-B knockdown in T47D cells did not lead to significant defects in cell proliferation or cell cycle progression (Fig. 7, A and B). The obvious disparity in the knockdown effects of NELF-B versus the other NELF subunits could be explained by insufficient depletion of NELF-B in T47D cells. Alternatively, this could reflect different degrees of functional importance for individual NELF-B subunits in regulating cell cycle-related gene expression.

The computational analysis of the exon array data uncovered many potential exon targets of NELF whose expression is significantly affected by knockdown of individual NELF subunits. In the case of the BSG gene, the effect of NELF on the abundance of the 5’ exon is most likely due to its preferential activation of one of the two alternative promoters. The effects of NELF knockdown on the abundance of the first two exons correlate well with its effects on RNAPII and histone density, as well as the activation-associated histone modifications. Given the close proximity of the two alternative promoters, this locus may serve as an excellent paradigm to identify the cis-elements for the promoter-specific effect of NELF. Further validation of the exon array data will offer new insights into the multifaceted nature of NELF in gene regulation.

In summary, the findings of our current work support a functional diversity of NELF-mediated gene regulation in human cells. The promoter-specific impacts of NELF on the densities of RNAPII, PIC, and various epigenetic markers underscore the importance of gene context in determining the functional outcome of the biochemical activity of NELF-mediated RNAPII pausing. Characterization of the exact cis-elements and trans-acting factors that dictate the effect of NELF is of great importance to a comprehensive understanding of NELF function in gene regulation.

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REFERENCES

1. Kadonaga, J. T. (2004) Cell 116, 247–257
2. Guenther, M. G., Levine, S. S., Boyer, L. A., Jaenisch, R., and Young, R. A. (2007) Cell 130, 77–88
3. Kim, T. H., Barrera, L. O., Zheng, M., Qu, C., Singer, M. A., Richmond, T. A., Wu, Y., Green, R. D., and Ren, B. (2005) Nature 436, 876–880
4. Muse, G. W., Gilchrist, D. A., Nechaev, S., Shah, R., Parker, J. S., Grissom, S. F., Zeitlinger, J., and Adelman, K. (2007) Nat. Genet. 39, 1507–1511
5. Zeitlinger, J., Stark, A., Kellis, M., Hong, J. W., Nechaev, S., Adelman, K., Levine, M., and Young, R. A. (2007) Nat. Genet. 39, 1512–1516
6. Glover-Cutter, K., Kim, S., Espinosa, J., and Bentley, D. L. (2008) Nat. Struct. Mol. Biol. 15, 71–78
7. Yamaguchi, Y., Takagi, T., Wada, T., Yano, K., Furuya, A., Sugimoto, S., Hasegawa, J., and Handa, H. (1999) Cell 97, 41–51
8. Yamaguchi, Y., Inukai, N., Narita, T., Wada, T., and Handa, H. (2002) Mol. Cell 22, 2918–2927
9. Marshall, N. F., Peng, J., Xie, Z., and Price, D. H. (1996) J. Biol. Chem. 271, 27176–27183
10. Fujinaga, K., Irwin, D., Huang, Y., Taube, R., Kurosu, T., and Peterlin, B. M. (2004) Mol. Cell Biol. 24, 787–795
11. Ivanov, D., Kwak, Y. T., Guo, J., and Gaynor, R. B. (2000) Mol. Cell Biol. 20, 2970–2983
12. Kim, J. B., and Sharp, P. A. (2001) J. Biol. Chem. 276, 12317–12323
13. Wu, C. H., Yamaguchi, Y., Benjamin, L. R., Horvat-Gordon, M., Washinsky, J., Enerly, E., Larsson, J., Lambertsson, A., Handa, H., and Gilmour, D. (2003) Genes Dev. 17, 1402–1414
14. Aida, M., Chen, Y., Nakajima, K., Yamaguchi, Y., Wada, T., and Handa, H. (2006) Mol. Cell Biol. 26, 6094–6104
15. Zhang, Z., Aikins, M., Gilmour, D. S., and Henderson, A. J. (2007) J. Biol. Chem. 282, 16981–16988
16. Kininisi, M., Isaacs, G. D., Core, L. J., Hah, N., and Kraus, W. L. (2009) Mol. Cell Biol. 29, 1123–1133
17. Aiyar, S. E., Sun, J. L., Blair, A. L., Moskaluk, C. A., Lu, Y. Z., Ye, Q. N.,
NELF-mediated Gene Activation in Human Cells

Yamaguchi, Y., Mukherjee, A., Ren, D. M., Handa, H., and Li, R. (2004) Genes Dev. 18, 2134–2146
18. Adelman, K., Kennedy, M. A., Nechaev, S., Gilchrist, D. A., Muse, G. W., Chinenov, Y., and Rogatsky, I. (2009) Proc. Natl. Acad. Sci. U. S. A. 106, 18207–18212
19. Lee, C., Li, X., Hechmer, A., Eisen, M., Biggin, M. D., Venters, B. J., Jiang, C., Li, J., Pugh, B. F., and Gilmour, D. S. (2008) Mol. Cell Biol. 28, 3290–3300
20. Gilchrist, D. A., Nechaev, S., Lee, C., Ghosh, S. K., Collins, J. B., Li, L., Gilmour, D. S., and Adelman, K. (2008) Genes Dev. 22, 1921–1933
21. Mandal, S. S., Chu, C., Wada, T., Handa, H., Shatkin, A. J., and Reinberg, D. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 7572–7577
22. Sims, R. J., 3rd, Belotserkovskaya, R., and Reinberg, D. (2004) Genes Dev. 18, 2437–2468
23. Narita, T., Yung, T. M., Yamamoto, J., Tsuboi, Y., Tanabe, H., Tanaka, K., Yamaguchi, Y., and Handa, H. (2007) Mol. Cell 26, 349–365
24. Sun, J., Blair, A. L., Aiyar, S. E., and Li, R. (2007) J. Steroid Biochem. Mol. Biol. 107, 131–139
25. Sun, J., Watkins, G., Blair, A. L., Moskaluk, C., Ghosh, S., Jiang, W. G., and Li, R. (2008) J. Cell. Biochem. 103, 1798–1807
26. Ding, N., Zhou, H., Esteve, P. O., Chin, H. G., Kim, S., Xu, X., Joseph, S. M., Friez, M. J., Schwartz, C. E., Pradhan, S., and Boyer, T. G. (2008) Mol. Cell 31, 347–359
27. Gardina, P. J., Clark, T. A., Shimada, B., Staples, M. K., Yang, Q., Veitch, J., Schweitzer, A., Awad, T., Sugnet, C., Dee, S., Davies, C., Williams, A., and Turpaz, Y. (2006) BMC Genomics 7, 325
28. Yeo, G. W., Xu, X., Liang, T. Y., Muotri, A. R., Carson, C. T., Coufal, N. G., and Gage, F. H. (2007) PLoS Comput. Biol. 3, 1951–1967
29. Zhang, Z., Lotti, F., Dittmar, K., Younis, I., Wan, L., Kasim, M., and Dreyfuss, G. (2008) Cell 133, 585–600
30. Thomas, M. C., and Chiang, C. M. (2006) Crit. Rev. Biochem. Mol. Biol. 41, 105–178
31. Li, B., Carey, M., and Workman, J. L. (2007) Cell 128, 707–719
32. Aiyar, S. E., Blair, A. L., Hopkinson, D. A., Bekiranov, S., and Li, R. (2007) Oncogene 26, 2543–2553