MOLECULAR CARCINOGENESIS 47:34–46 (2008)

Nucleolin Binds Specifically to an AP-1 DNA Sequence and Represses AP1-Dependent Transactivation of the Matrix Metalloproteinase-13 Gene

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Transcriptional regulation via activator protein-1 (AP-1) protein binding to AP-1 binding sites within gene promoter regions of AP-1 target genes plays a key role in controlling cellular invasion, proliferation, and oncogenesis, and is important to pathogenesis of arthritis and cardiovascular disease. To identify new proteins that interact with the AP-1 DNA binding site, we performed the DNA affinity chromatography-based Nucleotide Affinity Preincubation Specificity TEst of Recognition (NAPSTER) assay, and discovered a 97 kDa protein that binds in vitro to a minimal AP-1 DNA sequence element. Mass spectrometric fragmentation sequencing determined that p97 is nucleolin. Immunoblotting of DNA affinity-purified material with anti-nucleolin antibodies confirmed this identification. Nucleolin also binds the AP-1 site in gel shift assays. Nucleolin interacts in NAPSTER with the AP-1 site within the promoter sequence of the metalloproteinase-13 gene (MMP-13), and binds in vivo in chromatin immunoprecipitation assays in the vicinity of the AP-1 site in the MMP-13 promoter. Overexpression of nucleolin in human HeLa cervical carcinoma cells significantly represses AP-1 dependent gene transactivation of a minimal AP-1 reporter construct and of an MMP-13 promoter reporter sequence. This is the first report of nucleolin binding and transregulation at the AP-1 site.

Key words: AP-1; nucleolin; transactivation; NAPSTER; matrix metalloproteinase-13

INTRODUCTION

The activator protein-1 (AP-1) transcription factor is involved in coordinate regulation of gene expression events that control diverse cellular processes, including cellular invasion, cell cycle regulation, cell differentiation, tissue remodeling, DNA repair, apoptosis, oncogenesis, and metastasis [1–4]. AP-1 is a key mediator in the pathogenesis of cancer, arthritis, cardiovascular, and other disease states [2,4–10]. AP-1 binds and regulates numerous genes containing TGAc/gTCA consensus cis regulatory elements (called AP-1 DNA binding sites or AP-1 sites), which generally lie within gene promoter regions [11,12]. Proteins encoded by *jun* family and *fos* family proto-oncogenes compose the AP-1 dimer as Jun-Jun homodimers or as Jun-Fos heterodimers. The *jun* family consists of *c-jun*, *junB*, and *junD*, and the *fos* family consists of c-*fos*, *fra-1*, *fra-2*, and *fosB*. Many genes harboring AP-1 sites are transcriptionally activated in response to endogenous and extracellular stimuli, including hormones, growth factors, oncogenes, tumor promoters such as 12-O-tetradecanoyl phorbol-13-acetate (TPA), and many other carcinogenic agents [3]. Matrix metalloproteinases (MMPs) and genes in many other functional classes are regulated by AP-1 and implement physiological responses elicited by these agents [10]. MMPs play important roles in the degradation of extracellular matrix (ECM) molecules during wound healing, tissue remodeling, and angiogenesis, and in pathological processes including tumor invasion, metastasis, and arthritis [13,14].

Abbreviations: AP-1, activator protein-1; TPA, 12-O-tetradecanoyl phorbol-13-acetate; ECM, extracellular matrix; NCL, nucleolin; NAPSTER, Nucleotide Affinity Preincubation Specificity TEst of Recognition; MMP-13, matrix metalloproteinase-13; HA, hemaglutinin; Topo, topoisomerase-1; GAPDH, glyceraldehydes phosphate dehydrogenase; CMV, cytomegalovirus; β-gal, beta galactosidase; GALV, gibbon ape leukemia virus; NE, nuclear extract; ChIP, chromatin immunoprecipitation; PCR, polymerase chain reaction; bp, base pair; DMSO, dimethyl sulfoxide; YB-1, Y-box binding protein-1.

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Received 19 January 2007; Revised 26 April 2007; Accepted 27 April 2007

DOI 10.1002/mc.20358

Nucleolin (NCL) is an abundant and ubiquitous protein (see Reference [15] for review). NCL binds to several distinct nucleotide sequences in RNA and DNA [16,17]. NCL is a major component of the nucleolus, and is also present in the nucleoplasm and cytoplasm [16]. NCL has been implicated in numerous functions, including chromatin condensation and remodeling, processing and stabilization of RNA, and activation and repression of transcription [15–23].

The Nucleotide Affinity Preincubation Specificity TEst of Recognition (NAPSTER) microscale DNA affinity chromatography-based binding assay was devised to identify new sequence-specific DNA binding proteins [24,25]. Using NAPSTER, we previously identified a 97 kDa protein that binds to a consensus AP-1 DNA sequence [26]. We now report that p97 is nucleolin. Nucleolin binds in vitro and in vivo to the promoter sequence of the matrix metalloproteinase-13 (MMP-13) gene, in the vicinity of the AP-1 site. Overexpression of nucleolin represses transactivation from a minimal AP-1 reporter sequence, and from an MMP-13 promoter reporter sequence [27,28].

MATERIALS AND METHODS

Reagents

Reagents and supplies not described here were purchased from vendors cited [25,29].

Antibodies

Anti JunD antibodies (αJunD; [29]), rabbit anti NCL antibody (αNCL; Catalog #sc-8031), αhemaglutinin (αHA; Cat. #sc-805), and αTopoisomerase I (Cat. #sc-10783), were from Santa Cruz Biotechnology (Santa Cruz, CA). A second rabbit αNCL antibody was from Medical Biological Laboratories Co. Ltd. (MBL, Nagoya, Japan; Cat. #M019-3). αGlyceraldehyde phosphate dehydrogenase (GAPDH) was a mouse monoclonal from Research Diagnostics, Inc. (Flanders, NJ; Cat. #RDI-TRK5G4-6C05). Rabbit nonimmune IgG was from Active Motif (Carlsbad, CA). Peroxidase conjugated anit-rabbit and anti-mouse secondary antibodies were from Calbiochem (San Diego, CA).

Immunoblotting Analyses

Western immunoblotting was performed as described [25,26,29]. Immunoblotting with αNCL and with αJunD were performed at an antibody concentration of 0.5 μg/ml. αTopoisomerase I and αHA-tag were used at 0.2 μg/ml. αGAPDH was used at 0.4 ng/ml.

Plasmids

pcDNA3.1(+) was from Invitrogen (Carlsbad, CA). HA-tagged full length NCL expression construct pNtag4 under the control of the constitutive cytomegalovirus (CMV) promoter was a gift of Dr. N. Maizels (U. Washington). pSVβgal plasmid harboring a full length beta galactosidase (β-gal) gene was from Promega (Madison, WI). The CMV expression construct pcDNAc-Fos, which harbors a full length human c-*fos* gene insert, was a gift of Dr. N. Colburn (NCI-FCRDC, Frederick, MD). Wild-type (wt) and mutant (mut) AP-1 luciferase constructs harboring three tandem copies of the wt AP-1 sites (5′-agcca gagaaatagatgagtcaacagc-3′, AP-1 sequence underlined) or mut AP-1 sites (5′ agccagagaaatagag**g**gagtc-**t**acagc-3′, AP-1 sequence underlined, mutations in bold) from the gibbon ape leukemia virus long terminal repeat (GALV-LTR) were constructed as described [25]. MMP-13 promoter reporter constructs containing wt and mut AP-1 sites were a gift of Dr. C. Brinckerhoff (Dartmouth College; [30]).

Cell Culture

Human HT29 adenocarcinoma cells and human HeLa cervical carcinoma cells were grown as described [25].

Nuclear Extract Preparation

Nuclear extracts (''NE'') from human HT29 cells and HeLa cells treated with TPA were made as described [24–26,29]. NE from human MCF-7 breast cancer cells was purchased from Active Motif.

NAPSTER AP-1 DNA Binding Assays

NAPSTER assays were performed with streptavidin beads coupled to double-stranded AP-1 oligos as described [26]. Beads attached to the wt GALV oligo harbored a 27bp 1X GALV sequence, the same as that which is inserted into the GALV luc reporter constructs. Other assays employed a sequence from the human MMP-13 promoter, 5′-CCTATCCA-TAAGTGATGACTCACCATT-3′.

NAPSTER employed three samples in which NE was subjected to batchwise chromatography with GALV or MMP-13 DNA beads. Sample I, direct mixing of AP-1 DNA beads with NE; samples II and III, AP-1 DNA mixing of beads with NE after preincubation with 2.5-fold molar excess of wt or mutant (mut) AP-1 DNA oligo in solution, respectively. Mutated GALV and MMP-13 oligos harbored two mutations in the AP-1 site: 5′-**G**GAGTC**T**-3′, mutations in bold.

Purification of p97

1.8 mg HT29 NE was dialyzed against buffer Z and subjected to batchwise affinity chromatography by pre-clearing with 120 μg of mut AP-1 DNA conjugated to wt GALV AP-1 NAPSTER beads in the presence of 3.6 μg/ml polydI/dC. Samples were incubated for 60 min on a rotating clip wheel at 4°C, and centrifuged for 60 s at 9500*g*. Mut beads were discarded. Supernatant samples were divided into three equal samples I, II, and III, containing

600 μg each of NE protein. Samples II and III were subjected to pre-incubation with 50 μg of wt (sample II) or mut (sample III) unconjugated GALV AP-1 oligos followed by DNA affinity chromatography with wt AP-1 DNA beads containing 20 μg of wt AP-1 DNA. Samples were incubated and washed as described for NAPSTER analyses [24].

Pellets for samples I, II, and III were subjected to SDS–PAGE [26,29]. Samples were electrophoresed overnight at 5 mA in a minigel apparatus (BioRad Laboratories, Hercules, CA), such that the 30 kDa molecular weight marker reached the bottom of the gel. The gel was stained with Coomassie brilliant blue. Gel bands corresponding to purified 97 kDa protein species in samples I and III, and to that Rf position in sample II (which lacked stainable quantities of p97 protein), representing about 200–400 fmol of protein, were excised for further analyses.

Mass Spectrometric Mass Spectrometry Fragmentation Sequencing Analysis of p97

Gel bands were submitted for mass spectrometric mass spectrometry fragmentation sequencing analyses (''MS/MS sequencing'') at the Yale HHMI Biopolymer Laboratory/W.M. Keck Foundation Biotechnology Resource Laboratory (http://info.med.yale.edu/wmkeck/prochem). Proteins were trypsinized for 18 h, eluted, and analyzed by MS/MS. Liquid LC-MS/MS chromatography was used for sample I of p97 and nanospray MS/MS was used for samples II and III. All spectra were searched manually and by using the Mascot peptide mass fingerprint algorithm for protein identification (http://www.matrix-science.com/search_form_select.html).

Gel Shift Assays

Electrophoretic mobility shift assays (gel shift assays were performed with NE from human MCF-7 breast cancer cells as described [29].

Transactivation Assays

2×10^5 adherent HeLa cells were plated onto 6 well 35 mm dishes. Twelve hours after plating, luciferase reporter constructs harboring wt or mut AP-1 sites were transiently co-transfected with 1 μg pNtag4, HA-tagged NCL CMV expression construct, and 600 ng of pSVβgal expression vector, as described [25]. Cells that received no transfected pNtag4 were transfected with 1 μg pcDNA3.1(+) control vector (InVitrogen). Ten nanograms of c-fos expression construct was transfected in some experiments. Cells were harvested at 24 h post-transfection and assayed for luciferase and β-gal activities [25].

Chromatin Immunoprecipitation Assays

HeLa cells were treated for 24 h with 100 ng/ml TPA. Chromatin was prepared and assayed in chromatin immunoprecipitation (ChIP) assays using a ChIP-IT kit (Active Motif) according to the kit protocol. Five microgram chromatin samples were used for each immunoselection. Chromatin was precleared with 100 μl of Protein G beads for 90 min, immunoselected, and processed as described in the protocol. It was then subjected to polymerase chain reaction (PCR) amplification with 1 unit of Platinum Taq polymerase per reaction (Invitrogen), using the primers indicated in Table 1. Polyacrylamide gel electrophoresis of PCR products in Tris-borate EDTA buffer was performed in 10% native PAGE gels with half of the reaction mix. Gels were visualized with a FluorChem 8900 imaging system (Alpha Innotech, San Leandro, CA) after staining for 5 min in 1 μg/ml ethidium bromide in water.

RESULTS

p97, a New Protein That Binds the AP-1 DNA Binding Site

NAPSTER is an analytical DNA affinity chromatography-based method used to identify new sequence-specific DNA binding proteins in a rapid microscale assay. In the NAPSTER assay, nuclear extracts (NE) either directly undergo AP-1 DNA affinity chromatography (Sample ''I'' in all NAPSTER experiments) or are first preincubated with excess soluble wt oligo (Sample ''II'') or mutated AP-1 oligo (Sample ''III'') prior to chromatography. Mutated AP-1 sequences contain mutations that have been shown in previous studies to abrogate DNA binding and transactivation by AP-1 proteins [12,24,26,29]. Batchwise microscale DNA affinity chromatography is then performed, by means adapted from Lee et al., with numerous modifications [11,24,26]. Samples from NAPSTER isolation are then subjected to SDS–PAGE and Western immunoblotting with antibodies against the proteins of interest. Using NE from human HT29 cells, we observe specific binding of all seven Jun and Fos AP-1 subunits to the AP-1 DNA binding site [25].

In previously published studies, we had identified a 97 kDa protein that binds specifically to the AP-1 DNA binding site in NAPSTER [26]. In the current work described herein we purified p97 protein from crude HT29 nuclear extracts so that it could be identified by mass spectrometric mass spectrometry fragmentation sequencing analyses (''MS/MS sequencing'').

p97 protein was purified by a three-step protocol from HT29 NE in which NE was pre-cleared with AP-1 DNA affinity beads containing a mutated AP-1 site, followed by NAPSTER purification with wt AP-1 DNA affinity beads, and SDS–PAGE. As shown in the Coomassie-stained gel in Figure 1, NAPSTER samples I and III but not sample II reproducibly contain p97 protein. A band of approximately 120 kDa also exhibited partially specific binding, but this was not consistently detected. p97 protein bands from samples I and III, and a gel slice at the corresponding

Table 1. MS/MS Fragmentation Sequencing Analyses and Identification of p97 as Nucleolin (*NCL*)

M+H	Mascot match	Manual match	Protein ID	Sequence	Comments
Sample I					
1323.63	Yes		Nucleolin	EAoxMEDGEIDGNK	
995.53	Yes		Nucleolin	NSTWSGESK	
1307.63	No	Yes	Nucleolin	EAMEDGEIDGNK	
1322.77	Yes		Nucleolin	GLSEDTTEETLK	
1000.63	Yes		Nucleolin	NDLAVVDVR	
1178.69	Yes		Nucleolin	EVFEDAAEIR	
1160.69	Yes		Nucleolin	SISLYYTGEK	
937.49	Yes		Nucleolin	TGISDVFAK	
1112.65	Yes		Topo I	AEEVATFFAK	
2200.26	Yes		Nucleolin	GLSEDTTEETLKESFDGSVR	
940.61	No	Yes	Nucleolin	GIAYIEFK	
1755.11	No				Poor ion fragmentation
1510.85	No	Yes	Nucleolin	SATEET[IL][QK]EVFEK	
1777.05	No	Yes	Nucleolin	KFGYVDFESAEDLEK	
1648.89	Yes		Nucleolin	FGYVDFESAEDLEK	
1561.85	Yes		Nucleolin	GFGFVDFNSEEDAK	
1594.91	Yes		Nucleolin	GYAFIEFASFEDAK	
1761.03	No	Yes	Nucleolin	SYSATEETLQEVFEK	
2211.36	Yes		Trypsin		Enzyme used for digestion
Sample II					
1189.73	No	No		DND[IL]M[IL]VR	No match in database
1408.79	No	Yes	DNAliga-selII	AAVWEITGAEFSK	Top Mascot and manual match
1420.79	No	No		T[QK][QK][IL]	Unable to make reasonable match
1383.79	No	No		Y[QK]EGA	Too short for meaningful match
1020.59	No	No			Insufficient fragmentation
1127.59	No	No			Insufficient fragmentation
1023.53	No	No			Very complex spectra
1175.71	No	No		[FoxM]ED	Too short for search
Sample III					
1160.71	No	Yes	Nucleolin	SISLYYTGEK	
1178.69	Yes		Nucleolin	EVFEDAAEIR	
1322.77	Yes		Nucleolin	GLSEDTTEETLK	
1510.85	No	Yes	Nucleolin	TEET[IL][QK]EV	Sequence tag matches nucleolin
1648.93	Yes		Nucleolin	FGYVDFESAEDLEK	
1754.11	No	Yes	Topo I	GPVFAPPYEPLPENVK	
1561.67	Yes		Nucleolin	GFGFVDFNSEEDAK	
937.59	No	Yes	Nucleolin	TGISDVFAK	
1420.89	No	No			Unable to call reasonable sequence
1594.91	Yes		Nucleolin	GYAFIEFASFEDAK	
1201.67	No	Yes	keratin		Probable contaminant of sample
1425.83	No	No			No sequence could be called
1000.65	Yes		Nucleolin	NDLAVVDVR	

MS/MS data for samples I, II, and III isolated from Coomassie stained bands run in SDS–PAGE gels (Fig. 1) are shown. Measured molecular weights (''M + H'') of all peptide fragments are shown in Column 1. Identification of matches in the Mascot Peptide mass fingerprint algorithm are designated in Column 2 (''Mascot Match''). When Mascot Matches were not obtained, manual matches were sought by determining amino acid sequence based upon the difference in masses, and then performing protein Blast homology searches to identify protein matches. Protein identities (''Protein ID'') are designated in Column 4, and peptide sequences are designated in column 5. In column 5, row 1, ''oxM'' designates oxidized methionine. Bracketed amino acids in Column 5 (e.g., [IL]) indicate ambiguous assignments of amino acid identity (in this case residue assignment is either to I or L). Comments in column 6 indicate additional information about data obtained.

R_f position from sample II, were excised and subjected to in-gel tryptic digestion followed by MS/MS sequencing. Since samples I and III contain p97 whereas sample II lacks p97, we predicted that proteins of the same identities would be found in samples I and III but would be lacking in sample II.

Sixteen of the nineteen peptides generated from sample I matched to the protein nucleolin (Table 1). A single peptide in sample I matched to topoisomerase I. Sample I was thus designated as nucleolin (''NCL''). Sample II contained only a small amount of Coomassie-stained protein. Of the eight peptides

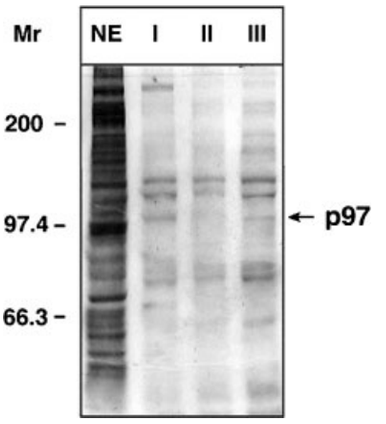

Figure 1. NAPSTER purification of Coomassie-stained p97 protein specifically bound to the AP-1 DNA sequence. p97 protein from NAPSTER samples I, II, and III were each purified and subjected to Coomassie staining from 600 μg of HT29 NE starting material as described in Materials and Methods. I, no competitor oligo; II, preincubation with wt AP-1 oligo; III, preincubation with mut AP-1 oligo. NE, 30 μg of nuclear extract, directly loaded.

isolated and analyzed from sample II, no matches to NCL were found. Seven peptides were present in quantities insufficient to determine their identities, and a single peptide matched to DNA ligase III. Since two peptide matches to the same protein are required to designate a sample as a given protein, no protein designation was made for sample II. For sample III, 9 of the 13 isolated peptides matched to nucleolin either by Mascot or manual inspection. One of the remaining four peptides matched DNA topoisomerase I, one matched to keratin, and two peptides had no matches. Sample III, like sample I, was thus designated as NCL. Based on these collective data we identified p97 as nucleolin.

Nucleolin Binds the AP-1 DNA Sequence in NAPSTER Assays as Detected by Immunoblotting With Anti Nucleolin Antibodies

Immunoblotting of NAPSTER samples from HT29 nuclear extracts with anti nucleolin (αNCL) antibodies was performed to confirm that nucleolin binds specifically to the AP-1 DNA sequence. As shown in Figure 2A (left panel), samples I and III contain a 97 kDa species that is recognized by αNCL, whereas sample II does not. A second independently generated αNCL antibody also recognizes the 97 kDa band in NAPSTER samples I and III (not shown). These data confirm that p97 is NCL. As expected, AP-1 proteins encoded by the *jun* family of proto-oncogenes also bind the AP-1 site in NAPSTER (Fig. 2A, JunD middle panel, and data not shown). NAPSTER with an antibody against topoisomerase I (TopoI), for which a single peptide match was found in each of samples I and III, shows TopoI binding to the DNA, but its binding is not specific, since it is not competed by preincubation with excess wt or mut AP-1 oligo (Fig. 2A, right panel). Specific binding of NCL to the AP-1 site is also observed using NE from human HeLa cervical carcinoma cells, the latter of which is a readily transfectable cell line amenable to gene expression studies (Fig. 2B). Binding of NCL to the AP-1 site using NE from HT29 and HeLa cells was observed in 47 independent NAPSTER assays. Mutation of the GALV AP-1 oligo abrogates specific binding of NCL and AP-1 when the core 7 bp AP-1 sequence is mutated, but not when sequences along the length of the flanking regions are mutated (not shown). NCL and AP-1 also bind AP-1 oligos from the MMP-13 promoter, which contains flanking sequences different from the GALV AP-1 oligos (Fig. 5A and data not shown). Taken together, these data indicate a requirement for the core AP-1 site but not flanking sequences for NCL and AP-1 protein binding to the GALV AP-1 oligo.

NCL has previously been reported to bind to single-stranded DNA [31,32]. To rule out the possibility that NCL is binding to single-stranded DNA that may be attached to the beads in contaminating quantities, we prepared double-stranded AP-1 DNA by annealing the biotinylated DNA strand with a fivefold molar excess of the complementary strand, prior to coupling the double-stranded oligo to the beads. DNA prepared in this manner bound NCL in equal or greater quantities than DNA that had been annealed at an equimolar ratio of the biotinylated to non-biotinylated strands (Fig. 2A, left panel; Fig. 2B). Furthermore, NCL did not bind specifically to either of the two single strands when they were biotinylated, coupled to streptavidin beads, and tested separately by NAPSTER (not shown). Therefore NCL binds to a double-stranded AP-1 DNA sequence.

Nucleolin Binds to the AP-1 Site in Gel Shift Assays

In addition to NAPSTER we performed gel shift assays with NE from human MCF-7 breast cancer cells (Fig. 2C). The AP-1 shift is competed by preincubation with wt but not mut unlabeled AP-1 oligo. Preincubation of NE with αc-Jun causes a supershifted band to appear. Preincubation of NE with αNCL significantly blocks the AP-1 shift, whereas non-immune IgG does not. Preincubation of NE with a second independent antibody against NCL also decreases the signal intensity (not shown). These data demonstrate that NCL binding to the AP-1 site is detectable by both NAPSTER and gel shift methods.

Nucleolin Represses Transactivation of AP-1-Dependent Gene Expression

To examine the functional consequences of nucleolin/AP-1 sequence interaction, we generated AP-1 luciferase reporter constructs that harbor AP-1 DNA binding sequences identical to those used in NAPSTER assays [25]. Constructs were generated by subcloning a wt AP-1 site or an AP-1 site with inactivating mutations in it (mut). HeLa cells were transiently co-transfected with wt or mut minimal

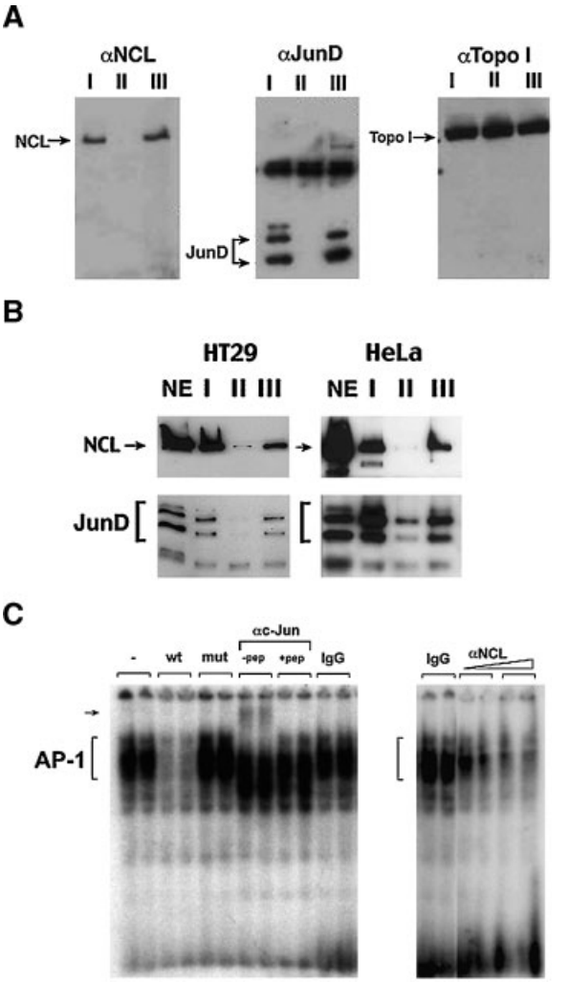

Figure 2. Nucleolin binds in vitro to the AP-1 site. (A) p97 is nucleolin by immunoblotting analyses of nuclear proteins bound to AP-1 DNA in NAPSTER. NASPTER was performed with HT29 NE using the minimal GALV AP-1 sequence. Immunoblotting of samples I, II, and III, as defined in the legend of Figure 1 was performed with antibodies against the antigens indicated above each panel. Left panel, αNCL (from Santa Cruz Biotechnologies); middle panel, αJunD; right panel, αTopoisomerase I. Assays were performed with 300 μg of input NE protein for each of samples I, II, and III for samples immunoblotted with αNCL, and αTopoI, and with 30μg for αJunD. (B) NCL from human HeLa cervical carcinoma cells binds AP-1 DNA in NAPSTER. HT29 and HeLa NE samples containing 300 μg of protein were subjected to NAPSTER analyses for each of samples I, II, and III, followed by Laemmli SDS–PAGE and immunoblotting with αNCL (top row), or αJunD (bottom row). ''NE,'' 30 μg of directly loaded HT29 or HeLa NE. AP-1 streptavidin beads used in this experiment were prepared using AP-1 DNA that had been annealed using a 5:1 ratio of the unbiotinylated strand to the biotinylated strand. (C) Nucleolin binds the AP-1 site in gel shift analyses. Left panel: ''-'', no competitor oligo; ''wt,'' wild-type AP-1 competitor oligo; ''mut,'' mutated competitor AP-1 oligo (same mutations as those used for NAPSTER in Figures 1 and 2). ''αc-Jun,'' 1 μg αc-Jun preincubated with MCF-7 NE. −pep, no peptide antigen competitor preincubation; ''+pep,'' c-Jun peptide competitor antigen preincubated with αc-Jun prior to gel shift assay as described [29]. Arrow indicates position of c-Jun supershift. ''IgG,'' 2 μg of non-immune IgG (Upstate Cell Signaling Solutions, Lake Placid, NY) preincubated with MCF-7 NE prior to addition of labeled probe. Right panel, ''IgG,'' 5 μg of non-immune IgG. Triangle denotes increasing concentrations of anti NCL (''αNCL'') antibody (Active Motif, Inc.) preincubated with MCF-7 NE. Left bracket: 2 μg αNCL; right bracket: 5 μg αNCL. Antibody preincubations were performed at RT for 60 min. Left and right panels were two autoradiograms with the same exposure time from the same experiment.

AP-1 luciferase reporter constructs, a NCL overexpression construct, and a constitutive β-gal expression construct. Cells were then treated with TPA or with dimethyl sulfoxide (DMSO) control solvent and analyzed for luciferase activity. TPA enhances by 3.5-fold AP-1 transactivation of the wt AP-1 reporter construct (Fig. 3A), but has no effect upon the mut AP-1 reporter construct. Overexpressed NCL represses TPA-induced AP-1 transactivation of the wt AP-1 reporter by 72%. NCL also mildy represses transactivation even in the absence of TPA, by 23% (not shown). NCL does not repress transactivation of the mutant AP-1 reporter in TPA-treated or untreated cells, demonstrating that NCL repression is dependent upon the AP-1 DNA sequence. Luciferase activity of the mutated minimal AP-1 reporter construct is sufficiently high that repression would have been readily detectable had it occurred (700–2000 U of raw luciferase activity vs. background levels of 50–80 U). Expression of transfected NCL was confirmed by immunoblotting with αHA-tag antibodies (for detection of HA-tagged NCL; Fig. 3B), and with αNCL (Fig. 3C). Equal loading of protein from lysates of transfected cells was verified by Western immunoblotting with anti GAPDH antibody (not shown). Total intracellular levels of NCL protein, detected by immunoblotting with αNCL, increase at least 1.7-fold as a consequence of NCL overexpression. This moderate increase is expected in light of the fact that NCL is a highly expressed endogenous protein. Overexpressed HA-NCL in Figure 3C appears as a band with slightly less mobility than the endogenous protein in SDS–PAGE, most likely because of the HA-tag present on the overexpressed molecule.

As for TPA-induced AP-1 transactivation, nucleolin represses transactivation induced by overexpressed AP-1 components. Overexpressed human c-*fos* from pcDNAc-*fos*, a CMV c-*fos* overexpression construct, causes 2.5-fold induction of transactivation of the wt AP-1 reporter, but has no effect on the mutated AP-1 reporter (Fig. 4A). NCL causes 73% repression of c-Fos mediated transactivation of the wt AP-1 reporter. Overexpression of HA-NCL and c-Fos proteins expressed by the CMV NCL and CMV c-*fos* overexpression constructs were verified by immunoblotting (Fig. 4B and C). NCL overexpression does not downregulate expression of endogenously expressed c-Fos protein, nor does it downregulate protein expression from the CMV promoter (not shown). Equal loading of protein from lysates of transfected cells was verified by Western immunoblotting with anti GAPDH antibody (not shown). NCL also represses transactivation of a wt minimal AP-1 reporter construct induced by a transfected *junD* overexpression construct, by 78% (not shown).

Several independent measures of cell viability after transfection of NCL were used to assess gene

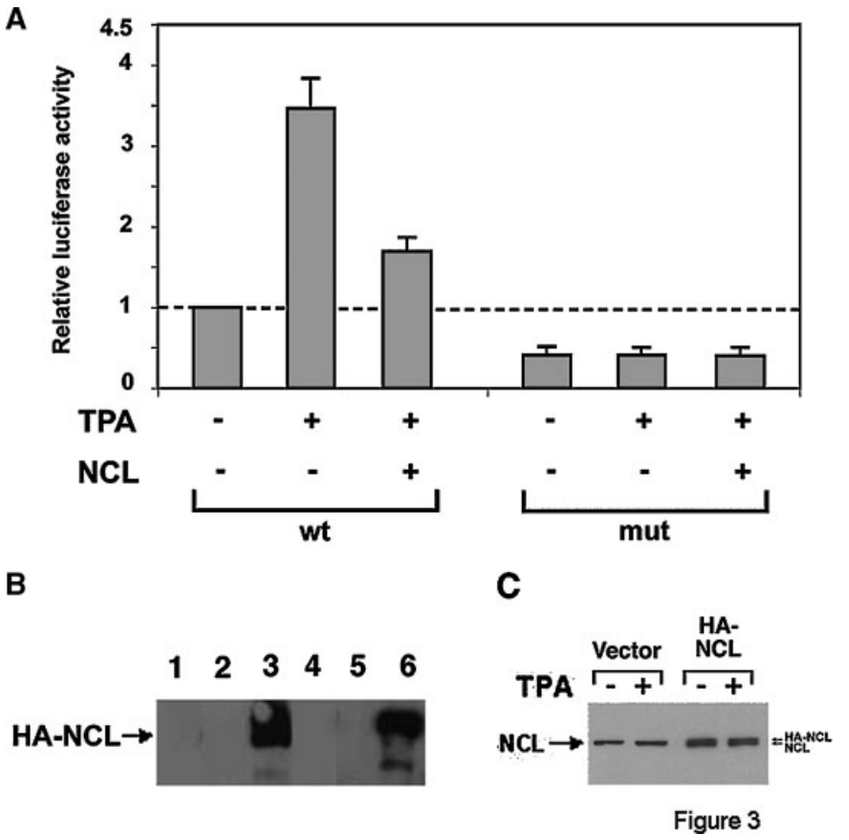

Figure 3. NCL represses transactivation of a minimal AP-1 reporter construct induced by TPA in HeLa cells. (A) NCL represses AP-1-dependent gene transactivation. HeLa cells (2×10^5) were transiently co-transfected with 1 μg of pNtag4 NCL overexpression construct or pcDNA3.1 (+) vector control plasmid, with 2 μg of wild-type (3XwtAP-1-luc) or mutant (3XmutAP-1-luc) GALV AP-1 luciferase reporter constructs, and with 0.6 μg pSVβgal β-galactosidase (β-gal) expression construct. Transfection of pcDNA3.1(+) control vector or of pNTag4 are indicated in the NCL row under the bar graphs with ''−'' and ''+'', respectively. Transfectants were treated with TPA or DMSO 4 h after transfection and were harvested 24 h later (''+'' or ''−'' respectively in TPA row below bar graphs). Cell lysates from independently tranfected duplicate samples were generated and assayed for luciferase and β-gal activity as described [25]. Data were normalized to β-gal expression. Error bars represent standard error of the mean (SEM) of 14 independent experiments for wt and 7 independent experiments for mut transfectants, each performed in duplicate. Statistical significance of differences in relative luciferase activity between wt 3XAP-1 luciferase reporter transfectants with or without TPA or nucleolin were assessed using the statistical test for analysis of variance (ANOVA test, in which the minimum significance level was set at P value of 0.05). Calculated values for TPA-treated wt 3XAP-1 transfectants were compared to corresponding samples within the test group (e.g., compared to wt 3XAP-1 transfected, TPA-untreated; wt 3XAP-1 transfected, TPA-treated + NCL; mut 3XAP-1 transfected, TPA-treated), and were significant to $P < 0.001$. Percentage repression was calculated according to the formula:

$$100 \times \frac{(\text{Activity of wt reporter in TPA-treated cells}) - (\text{Activity of wt reporter plasmid in TPA-treated cells with pNtaq4})}{(\text{Activity of wt reporter in TPA-treated cells}) - (\text{Activity of wt reporter plasmid in TPA-treated cells})}$$

where the values are all in units of relative activity, and where the relative activity of wt reporter in TPA-untreated, pcDNA3.1(+) transfected cells is defined as 1.0. (B) NCL overexpression in transient transfectants. Overexpression of NCL was confirmed in transient pNtag4 transfectants versus pcDNA3.1(+) control transfectants by Western immunoblotting of 10 μl of lysate from each transfectant sample with anti HA-tag antibody. The identities of samples numbered 1 through 6 correspond to the identities of the labeled bar graphs in (A), from left to right. (C) NCL overexpression increases levels of total nucleolin protein. Cells transfected with 1 μg pNtag4 or with pcDNA vector control were harvested, whole cell lysates were generated, and 10 μg of total protein were immunoblotted with αNCL antibody.

toxicity as a possible mechanism of transrepression. Counting of cells in a hemocytometer after transfection with NCL, and immunoblotting of equal volumes of cell extracts with αGAPDH antibodies, indicate little or no change in cell number as a function of expression of the introduced genes (not shown). Trypan blue exclusion assays also demonstrated 97% viability of TPA-treated cells after transfection of NCL, compared to 98% viability of TPA-treated cells transfected with pcDNA3.1(+) control vector. Therefore AP-1 transrepression does not occur through a non-specific toxic mechanism.

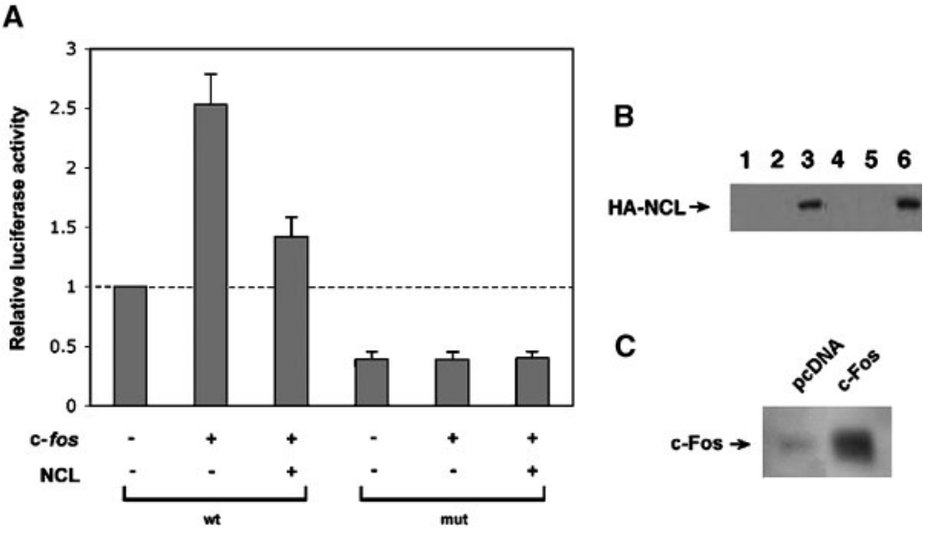

Figure 4. NCL represses transactivation induced by c-fos transfected into HeLa cells. (A) Cells were co-transfected with 10 ng of pcDNAc-fos, plus 1 μg of pNtag4 nucleolin overexpression construct or pcDNA3.1 (+) vector control plasmid, with wild-type or mutant 3XAP-1-luc AP-1 reporter constructs, and with 0.6 μg of pSVβgal overexpression construct. All data were normalized to β-gal expression. c-*fos* row: ''−,'' no c-*fos*; ''+,'' 10 ng c-*fos*. NCL row: 1 μg pcDNA 3.1(+) indicated by ''−,''1 μg pNTag4 indicated by ''+.'' Each value represents the mean ± SEM of five independent experiments, each performed in duplicate and is expressed relative to the control 3XwtAP-1-luc activity in the absence of c-*fos* and without any TPA treatment. Calculated values for *fos*-transfected wt 3XAP-1 transfectants were compared to corresponding samples within the test group (e.g., wt 3XAP-1 transfected, *fos*-untransfected; wt 3XAP-1 transfected *fos*-transfected + NCL; mut 3XAP-1 transfected *fos*-transfected), and were significant to $P < 0.01$, using the ANOVA test. (B) Confirmation of NCL overexpression by immunoblotting analyses with anti HA-tag antibodies. The identities of samples numbered 1 through six correspond to the identities of the labeled bar graphs in (A), from left to right. (C) Transfection of c-fos increases total intracellular levels of c-Fos protein. Cells transfected with pcDNAc-Fos or with pcDNA3.1(+) vector control were harvested following 24 h treatment with 100 ng/ml TPA, whole cell lysates were generated, and 10 μg of total protein were immunoblotted with anti c-Fos antibody.

Collectively, these data demonstrate that NCL specifically represses transactivation of AP1-dependent gene expression of a minimal AP-1 reporter construct.

Nucleolin Binds to the MMP-13 AP-1 Target Gene Promoter In Vitro and In Vivo

MMP-13 is an endogenous AP-1 target gene that encodes an ECM degrading endopeptidase with important roles in tumor invasion and tissue remodeling [14,33]. The MMP-13 promoter contains an AP-1 binding site [34]). As described above, NCL from HeLa nuclear extracts binds in vitro to a minimal AP-1 site. NCL is readily detected in the nucleoplasm of HeLa cells, and overexpression of NCL does not alter the levels of expression or the subcellular localization of any of the seven AP-1 subunits (data not shown). It also does not alter the abundance of post-translationally modified forms of the AP-1 protein subunits, such as phosphorylated c-Jun protein (not shown). Based on these collective data we hypothesized that NCL binds the MMP-13 AP-1 target gene promoter sequence and regulates transactivation in a manner that depends on the AP-1 site.

To determine whether NCL interacts with the MMP-13 sequence in vitro and in vivo, we performed NAPSTER and ChIP analyses with DNA sequences from the human MMP-13 promoter. NAPSTER was performed with an oligo coupled to streptavidin beads, derived from a human MMP-13 promoter sequence containing the AP-1 site. NCL binds to the MMP-13 sequence, and mutational inactivation of the AP-1 site blocks its binding to the sequence (Fig. 5A, upper panel). JunD also binds specifically to the AP-1 site in the MMP-13 sequence (Fig. 5A, lower panel).

For ChIP analyses, PCR primers were designed that amplified a region of the MMP-13 promoter that contains the AP-1 site. PCR primers were designed that flanked the AP-1 site within 64 bp upstream and 78 bp downstream of the AP-1 site (Table 2). A PCR product of the expected size (142 bp) is reproducibly observed using chromatin immunoselected with αNCL antibodies. This signal is of significantly greater intensity than that which is observed when chromatin is immunoselected with control non-immune IgG (Fig. 5B). Similar results were observed in five independent experiments using three independent chromatin preparations. Immunoselection with a second, independently derived antibody against NCL also yields a 142 bp PCR signal that is of greater intensity than the signal that is obtained with non-immune IgG (not shown). Immunoselections

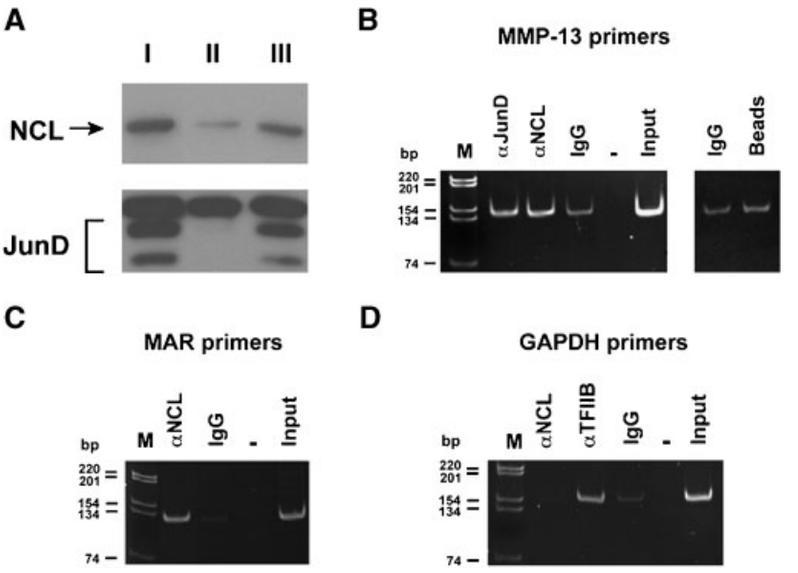

Figure 5. NCL binds to the MMP-13 promoter sequence in vitro and in vivo. (A) NCL and JunD bind to the MMP-13 promoter sequence in a manner that depends upon the AP-1 sequence. NAPSTER was performed with 150 μg NE from TPA-treated HeLa cells and with 10 μg of MMP-13 oligo on DNA affinity beads. Preincubations were performed with 25 μg of unconjugated wt (sample II) or mut (sample III) MMP-13 oligo. αNCL, upper panel; αJunD, lower panel. In addition to the two JunD bands that bind the site, a band that migrates more slowly binds non-specifically, since it is present in all three samples. (B) NCL and JunD bind to the MMP-13 promoter in chromatin immunoprecipitation assays. Immunoselections were performed with αNCL, αJunD, and nonimmune IgG, respectively. ''–,'' PCR amplification performed with H_2O only, without antibodies or IgG (control for PCR background contamination); ''input,'' 5 ng of unselected chromatin subjected to PCR with primers flanking the AP-1 site within 64 bp upstream and 78 bp downstream of the AP-1 site within the MMP-13 promoter. M, markers; bp, base pairs. (C) Nucleolin binds to the matrix attachment region sequence. All samples were PCR amplified with primers that flank within 62 bp upstream and 60 bp downstream of the NCL binding site within the human matrix attachment region (MAR). (D) Nucleolin does not bind to the GAPDH promoter region containing a TFIIB transcription factor binding site. αTFIIB, imunoselection with antibody against transcription factor TFIIB. All samples were PCR amplified with primers that flank within 120 bp upstream and 46 bp downstream of the TFIIB binding site (TATA box) within the human GAPDH promoter.

performed with αJunD yield significantly more PCR product than control IgG, showing that JunD also binds to the MMP-13 promoter sequence. The signal intensity observed for samples incubated with beads in the absence of non-immune IgG is equal to that of samples selected with beads in the presence of non-immune IgG, indicating that the PCR signal detected in IgG samples is attributable to background binding to beads. These data show that NCL binds to the MMP-13 promoter in the vicinity of the AP-1 site. In vitro NAPSTER studies were predictive of in vivo ChIP assay results.

A number of control studies were performed to validate these results. PCR signals for ChIP selections performed with αNCL and αJunD increase linearly as a function of the increased PCR cycle number, and as a function of the quantity of input chromatin (not shown), indicating that observed differences in signal intensity occur within the linear range of detection. NCL is detectable by Western immunoblotting of samples immunoprecipitated with αNCL antibody under ChIP conditions (not shown). Immunoselection with αNCL yields more ChIP PCR product than nonimmune IgG using PCR primers

Table 2. PCR Primers Used for Chromatin Immunoprecipitation Analyses

Primer set	Primer sequence	Annealing temp.	PCR product size
MMP-13	For 5′-CCACGTAAGCATGTTTACCTTCAAGTGAC-3′ Rev 5′-GATGCATCTTGAATGGTGATGCCTGG-3′	63°C	142 bp
GAPDH	For 5′-TACTAGCGGTTTTACGGGCG -3′ Rev 5′-TCGAACAGGAGGAGCAGAGAGCGA-3′	59°C	166 bp
MAR	For 5′-GGCATTTTACAATGGGAAAATGATG-3′ Rev 5′-CACTGGAATTTTTTTGTGTGTATGG-3′	58°C	122 bp

For, forward primers; rev, reverse primers.

that amplify a region of the matrix attachment region (MAR), thus showing binding of NCL to a previously characterized NCL DNA binding sequence (Fig. 5C). Enhanced PCR signal intensity is not observed in αNCL immunoselections compared to control IgG selections employing primers within the MMP-13 coding region 2.6 kb downstream of the of the promoter sequence, indicating that NCL does not bind to this region (not shown). NCL does not bind to sequences within the GAPDH gene promoter, which has no AP-1 sites, since the intensity of the PCR signal detected for αNCL immunoselected samples amplified with primers for the GAPDH gene is not greater than that which is detected for samples immunoselected with non-immune IgG (Fig. 5D). However, binding of the TFIIB transcription factor to its binding site within the GAPDH amplified sequence is detected. Taken together, these data show that NCL binds in vivo to the MMP-13 promoter, in the vicinity of the AP-1 site.

Overexpression of Nucleolin Represses Transactivation of the MMP-13 Promoter

TPA induces transactivation of a human MMP-13 luciferase promoter reporter construct containing a single AP-1 site by 2.8-fold (Fig. 6). Overexpressed NCL represses 93% of TPA-induced transactivation of the MMP-13 promoter. Mutational inactivation of the AP-1 site abrogates TPA induction of MMP-13 transactivation, and it abrogates NCL repression. Luciferase activity of the mutated MMP-13 construct is sufficiently high that repression would have been readily detectable had it occurred (300–1500 U of raw luciferase activity for the mutated constructs, vs. 50–80 U of background activity).

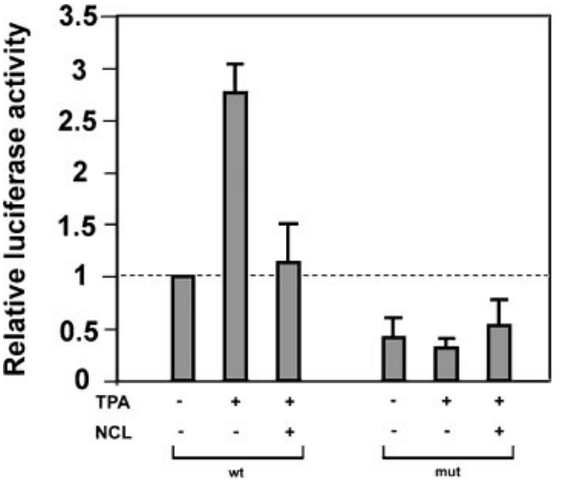

Figure 6. NCL represses MMP-13 transactivation. (A) NCL represses MMP-13 transactivation via the AP-1 site. HeLa cells were transfected with 1 μg of wt or mut human MMP-13 promoter luciferase reporter constructs (5223 bp), along with 1 μg of pNtag4 NCL overexpression construct or pcDNA3.1(+) vector control construct (for ''–'' NCL samples). Samples were treated with 100 ng/ml TPA or with DMSO control solvent for 24 h prior to harvest. Each value represents the mean ± SEM of seven independent experiments, each performed in duplicate and is expressed relative to the control wt MMP-13 luciferase activity in the absence of NCL and without any TPA treatment. Calculated values for TPA-treated wt MMP-13 transfectants were compared to corresponding samples within the test group (e.g., wt MMP-13 transfected, TPA-untreated; wt MMP-13 transfected, TPA-treated + NCL; mut MMP-13 transfected, TPA-treated), and were significant to $P < 0.001$ using the ANOVA test.

DISCUSSION

DNA binding of NCL has been shown to regulate gene expression at several transcriptional promoter motifs [16,35–38]. These include the bone-specific osteocalcin promoter element OC I, the human papilloma virus-18 enhancer, and the B sequence motif within the alpha-1 acid glycoprotein promoter [35,36,38]. NCL has been shown to activate transcription from Eμ, Fp, HPV-18, and c-*myc* promoter and enhancer sequences, and represses transcription from the alpha-1 acid glycoprotein promoter [35,37,38]. Nucleolin is required for polymerase I transcription of rDNA, and it functions as a histone chaperone for remodeling of nucleosomes in chromatin [22,39]. This is the first report of NCL binding to an AP-1 site and controlling AP1-dependent gene expression. Whereas NCL has been shown to post-transcriptionally upregulate matrix metalloproteinase-9 (MMP-9), this is the first report of NCL transcriptional regulation of a matrix metalloproteinase.

A number of bZIP proteins of the AP-1 superfamily bind specifically to the minimal seven base pair AP-1 consensus sequence, and regulate transactivation [40–48]. Non-bZIP transcription regulatory proteins have been found to repress AP1-dependent transactivation [38,49–58], (see Reference [59] for review). Binding of non-bZIP proteins to the AP-1 site has not been demonstrated, and in many instances has been ruled out. Recently we reported that Y-box binding protein-1 (YB-1) binds to the AP-1 site and also represses AP-1 transactivation [25]. NCL and YB-1 have been found together in several large multi-component ribonucleoprotein complexes, and nucleolin is detected in complexes with YB-1 in GST pulldown assays [60–62]. Future experiments will determine whether YB-1 and NCL form a ternary complex at the AP-1 site. To our knowledge, YB-1 and NCL are the first non-bZIP AP-1 DNA binding proteins outside the AP-1 superfamily that bind the AP-1 site and repress AP-1 transactivation.

The original purifications of AP-1 proteins were performed in groundbreaking studies in 1987 using purification schemes employing AP-1 DNA affinity chromatography [11,12]. Whereas AP-1 was the dominant species in these purifications, NCL binds the AP-1 DNA in greater abundance than AP-1 proteins in NAPSTER purifications, since NCL is visible by Coomassie staining, whereas AP-1 species are only visible upon Western Immunoblotting

analyses (Fig. 1 and data not shown). One of the original AP-1 purification schemes used HeLa cell nuclear extracts [11], which have readily detectable NCL AP-1 DNA binding activity in our hands. It is possible that NCL was isolated in the original work, in which a minor silver-stained species was found at 116 kDa from HeLa cells that co-purified with AP-1 [11]. p116 was not pursued or identified. Or it may be that p116 is poly ADP (ribose) polymerase, a common contaminant in DNA affinity purified protein fractions [63]. It is also possible that NCL was lost in the original purifications. Whereas our purification protocol consists of only two steps, the original purifications consisted of multiple steps performed before the AP-1 DNA affinity chromatography step, including ammonium sulfate precipitation, molecular sieve chromatography, heparin affinity chromatography, and stepwise KCl elution after DNA affinity chromatography. These additional steps may have removed NCL from these preparations.

Binding of NCL to the AP-1 DNA sequence is independent of the binding of AP-1 proteins to the AP-1 site, since immunodepletion of AP-1 with anti PAN Jun/Fos antibodies has no effect upon the binding of NCL to the AP-1 DNA sequence [26]. Furthermore, since we see NCL binding in NAPSTER in excess relative to AP-1 protein binding, binding of NCL would necessarily occur without AP-1 proteins. The possibility that NCL binds the AP-1 site along with other proteins, such as YB-1, has not been ruled out, and is under investigation.

MMP-13 belongs to the large family of MMP enzymes, and has broad substrate specificity for ECM macromolecules [14,33]. MMP-13 expression in healthy tissues is restricted to circumstances in which rapid remodeling of ECM within target tissue is required [64], particularly in development and tissue remodeling in bone and cartilage [65]. MMP-13 is also upregulated in capillary tube formation in vitro, and may play an important role in endothelial cell invasion during angiogenesis [66,67]. Elevated MMP-13 activity outside of these venues is pathological. It causes connective tissue destruction in arthritis [65]. It is observed in many cancerous tumors, and is associated with invasive, metastatic behavior and poor disease prognosis, and it also mediates mammary carcinogenesis [14,33,68]. Overexpression of MMP-13 enhances invasiveness, and targeted inhibition of MMP-13 inhibits invasion and tumor growth [27,64]. Transgenic mice overexpressing *jun* or *fos* AP-1 subunits or with targeted knockout of *jun* or *fos* AP-1 subunits have demonstrated that AP-1 mediates invasion and all of the above biological processes [2,14,33,67–70]. Transgenic overexpression of *jun* and *fos* upregulates expression of MMPs and targeted disruption downregulates expression of MMPs. C-*fos* overexpression in transgenic mice induces MMP-13 expression and causes osteosarcomas, whereas c-*fos* knockout mice show significantly reduced MMP-13 induction [71,72]. Ablation of the junB gene in endothelial cells blocks transactivation of the MMP-13 promoter and inhibits capillary tube morphogenesis [67]. These and other studies demonstrate that MMPs including MMP-13 are key downstream AP-1 target genes that implement matrix degradation in invasion and a range of other physiological and pathological processes.

Induced expression of MMP-13 in cells requires binding of AP-1 to its binding sequences within the MMP-13 promoter [69,70]. Since AP-1 plays important roles in mediating tumor invasion, tissue remodeling, wound healing, angiogenesis, arthritis, and other processes, and since NCL represses transactivation of the AP-1 sequence, it is logical to hypothesize that NCL may mediate one or more of these processes. Future experiments will determine whether NCL mediates some of these processes by binding and downregulating AP-1 transcriptional activity within the MMP-13 promoter.

ACKNOWLEDGMENTS

This work was supported by NIH grant CA73783 to LB; by a Minority Supplement to the grant from the NIH Comprehensive Minority Biomedical Branch to JCT; and by grants to LB from the Elsa U. Pardee Foundation, the Max and Victoria Dreyfus Foundation, and the Texas A & M HSC Research Enhancement Program. We are grateful to Dr. Geoffrey Kapler, Dr. David Giedroc, Dr. Julian Leibowitz, and Dr. Van Moudrianakis for evaluation of the manuscript, to Dr. Larry Dangott, Kathryn Peebles and Dave Shahani for experimental and administrative assistance, and to Barry Datlof for editorial assistance.

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