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Human Hematopoietic Cell Specific Nuclear Protein MNDA Interacts With the Multifunctional Transcription Factor YY1 and Stimulates YY1 DNA Binding

Jingping Xie, Judith A. Briggs, and Robert C. Briggs*

Department of Pathology, Vanderbilt University Medical School, Nashville, Tennessee 37232

Abstract The human myeloid nuclear differentiation antigen, MNDA, is expressed only in myelomonocytic and a subset of B lymphoid hematopoietic cells. MNDA is uniformly distributed throughout the interphase cell nucleus and associates with chromatin, but does not bind specific DNA sequences. We recently demonstrated that MNDA binds nucleolin and nucleophosmin/NPM/B23 and both of these nuclear proteins bind the ubiquitous zinc finger transcription factor YY1. Investigations of the possible effect of MNDA on the interaction between YY1 and NPM, showed that MNDA bound YY1 directly under both in vitro and in vivo conditions. The MNDA-YY1 interaction enhanced the affinity of YY1 for its target DNA and decreased its rate of dissociation. The N-terminal half (200 amino acids) of MNDA was sufficient for maximum enhancement of YY1 DNA binding and a portion of this sequence was responsible for binding YY1. MNDA participated in a ternary complex with YY1 and the YY1 target DNA element. The results show that MNDA affects the ability of YY1 to bind its target DNA sequnce and that MNDA participates in a ternary complex possibly acting as a cofactor to impart lineage specific features to YY1 function. J. Cell. Biochem. 70:489–506, 1998.

Key words: hematopoiesis; protein interaction; EMSA; nucleolin; nucleophosmin/NPM/B23

A large number of tissues and cells have been examined using immunoblotting and immunohistochemistry to establish that human myeloid cell nuclear differentiation antigen (MNDA) expression is restricted to hematopoietic cells [Briggs et al., 1994a,b; Cousar and Briggs, 1990; Goldberger et al., 1986; Kao et al., 1996, 1997; Miranda, Briggs, Jensen, Kinney, Cousar, unpublished communications]. Analysis of normal bone marrow cells showed that the level of MNDA expression increases during maturation of myeloid cells beginning in the promyelocyte and becoming maximal in the granulocyte [Cousar and Briggs, 1990; Miranda et al., 1998b]. MNDA is also expressed in monocytes and activated histiocytes and a subset of B lymphocytes localized in the mantle zone of lymphoid tissues (Miranda, Briggs, Jensen, Kinney, Cousar, unpublished communications). In reactive processes, a greater number of positive mantle zone cells with higher level of expression is observed. MNDA expression appears generally to be retained following neoplastic transformation of MNDA positive cells (Miranda, Briggs, Jensen, Kinney, Cousar, unpublished communications). In certain neoplasms (e.g., acute promyelocytic leukemia) the level of MNDA expression exceeds that of the normal cell counterpart suggesting that MNDA might be involved in the pathogenesis of some hematopoietic cell neoplasms (Miranda, Briggs, Jensen, Kinney, Cousar, unpublished communications). MNDA function appears associated with maturation and/or activation of human myelomonocytic cells and a subset of B lymphocytes.

The presence of a 200 amino acid (aa) conserved C-terminal sequence, regulation of expression by interferon α and gene mapping have identified MNDA as a member of the *gene 200 cluster* family [Briggs et al., 1992, 1994a; Burrus et al., 1992; Dawson et al., 1995]. Characteristics of MNDA, including lineage and stage specific expression, nuclear localization [Cousar and Briggs, 1990; Goldberger et al., 1986], association with chromatin [Duhl et al., 1989; Gaczynski et al., 1990] and binding to

J. Xie's current address is National Institutes of Health, National Institute of Dental Research, Bethesda, MD 20892.

*Correspondence to: Dr. Robert C. Briggs, Department of Pathology, TVC 4918A, Vanderbilt University Medical School, Nashville, TN 37232-5310. E-mail: bob.briggs@mcmail.Vanderbilt.edu

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nuclear proteins [Xie et al., 1995, 1997b] are consistent with the possibility that MNDA is involved in regulatory mechanisms that effect lineage specific gene expression. A second human gene, AIM2, related to MNDA, was recently identified and found to be expressed preferentially in hematopoietic tissues [DeYoung et al., 1997]. On the other hand, constitutive, tissue/cell specific expression of the mouse 200 cluster genes has not been reported. Although, one of the mouse gene products, D3, was induced by interferon in a cell specific manner [Tannenbaum et al., 1993]. The product of the mouse gene p202 has been studied extensively and shown to bind a number of nuclear proteins and transcription factors, NF-κB p50 and p65, AP-1 c-Fos and c-Jun, Rb, E2F, and the murine homolog of the human p53-binding protein 1 [Datta et al., 1996; Min et al., 1996]. The binding of p202 to these proteins influences their transcriptional regulatory activities and is believed to be related to the growth suppressing effect of p202 when it is overexpressed [Datta et al., 1996; Min et al., 1996].

MNDA binds itself, nucleolin, nucleophosmin (NPM/B23) and the NPM-MLF chimera, a product of a fusion gene associated with the t(3;5) found in acute myeloid leukemia and myelodysplastic syndrome [Xie et al., 1995, 1997a,b]. The ability of MNDA and p202 to bind specific nuclear proteins is consistent with the possibility that these proteins generally act as coregulators altering gene expression through their interactions with transcription factors or other nuclear proteins.

In the present investigation, MNDA was observed to interact directly with the zinc finger transcription factor YY1 and stimulate its ability to bind specific DNA. The effect on DNA binding depended on the direct interaction between MNDA and YY1. A ternary complex of MNDA, YY1 and the YY1 target DNA was formed which most likely accounts for the ability of MNDA to increase the association rate and also decrease the rate of YY1 dissociation from DNA. The results deomonstrate that MNDA forms a complex with YY1 affecting the interaction between YY1 and the target YY1 DNA element.

MATERIALS AND METHODS

Cell Lines and Nuclear Protein Extracts

HeLa cells were grown in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% defined bovine calf serum (Hyclone Laboratories, Logan, UT), 2 mM glutamine and antibiotics. Cos 7 cells were grown in the same medium, but with 10% fetal bovine serum (Life Technologies, Inc.). MNDA expressing human leukemic U937 as well as MNDA negative K562 cells were cultured in RPMI 1640 with 15% fetal bovine serum as previously described [Goldberger et al., 1986]. Nuclear protein extracts of both HeLa and U937 cells were prepared as described by Dignam et al. [1983] except that the U937 cells were initially lysed using hypotonic conditions as described by Hallick and Namba [1974].

SDS Polyacrylamide Gel Electrophoresis (PAGE), Western Blot, and Far Western Assays

SDS-PAGE was performed as described by Laemmli [1970] and proteins were electrotransferred onto nitrocellulose membranes as described by Towbin et al. [1979] for Western/immunoblot assay. Anti-MNDA rabbit polyclonal and rat monoclonal antibodies were obtained from Calbiochem (San Diego, CA). MNDA antibodies obtained from Chemicon (Temecula, CA) and Stratagene (LaJolla, CA) were equally effective. Anti-YY1 polyclonal antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The procedures for preparing blots and detection of protein-protein binding by the Far Western assay were described in detail in a previous report [Xie et al., 1995].

Expression Constructs and Deletion Mutations Used for Transfection

The full length MNDA coding sequence minus the AUG initiation codon was excised from pQE30-MNDA, described earlier [Xie et al., 1995], and inserted into pSG424 expression vector [Sadowski and Ptashne, 1989]. This construct, designated pSG424-MNDA, expresses a Gal4 DNA binding motif (aa 1-147) fused to the N-terminus of the MNDA protein. An additional polylinker site was inserted immediately downstream of the MNDA stop codon to aid in the production of mutant constructs. Wild type MNDA was expressed by insertion of the MNDA coding sequence including the initiation codon into pSG5 (Stratagene), designated pSG5-MNDA and pBK-RSV (Stratagene), designated pBK-MNDA. Constructs with the *MNDA* coding sequence inserted in an antisense orientation were prepared for use as negative controls.

Expression and Purification of Recombinant Protein Products

pQE30-MNDA, described previously [Xie et al., 1995], was used to prepare his-tagged recom-

binant MNDA. C-terminal deletion mutations were prepared from pQE30-MNDA using the Erase-a Base System (Promega). A GST-MNDA fusion construct was prepared by insertion of the MNDA coding sequence minus the AUG codon into pGEX-3 X (Pharmacia, Piscataway, NJ). pGST-MNDA was then used as a template for inverse long distance PCR employing a proof reading polymerase (Pfu, Stratagene) to obtain eight mutated plasmid constructs containing sequentially deleted blocks of sequence encoding approximately 50 amino acids. To prepare the mutant PCR products, eight pairs of primers in opposite orientation, flanking the region to be deleted, were designed with the aid of Oligo® software (National Biosciences, Inc.) which would result in continued in-frame translation of the MNDA after blunt ligation of the PCR product.

All constructs were tested for ability to express the expected sized products by electrophoretic or Western blot analysis of appropriate bacterial (BL21 host cell) or affinity purified mammalian cell lysates. Precise location of random 3′ terminal deletion points in the constructs generated using the Erase A Base System were determined by sequencing. Specified mutations were verified by loss/gain of unique restriction sites and/or expected alteration in the size of PCR products relative to nonmutated constructs.

Evaluation of MNDA Sequence Specific DNA Binding Activity

Immobilized native or recombinant his-tagged MNDA [Xie et al., 1995] was used to select target DNA binding elements from pools of degenerate oligonucleotides (n = 16 and n = 25) flanked by defined cloning sequences [Mao et al., 1994; Wright and Funk, 1993]. Six rounds of cyclic amplification and selection were performed with screening of amplified products beginning after three rounds using electrophoretic mobility shift assay (EMSA) [Mao et al., 1994; Wright and Funk, 1993]. Purified recombinant Ga14-MNDA containing the Ga14 DNA binding domain (described above) was used as a positive control.

Expression and Purification of Recombinant Proteins

Production and purification of his-tagged MNDA and its derivatives were described earlier [Xie et al., 1995]. Expression of GST-tagged MNDA, the deletion mutants or GST-YY1 (the pGEX/YY1 construct kindly provided by Dr. Edward Seto, University of Texas Health Science Center, San Antonio, TX) was induced by addition of 0.5 mM IPTG. After 4 h of induction, the bacteria were pelleted and resuspended in PBS supplemented with 2 mM EDTA, 1 mM PMSF and 0.5% Triton X-100 (4 ml per 100 ml culture volume). The cells were disrupted by sonication and centrifuged to remove cellular debris. The supernatant was incubated with 100 µl prewashed glutathione Sepharose® 4B beads (Pharmacia) for 1 h at 4°C. The beads were then washed five times with 10 bed volumes of binding buffer. Purity of the GST fusion proteins was greater than 90% as assessed by Coomassie blue stained SDS PAGE gels. Glutathione bead immobilized GST-fusion proteins and GST without a fusion partner were used directly in co-precipitation assays. Proteins used in EMSA were eluted from the glutatione beads with 10mM reduced glutatione (Sigma, St. Louis, MO) in PBS, 2 mM EDTA, 0.1% Triton X-100 and then dialyzed against PBS, 2 mM EDTA, 0.1% Triton X-100 to remove residual glutatione prior to concentration using Centricon-10 (Amicon Inc., Beverly, MA). Recombinant Sp1 was produced from pBK-RSV-Sp1 (kindly provided by Dr. Michael Waterman, Vanderbilt University, Nashville, TN) using the same procedure and used without purification.

In Vitro Transcription Coupled Translation

pSG5-MNDA, pBK-RSV-Sp1, and pCMV-YY1 (kindly provided by Dr Y. Shi, Harvard Medical School, Boston, MA) were used as templates for in vitro translation of MNDA, Sp1, and YY1. One µg of supercoiled plasmid in 50 µl reaction volume was used in the TNT® T7 RNA polymerase coupled rabbit reticulocyte lysate system (Promega; yield of 1–2 µg translated protein per 50 µl reaction). ^{35}S-methionine was added to reactions to label the synthesized protein for coprecipitation and Far Western assays.

Coprecipitation of Endogenous and In Vitro Translated Proteins

Affinity matrices used in coprecipitation experiments included anti-MNDA monoclonal antibody coupled to beads (McAb-beads) [Hudson et al., 1988] or McAb-beads preincubated with his-tagged rMNDA as described previously [Xie et al., 1995]. The immobilized GST fusion proteins were prepared as discussed above. Nuclear

extracts or in vitro translated proteins were incubated with the affinity matrix in binding buffer (10 mM Tris-HCl, pH 7.5, 100 mM KCl, 10% glycerol, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.1% NP-40, 1 mM DTT) for 18 h at 4°C with constant rotation. The beads were washed five times with a 50- to 100-fold volume of binding buffer and then heated in SDS gel sample buffer to solubilize bound proteins for SDS-PAGE. Proteins were detected by Western blot or autoradiography.

Assays for Detection of an In Vivo Interaction Between MNDA and YY1

Cos 7 cells were transfected with pSG424-MNDA or pSG5-MNDA to express Gal4-MNDA or wild-type MNDA. MNDA and associated proteins were isolated by co-immunoprecipitation from cell lysates with McAb-beads. The precipitated proteins were examined for the presence of both MNDA and YY1 by Western blot. Cos 7 cells transfected with the antisense MNDA construct served as the negative control. The same procedures were used to isolate MNDA and associated proteins from U937 cell nuclear protein extracts by incubation with McAb-beads. A nuclear protein extract prepared from MNDA negative K562 cells served as the negative control.

The mammalian two hybrid system [Liu and Green, 1990] involved the cotransfection of pSG424-MNDA, a YY1-VP16 expression contruct (pCMV-YY1-VP16, kindly provided by Dr. Y. Shi, Harvard Medical School, Boston, MA) and pGL2-Gal4 into Cos 7 cells. Controls included replacement of the Gal4-MNDA with pSG424 (Gal4 only) or pSG5-MNDA (MNDA only). All transfections consisted of mixtures containing 2 µg of each DNA and were normalized by addition of the pSV-β-gal plasmid.

Electrophoretic Mobility Shift Assay (EMSA)

The DNA probe containing a YY1 consensus binding site used for EMSA was obtained from Santa Cruz Biotechnology Inc. (5′-CGCTCCGCGGCCATCTTGGCGGCTGGT-3′). A DNA probe for Ets binding (5′-GATTACTTACTACAAAGAGGAACTGACCACTTTCT-3′) was also used. Binding reactions included nuclear protein extract (0.5 µg protein), 1.0 µl in vitro translated product or purified recombinant protein as indicated. Reactions were performed in a 20 µl volume containing 100 mM KCl, 20 mM Hepes (pH 7.9), 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, 4% Ficoll, 3 µg of poly(dI-dC) and 0.25 ng of purified, labeled DNA probe (specific activity, 2×10^6 cpm/ng). In competition assays, cold ds oligonucleotide at 50-fold molar excess was added to the reaction mixture prior to addition of the labeled probe. Incubation times are indicated in the figure legends. When purified recombinant proteins were tested, 10 µg of BSA was added to the reaction mixture as carrier protein. DNA-protein complexes were resolved on 6% polyacrylamide gels in Tris-glycine buffer, pH 8.3 containing 1 mM EDTA (TGE) and complex formation was quantified using public domain image analyzing software (NIH Image, developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image).

YY1 DNA Element Affinity Matrix Preparation and DNA-Protein Complex Binding

Ten nmol of sense YY1 oligonucleotide was biotinylated using reagents and procedures recommended in the FluoroAmp® T4 Kinase Biotin Oligonucleotide Labeling System (Promega). The biotinylated oligonucleotide was purified by ethanol precipitation and Sephadex® G-50 column chromatography before annealing to unlabeled, antisense oligonucleotide. The annealed product was gel purified and immobilized on streptavidin agarose beads (Pierce Chemical Co., Rockford, IL) at 140 ng DNA/µl beads. Oligonucleotide beads (1 µl) were incubated with indicated amounts of HeLa or U937 nuclear extract with or without addition of rMNDA in a 100 µl reaction cocktail containing 7.5 µg poly(dI-dC) in EMSA buffer. After a 15 min incubation (22°C), the reaction was terminated by the addition of 1 ml cold 0.5 × TGE buffer and then quickly washed two times with same buffer. The bound protein was solublized in SDS gel buffer and analyzed by Western blot.

RESULTS

Evaluation of MNDA Sequence Specific DNA Binding Activity

Repeated attempts to select specific DNA binding elements from pools of random oligonucleotides recognized by MNDA failed to produce a consensus site. Length of the random sequence was varied, source of MNDA (nuclear protein extract or rMNDA) and selection matrix (McAb-beads or Protein A beads) were tested. Each experiment included a positive

control sequence specific DNA binding protein (Gal4 binding domain fused to the MNDA) that was effective in selecting specific DNA sequence as detected by EMSA after four rounds of amplification/selection (data not shown).

MNDA Interacts With YY1 Both In Vitro and In Vivo

To directly test whether MNDA binds to YY1, coprecipitation experiments were completed using affinity matrices or McAb-beads. The results of experiments shown in Figure 1 confirmed that both endogenous YY1 in U937 nuclear protein extracts (Fig. 1A) and in vitro translated YY1 (Fig. 1B) bound rMNDA. Cos 7 lysates from MNDA transfected cells were used for immunoprecipitation of a native YY1-MNDA complex (Fig. 1C). Endogenous MNDA and YY1 found in U937 cell nuclei were also co-immunoprecipitated with McAb-beads (Fig. 1D). Recombinant Sp1 and in vitro translated Sp1 did not coprecipitate with MNDA (data not shown).

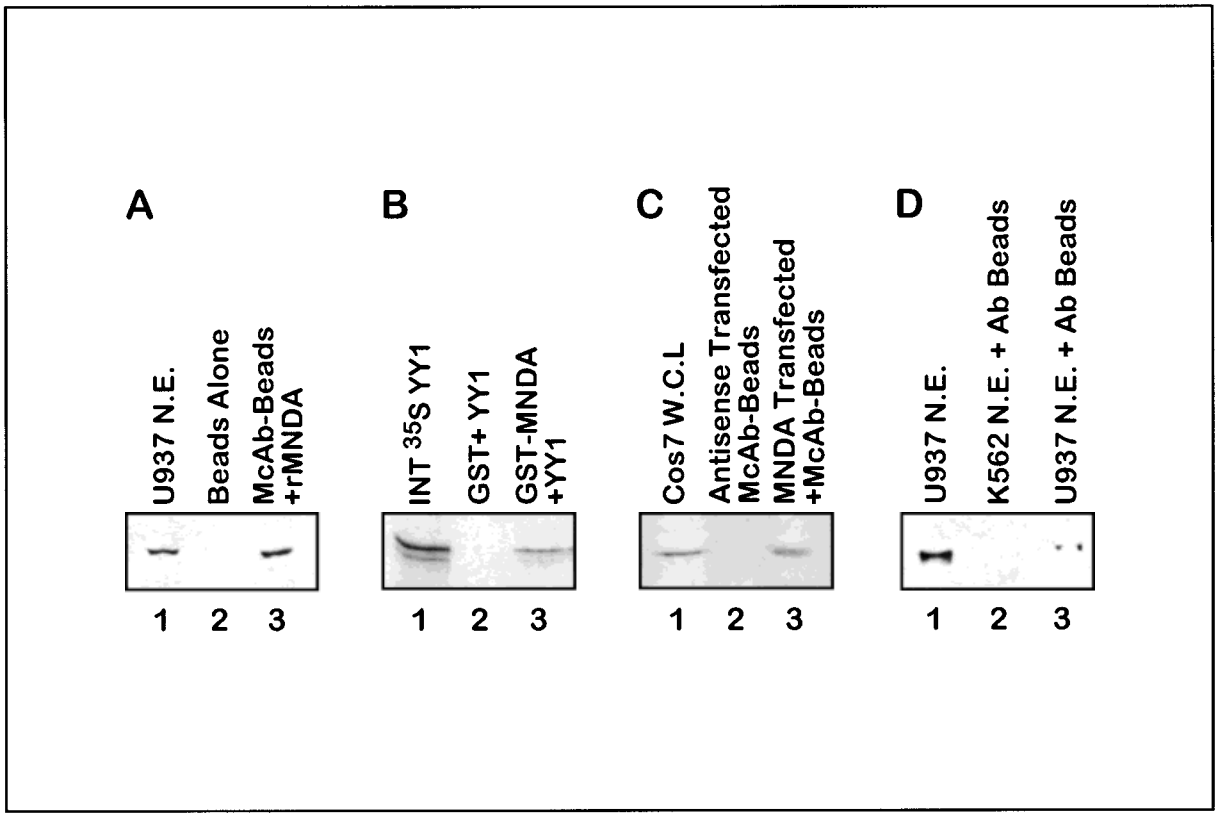


Fig. 1. MNDA binds YY1. **A:** rMNDA binds YY1 in a nuclear protein extract. Inactivated agarose beads (antibody free; **lane 2**) or McAB-beads (**lane 3**) were first precharged with excess purified rMNDA. After washing with PBS, beads were incubated with U937 nuclear protein extract (N.E.) containing 50 µg protein. Aliquots of MNDA coprecipitated proteins were separated by 8% SDS-PAGE. YY1 was detected on Western blots using a specific polyclonal antibody. **Lane 1** contains one-fifth of the original U937 nuclear proteins loaded on lanes 2 and 3. **B:** Immobilized GST-MNDA binds in vitro translated YY1. **Lane 1:** 1 µl of in vitro translated ^{35}S-met labeled YY1. In vitro translated YY1 coprecipitated after incubation with either immobilized GST (**lane 2**) or GST-MNDA (**lane 3**). The bound proteins were separated by SDS-PAGE and detected by autoradiography. **C:** Overexpressed MNDA is associated with endogenous YY1. Cos7 cells were transfected with an MNDA expression vector (pSG5-MNDA). Control cells were transfected with the same amount of pSG5-(-)MNDA antisense plasmid. Forty h posttransfection, cell lysates were prepared and incubated with McAb-beads. Immunoblots were used to detect YY1 protein coprecipitating with MNDA. **Lane 1:** Aliquot of Cos 7 lysate; **lane 2,** precipitated proteins from lysed Cos 7 cells transfected with the antisense construct; **lane 3,** precipitated proteins from MNDA transfected cells showing coprecipitation of YY1 with MNDA. In the absence of MNDA expression, no detectable YY1 bound the McAb-beads (lane 2). **D:** Endogenous MNDA is associated with endogenous YY1 in U937 cells. Nuclear protein extracts were prepared from MNDA expressing U937 cells and non-expressing K562 cells and incubated with McAb-beads. Endogenous YY1 found in the U937 nuclear protein extract or co-immunoprecipitating with MNDA was detected by immunoblot assay. YY1 detected in U937 cell nuclear protein extract (20 µg; **lane 1**) and co-immunoprecipitated with MNDA from U937 cell nuclear protein extract (200 µg; **lane 3**). No YY1 was precipitated with the McAb beads from MNDA negative K562 cell nuclear protein extracts (200 µg; **lane 2**).

Adding recombinant NPM [Xie et al., 1997b] did not affect the extent of the MNDA-YY1 interaction (data not shown). To further confirm that an interaction between MNDA and YY1 occurs in vivo, the mammalian two hybrid system was employed. Neither Gal4-MNDA nor YY1-VP16 alone (Fig. 2, lanes 2 and 3) were able to stimulate reporter activity over background level (lane 1), but cotransfection of Gal4-MNDA and YY1-VP16 activated the reporter gene by more than six-fold (lane 4). This magnitude of activation is MNDA dependent since replacing the Gal4-MNDA expression construct with only the Gal4 DNA binding domain failed to elevate reporter activity (lanes 5 and 6).

YY1 Binding Site in MNDA is Located Between aa 53 and 150

To determine the region within MNDA responsible for YY1 binding, eight sequential, internal deletion mutants were generated as schematically illustrated in Figure 3A. Coomassie blue stained, electrophoretically separated, purified mutated proteins from BL21 lysates are shown in Figure 3B. The Far Western assay was used to assess ^{35}S-met labeled in vitro translated YY1 binding to the intact and mutant MNDA proteins transferred to nitrocellulose membrane (Fig. 3C, identical to 3B except that one-fourth the amount of the material was electrophoretically separated). The result showed reduced binding in lanes 4 and 5 relative to other lanes indicating that the YY1 binding site is between aa 53-150. YY1 did not interact with GST alone or with the molecular weight markers (data not shown).

MNDA Stimulates YY1 Binding to the YY1 Target DNA Sequence

MNDA was pre-incubated with YY1 prior to analysis of YY1 DNA binding by EMSA. Nuclear protein extracts from both U937 and HeLa produced an EMSA shift pattern (Fig. 4A) using a 27 bp DNA probe containing the consensus YY1

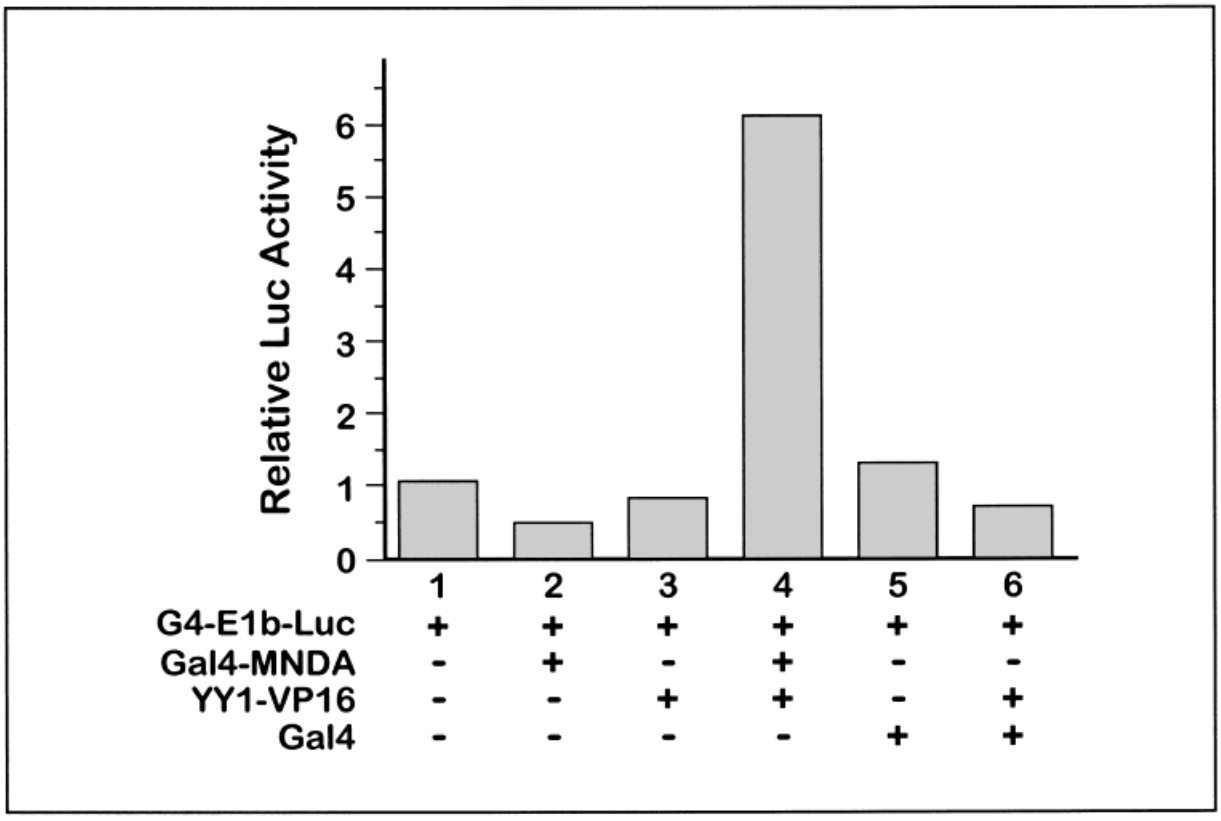


Fig. 2. The mammalian two hybrid analysis demonstrates the interaction between YY1 and MNDA in vivo. Two µg aliquots of each DNA was used to cotransfect Cos 7 cells. Carrier DNA was added to equalize total DNA used in each transfection. Luciferase activity is plotted for each transfection with different combinations of plasmids relative to the reporter control (**lane 1**). The data represents the average of independent duplicate experiments corrected for transfection efficiency. The variation between samples was less than 15%.

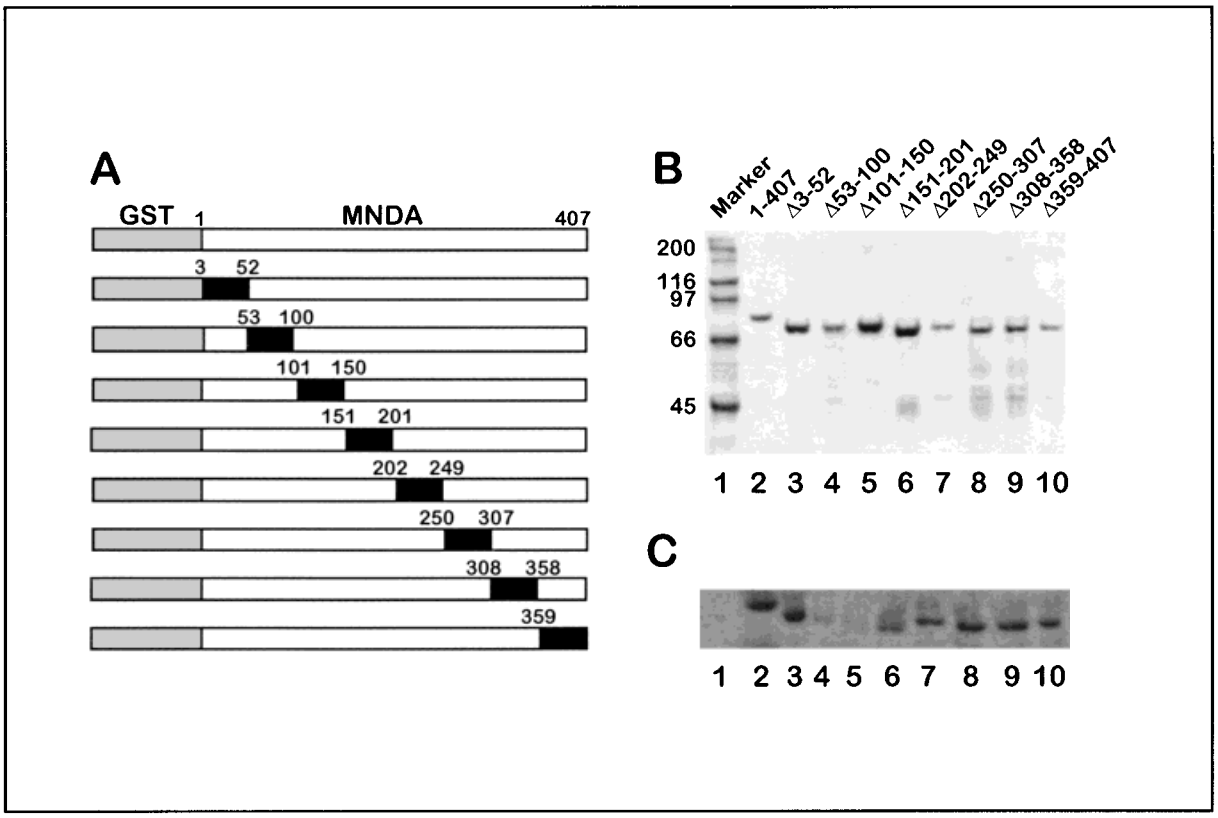
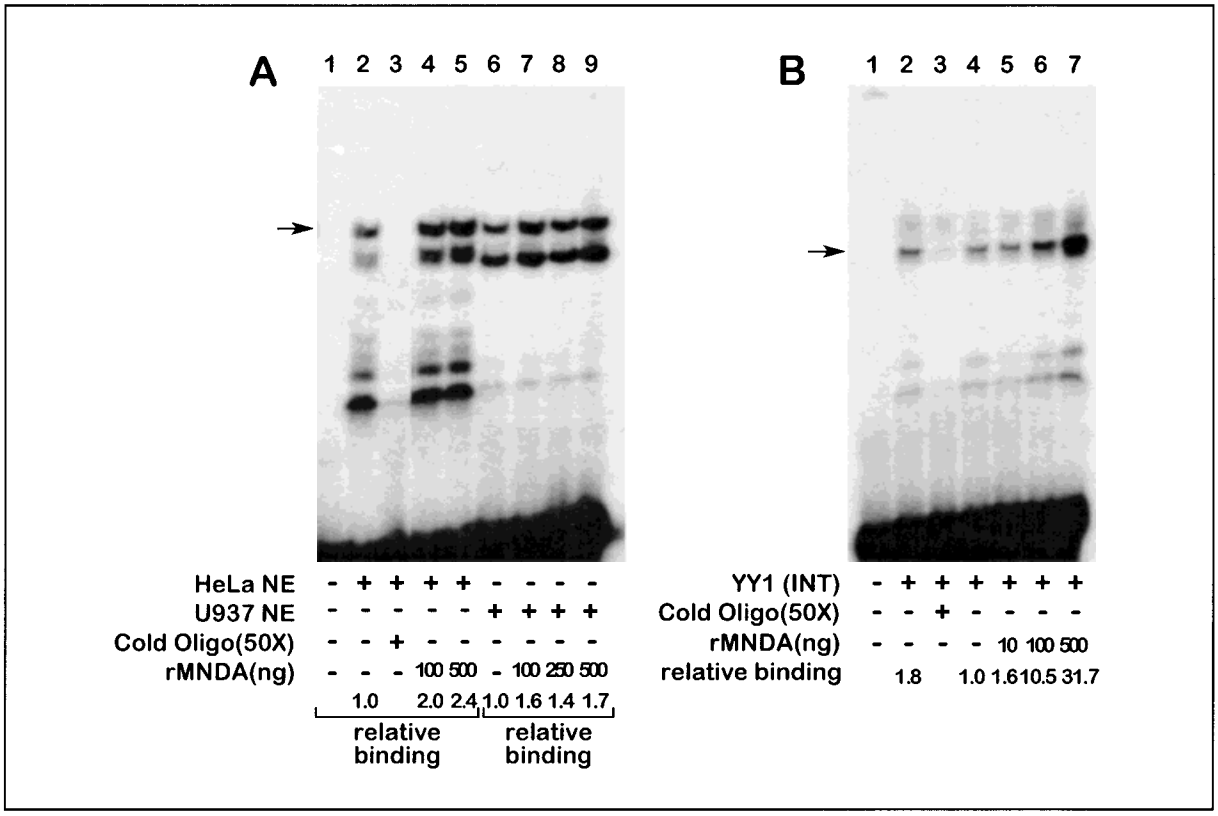


Fig. 3. Mapping the sites of MNDA interaction with YY1. **A:** Schematic representation of GST-MNDA (407 aa) and continuous internal deletion mutants with black regions showing sequence deleted between flanking aa. **B:** Commassie staining of 8% SDS-PAGE of purified GST-MNDA and the products of the deletion mutations. Each lane contains an equivalent fraction of the total glutathione-bead associated proteins (50 to 200 ng of proteins). **C:** Far Western analysis of YY1 binding to intact MNDA and products of deletion mutations (same lanes as in B). One-fourth the amount of each protein sample used in B was analyzed by incubating the renatured blot with in vitro translated, ^{35}S-Met labeled YY1. Bound YY1 was detected by autoradiography. Lanes 4 and 5 represent mutants with deletions of aa 53 to 100 and 101 to 150, respectively.

sequence, GCGGCCATC. Addition of rMNDA, increases the YY1 DNA shift band intensity (Fig. 4A, compare lanes 4 and 5 with lane 2 and lanes 7, 8, 9 with lane 6). When lower amounts of nuclear extract were used (less YY1), MNDA exhibited an even greater stimulation of YY1 DNA binding (data not shown). More DNA protein complex was observed with nuclear proteins from U937 relative to that from HeLa cells (compare lane 6 to lane 2). Overall, rMNDA addition to U937 nuclear protein extract produced less stimulation of YY1 DNA binding than when it was added to HeLa cell nuclear protein extracts. This is likely due to the presence of endogenous MNDA since a similar amount of YY1 was present in the U937 and HeLa nuclear protein extracts judged by Western blot (data not shown). To resolve the uncertain identity of multiple shift bands and eliminate the possible involvement of other nuclear proteins, the nuclear protein extract was replaced with in vitro translated YY1. Under these conditions, one shift band was observed and addition of rMNDA had a dramatic, concentration dependent, effect on YY1 DNA binding (Fig. 4B, compare lanes 5, 6 and 7 with lane 4). Since the reticulocyte lysate used to produce the in vitro translated YY1 also contains many cellular proteins, MNDA stimulation of YY1 DNA binding might also be mediated by a bridging protein in this assay. The use of highly purified recombinant GST-YY1 with rMNDA also stimulated YY1 DNA binding which argues against the idea of a bridging protein (Fig. 4C). The effect of MNDA on YY1 DNA binding was specific as it did not alter the Ets DNA shift (Fig. 4D).

MNDA-YY1 Protein-Protein Interaction Is Required to Stimulate YY1 DNA Binding

Confirmation of the MNDA-YY1 stimulatory effect on DNA binding was obtained by testing


A
1 2 3 4 5 6 7 8 9
HeLa NE - + + + + - - - -
U937 NE - - - - - + + + +
Cold Oligo(50X) - - + - - - - - -
rMNDA(ng) - - - 100 500 - 100 250 500
1.0 2.0 2.4 1.0 1.6 1.4 1.7
relative binding
relative binding
B
1 2 3 4 5 6 7
YY1 (INT) - + + + + + +
Cold Oligo(50X) - - + - - - -
rMNDA(ng) - - - - 10 100 500
relative binding 1.8 1.0 1.6 10.5 31.7

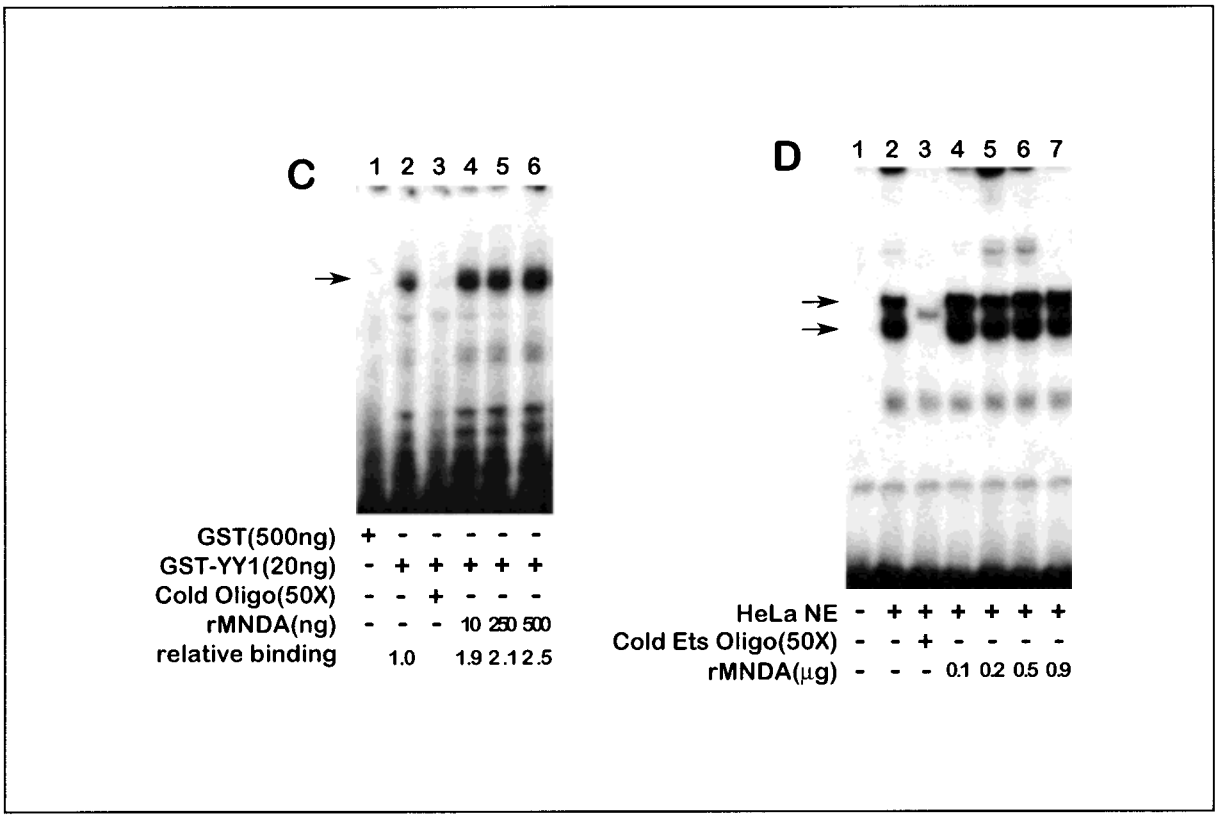

C
1 2 3 4 5 6
GST(500ng) + - - - - -
GST-YY1(20ng) - + + + + +
Cold Oligo(50X) - - + - - -
rMNDA(ng) - - - 10 250 500
relative binding 1.0 1.9 2.1 2.5
D
1 2 3 4 5 6 7
HeLa NE - + + + + + +
Cold Ets Oligo(50X) - - + - - - -
rMNDA(μg) - - - 0.1 0.2 0.5 0.9

Figure 4.

GST-MNDA internal deletion mutant proteins. As shown in Figure 5, GST control reactions (lanes 4 and 5) showed no effect on the basic shift band produced by YY1 alone (lanes 2 and 3). The stimulatory effect (lanes 6 and 7) was abolished when the GST-MNDA (Δ101–150; lacking sequence required for YY1 binding) was tested (Fig. 5, lanes 8 and 9), but testing of two other mutant proteins (Δ151–201 and Δ250–307) resulted in levels of stimulation comparable to the full length GST-MNDA (lanes 6, 7, and 10–13). These results are consistent with the MNDA-YY1 protein binding site mapping and provide definitive evidence that MNDA-YY1 protein interaction is required for the observed increase in YY1 DNA binding.

N-Terminal Half of the MNDA Protein Is Sufficient for Maximum Stimulation of YY1 DNA Binding

Since the domain responsible for binding YY1 is located in the N-terminal half of MNDA, the possibility that half of the MNDA could function alone in stimulating YY1 DNA binding was specifically examined. Two C-terminal deletion mutants (Δ200–407, lane 4 and Δ129–407, lane 3), shown in Figure 6A, were tested. The EMSA results (Fig. 6B) showed that the first 199 aa of MNDA were as effective as full length MNDA (Fig. 6B, compare lanes 3 and 4 with 7 and 8) while the the shorter N-terminal fragment (128 aa) lacking the complete region responsible for MNDA-YY1 binding was unable to stimulate YY1 DNA binding (Fig. 6B, compare lanes 5 and 6 with 1 and 2).

Fig. 4. MNDA stimulates YY1 DNA binding. **A:** Nuclear protein extracts (0.5 μg protein) from both HeLa (**lanes 2 to 5**) and U937 cells (**lanes 6 to 9**) were incubated with a 27 bp DNA sequence containing a YY1 binding site. Varying amounts of purified rMNDA (as indicated) were preincubated with nuclear protein extract for 15 min at room temperature before adding 1 μl DNA probe for an additional 5 min prior to loading. **Lane 1** is the control incubation containing probe only. The arrow points to a specific YY1 shift band as determined by comigration with a shift band generated using in vitro translated YY1. **B:** rMNDA stimulates in vitro translated YY1 DNA binding more dramatically than that observed with nuclear protein extracts in A. One μl of unlabeled in vitro translated (INT) YY1 was used in each reaction. Lane 1 represents a control in which the probe was incubated with reticulocyte lysate. **C:** MNDA stimulates highly purified YY1 DNA binding. Control purified recombinant GST (500 ng; **lane 1**) or 20 ng GST-YY1 (**lanes 2 to 6**) was tested. The amount of rMNDA added is indicated. **D:** MNDA does not affect Ets DNA binding. Nuclear proteins extracts (0.5 μg protein) from HeLa cells was incubated with a 35 bp DNA sequence containing an Ets binding site. Varying amounts of rMNDA were added as described above.

MNDA-YY1 Interaction Stimulates the YY1 DNA Association and Inhibits Dissociation

To investigate the mechanism involved in stimulating YY1 DNA binding, the on-rate and off-rate of binding in the presence and absence of MNDA was assessed. As shown in Figure 7A, in the absense of MNDA, YY1 DNA binding gradually increased with incubation time requiring approximately 15 min to reach maximum level (lanes 1 to 5). However, when the same amount of YY1 was preincubated with rMNDA, the maximal YY1 DNA binding level was reached within 1 min (lanes 6 to 10). These results show that MNDA greatly increased the YY1 DNA association rate. The off-rate of YY1 DNA binding with or without MNDA participation was analyzed by competition for the DNA in the pre-formed YY1 DNA complex by adding a 50-fold molar excess of cold YY1 oligonucleotide. The band intensity diminished at a slower rate in the presence of MNDA (Fig. 7B) indicating that MNDA inhibits the dissociation of YY1 from DNA. The requirement for an initial MNDA-YY1 interaction was further demonstrated when different amounts of rMNDA were preincubated with the target DNA and failed to stimulate YY1 DNA binding (Fig. 7C, compare lanes 2 to 4 with lane 1). The only notable difference between these samples was that some of the DNA probe remained at the top of gel (Fig. 7C, lanes 2–4) which might be due to MNDA nonspecific DNA binding and a tendency to self associate. In contrast, when MNDA was preincubated with the YY1 protein (compare lanes 5 to 7 with lane 1) before addition of the DNA probe, the stimulatory effect was again evident.

MNDA, YY1, and DNA Form a Complex

While the EMSA experiments (Figs. 4–7) demonstrated that MNDA stimulated YY1 DNA binding, a super shift band was not observed suggesting that MNDA was not present in the complex. To further test whether MNDA participated in a complex, but possibly disassociated from the complex during electrophoresis or did not alter the migration of the complex, a DNA affinity matrix was used to bind both YY1 and MNDA from a U937 nuclear protein extract. This approach resulted in detection of both YY1

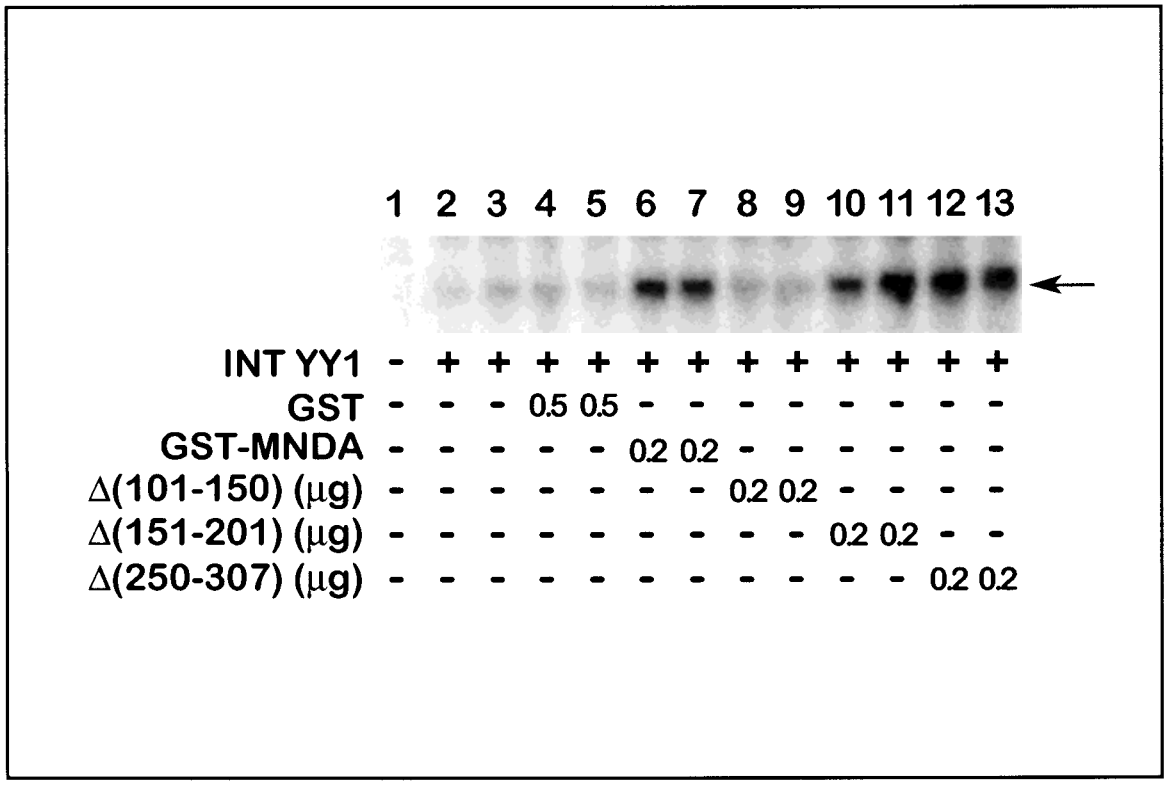


Fig. 5. The stimulated YY1 DNA binding is dependent upon a specific region of MNDA sequence. Purified GST, GST-MNDA, and selective products of internal deletion mutations were preincubated with in vitro translated YY1 and the effect on YY1 DNA binding was assessed by EMSA. GST-MNDA (**lanes 6 and 7**), but not GST alone (**lanes 4 and 5**) stimulated YY1 DNA binding; GST-MNDA (Δ101–150; **lanes 8 and 9**) is deficient in YY1 binding (see Fig. 4) and failed to enhance YY1 DNA binding. In contrast, other products of deletion mutations, GST-MNDA (Δ151–201) and GST-MNDA (Δ350–307) stimulated YY1 DNA binding to a level comparable to full length GST-MNDA (**lanes 10 to 13**).

and MNDA in the bound fraction (Fig. 8A,B) and showed that MNDA, YY1, and the YY1 target DNA form a ternary complex. When exogenous rMNDA was added to the MNDA negative HeLa nuclear protein extract, the immobilized YY1 target DNA coprecipitated YY1 with rMNDA in a concentration dependent manner (Fig. 8C, lanes 3, 4, and 5). Controls that included the DNA matrix without YY1 or naked beads retained rMNDA (Fig. 8C, lanes 7, 8, 9, 10) at a background level similar to that resulting in the control without beads (Fig. 8C, lane 6). This low background level appears to reflect either nonspecific binding of rMNDA to the tube surface or a small fraction of insoluble rMNDA.

DISCUSSION

The strict nuclear localization, lineage and stage specific expression, association with chromatin and interaction with other nuclear proteins, suggested that MNDA plays a role in regulating gene transcription. Since MNDA did not bind specific DNA sequences, but bound other nuclear proteins [Xie et al., 1995, 1997b], we speculated that MNDA functioned by modifying the activity of specific transcription factors, either modulating the binding of transcription activators/repressors to their target DNA or being involved in the communication between the transactivator and the basal transcription machinery. Non-DNA binding proteins, such as the viral proteins E1a [Liu and Green, 1994], VP16 [Preston et al., 1988], and the large T antigen of simian virus 40 (SV40) [Mitchell et al., 1987] as well as certain eukaryotic factors such as N-CoR [Chen and Evans, 1995; Horlein et al., 1995; Kurokawa et al., 1995] and RB [Chen et al., 1996], are well known regulators of gene transcription. MNDA binds a set of ubiquitous nuclear proteins and nucleolin and NPM are two of those proteins

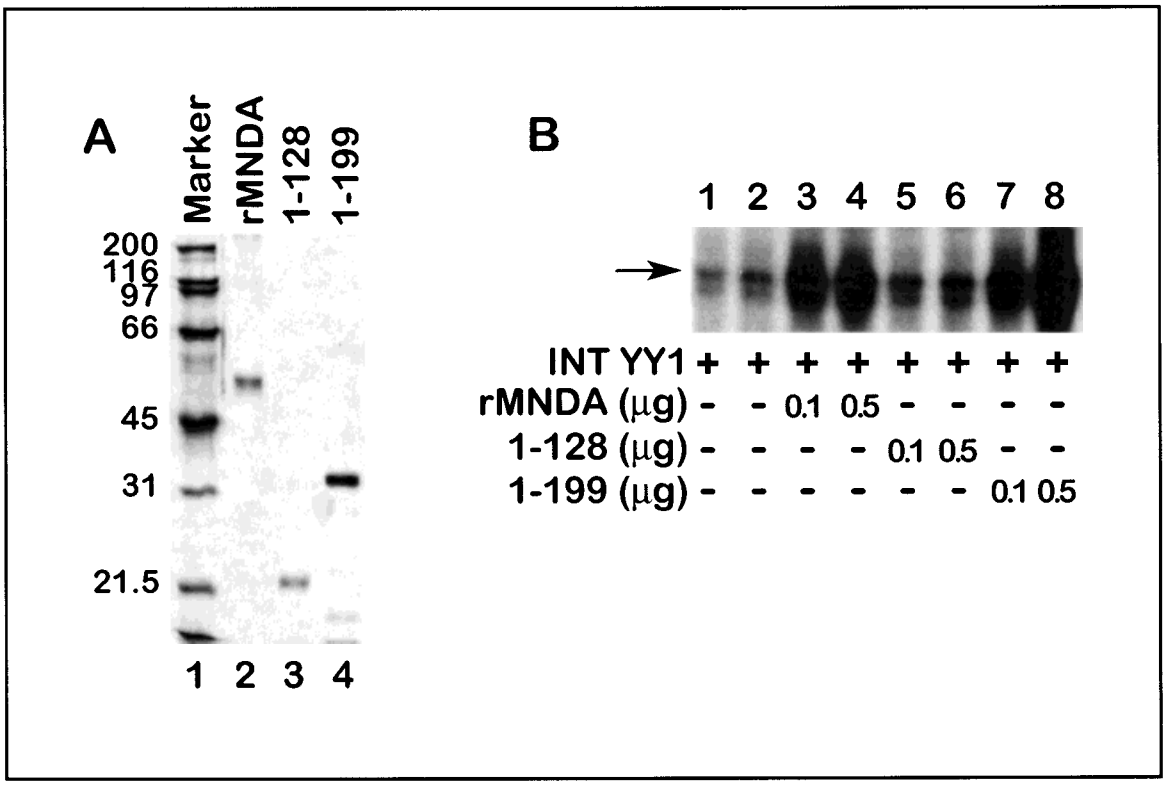
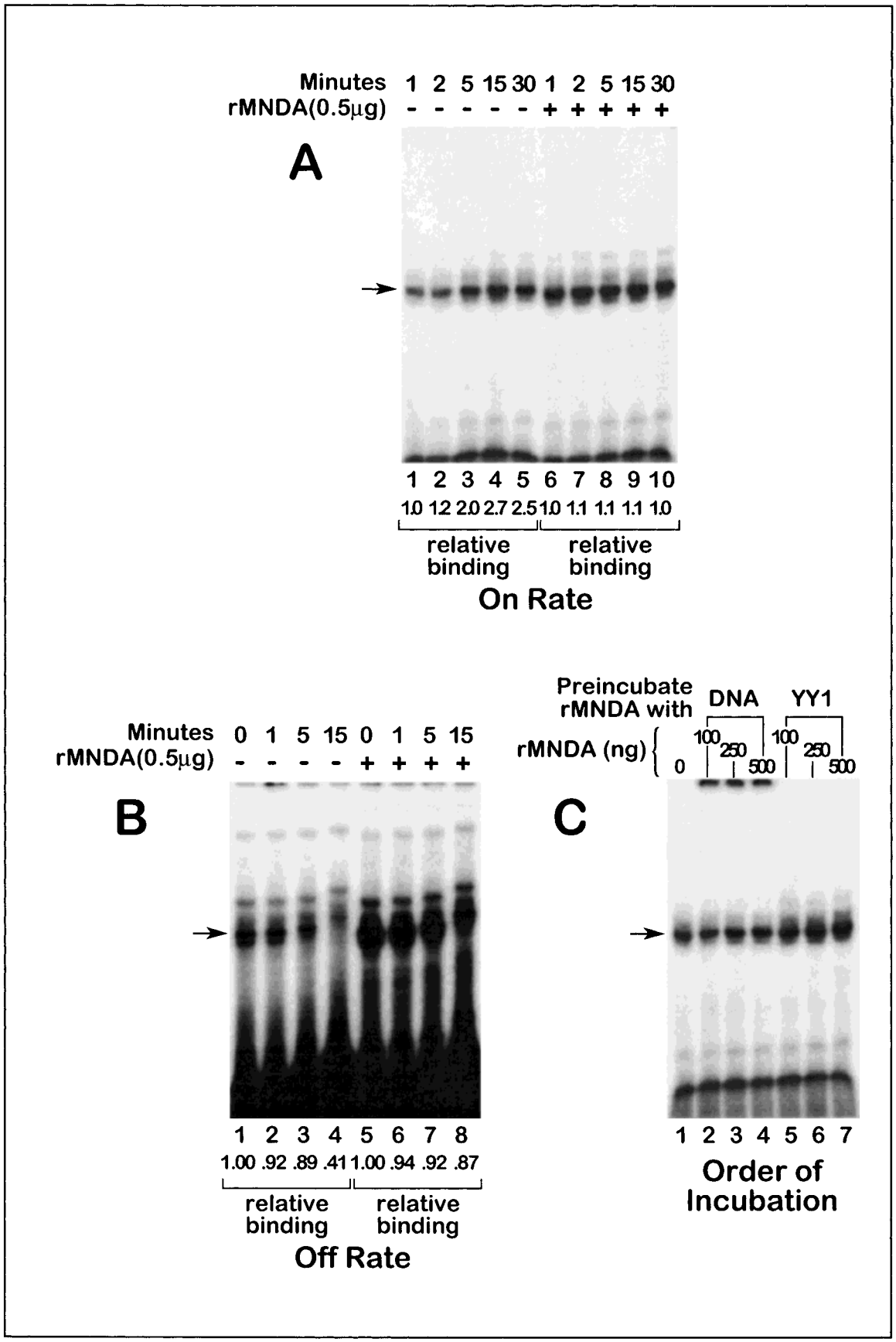


Fig. 6. Only the N-terminal half of MNDA is required to fully stimulate YY1 DNA binding. **A:** Coomassie blue staining of rMNDA (**lane 2**) and two C-terminal deletion mutants corresponding to the first 128 (**lane 3**) and 199 aa (**lane 4**) of MNDA. **B:** EMSA was used to demonstrate that the N-terminal 200 (**lanes 7 and 8**), but not the first 128 aa (**lanes 5 and 6**), was as effective as the whole length MNDA (**lanes 3 and 4**) in enhancing YY1 (in vitro translated product) DNA binding (compare to **lanes 1 and 2**). One μl of unlabeled in vitro translated (INT) YY1 was used in each reaction (20–40 ng protein).

[Xie et al., 1995, 1997b]. Noting that nucleolin and NPM interact with YY1, led to an investigation of the possible influence of MNDA on those interactions. Those experiments showed that MNDA was able to directly bind YY1 independent of the MNDA or YY1 interaction with NPM. YY1 is a ubiquitous, zinc-finger DNA binding protein implicated in the regulation of several cellular and viral genes [Basu et al., 1993; Bauknecht et al., 1992; Flanagan et al., 1992; Hahn, 1992; Hariharan et al., 1991; Natesan and Gilman, 1993; Park and Atchison, 1991; Riggs et al., 1993; Shi et al., 1991]. YY1 interacts with CREB/ATF proteins to repress CRE-dependent transcription of *c-fos* [Zhou and Engel, 1995] and it enhances the association of SRF with the SRE in the *c-fos* promoter [Natesan and Gilman, 1995]. YY1 has also been identified as a component of the nuclear matrix [Guo et al., 1995] and it binds other proteins including Sp1 and c-myc transcription factors, TATA binding protein associated factor, $TAF_{II}55$, TFIIB and the phosphoproteins, nucleolin and nucleophosmin/NPM/B23 [Chiang and Roeder, 1995; Inouye and Seto, 1994; Lee et al., 1993; Seto et al., 1993; Shrivastava et al., 1993; Usheva and Shenk, 1996; Yang et al., 1994].

Recombinant MNDA (both his- and GST-tagged) interacted with native YY1 from nuclear protein extracts as well as in vitro translated YY1. Coimmuno-precipitation of endogenous YY1 with MNDA from MNDA transfected Cos 7 whole cell lysates or from U937 cell nuclear protein extracts containing endogenous MNDA indicated that native MNDA is indeed associated with native YY1. Specificity of binding in these experiments was verified by inability to coprecipitate recombinant Sp1 with MNDA. The mammalian two hybrid system was used to definitively test the MNDA and YY1 interaction in vivo. Using mutagenesis coupled with immobilized coprecipitation and the Far Western approach, the MNDA-YY1 interaction site was mapped to aa 53 to 150 of MNDA.


Minutes 1 2 5 15 30 1 2 5 15 30
rMNDA(0.5μg) - - - - - + + + + +
A
1 2 3 4 5 6 7 8 9 10
1.0 1.2 2.0 2.7 2.5 1.0 1.1 1.1 1.1 1.0
relative binding
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B
1 2 3 4 5 6 7 8
1.00 .92 .89 .41 1.00 .94 .92 .87
relative binding
relative binding
Off Rate
Preincubate rMNDA with
DNA
YY1
rMNDA (ng)
0
100
250
500
100
250
500
C
1 2 3 4 5 6 7
Order of Incubation

Figure 7.

The EMSA experiments demonstrated MNDA stimulation of YY1 DNA binding. Other nuclear proteins associate with MNDA [Xie et al., 1995, 1997b] and in order to avoid interference from these proteins, a highly purified rMNDA was used to stimulate purified recombinant YY1 DNA binding. These results demonstrated that the direct interaction between MNDA and YY1 is the driving force for stimulating YY1 DNA binding. The failure of MNDA to produce a supershift complex in the EMSA has also been observed in other studies in which transcription factor-DNA binding has been augmented [Suzuki et al., 1993; Wagner and Green, 1993]. These observations are consistent with a catalytic mechanism where MNDA would function as a transient cofactor promoting YY1 DNA binding and once YY1 bound its target DNA, MNDA would disassociate from the complex. However, the failure to observe the MNDA, YY1, and DNA complex in EMSA could be a consequence of assay conditions. To resolve this uncertainty, a DNA protein binding assay (immobilized DNA protein binding assay) was used to examine the potential formation of the ternary complex. The results argued against a catalytic mechanism and for a stoichiometric mechanism where MNDA, YY1, and DNA form a complex. This is also consistent with all of the EMSA experiments which showed that an excess amount of rMNDA is required to reach maximum stimulation and prolonged incubation of MNDA with YY1 and its target DNA did not result in further elevation of YY1 DNA binding (data not shown). Interestingly, the ability of TFIIB to stimulate YY1 DNA binding in EMSA was also not associated with the generation of a super shift complex, but both TFIIB and YY1 were present in a complex that happened to exhibit the same migration characteristic as YY1 and DNA alone [Usheva and Shenk, 1996].

Analysis of MNDA internal and c-terminal deletion mutants, demonstrated that the region responsible for YY1 binding was also required for stimulating YY1 DNA binding. In addition, the N-terminal 199 aa region of MNDA was as effective as the full length MNDA in stimulating YY1 DNA binding. The N-terminal 200 aa sequence of MNDA is not highly conserved in other members of the gene family and the MNDA sequences between aa 53-150 has an especially low level of conservation with other members [Briggs et al., 1992, 1994a]. This region of sequence (aa 53-150) contains an extended basic amphipathic alpha helical region and an imperfect leucine zipper which are consistent with protein binding [Briggs et al., 1992]. The ability to stimulate YY1 DNA binding might be a functional activity of MNDA not found in other members of the *gene 200 cluster* family.

Several different mechanisms could account for the stimulation of transcription factor DNA binding initiated through protein-protein interaction. The enhancement may result from dissociating the transcription factor from other negative regulators as suggested for Rb activation of Sp1 DNA binding [Chen et al., 1994]. Facilitated multimerization accounts for HTLV Tax protein stimulation of bZIP protein DNA binding [Wagner and Green, 1993]. Alternatively, protein binding could change the conformation of the transcription factor and increase its affinity for target DNA sequences. MNDA binds YY1 directly, forming a ternary complex with YY1 and its target DNA which is a characteristic of inhibition by quenching [Latchman, 1996]. The same region of MNDA is responsible for both YY1 binding and stimulation of YY1 DNA binding which further indicates that MNDA directly alters the ability of YY1 to bind DNA. YY1 has not been reported to be capable of multimerization and MNDA stimulation does not alter the electrophoretic mobility of YY1 DNA complex, which indicates that MNDA does not promote multimerization of YY1. MNDA stimulation of YY1 DNA binding is attributable to an ability

Fig. 7. The MNDA-YY1 interaction stimulates YY1 DNA association rate, reduces dissociation rate, and the enhancement requires a preincubation of MNDA with YY1, but not with DNA. **A:** Association rate of in vitro translated YY1 with target DNA in the absence (**lanes 1 to 5**) or presence of purified rMNDA (**lanes 6 to 10**). Incubation times after probe addition at room temperature are indicated. **B:** Off-rate of YY1 DNA binding in the absence (**lanes 1 to 4**) or presence of MNDA (**lanes 5 to 8**). After a 15 min incubation with labeled probe, competition was initiated by adding a 50-fold molar excess of unlabeled DNA. Aliquots were withdrawn at the indicated times and loaded immediately on a 6% acrylamide gel under current. In both A and B, the in vitro translated YY1 was preincubated with or without MNDA for 15 min prior to addition of labeled probe. **C:** MNDA preincubated with YY1 protein, but not with DNA stimulates YY1 DNA binding. The YY1 target DNA (**lanes 2 to 4**) or in vitro translated YY1 (**lanes 5 to 7**) was preincubated with increasing amount of rMNDA for 15 min at room temperature. YY1 or DNA was then added to corresponding tubes and incubated for an additional 5 min before electrophoresis.

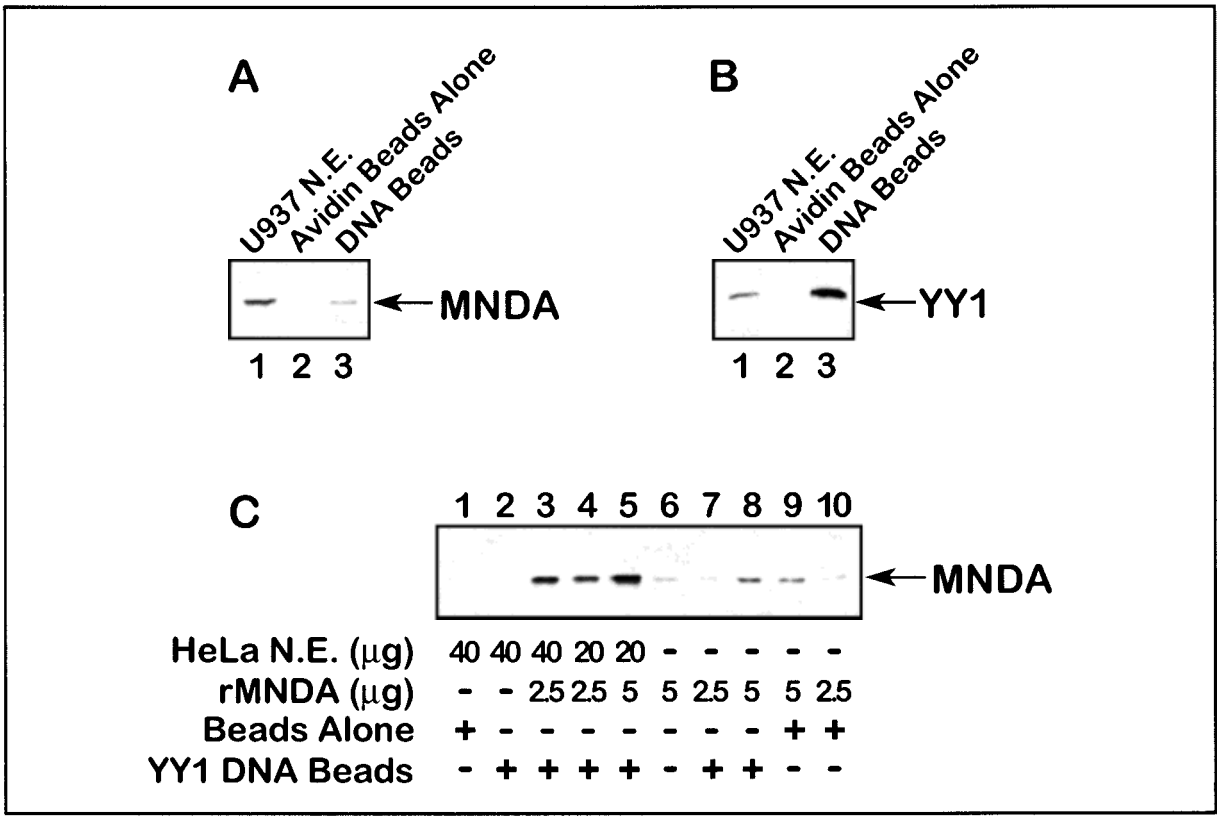

Fig. 8. MNDA and YY1 form a ternary complex with the target DNA. Biotinylated DNA containing the YY1 element was immobilized on streptavidin beads and incubated with MNDA positive U937 nuclear protein extracts (N.E.). Aliquots of bound proteins were separated on 8% acrylamide gels and analyzed for MNDA or YY1 by Western blot. **A:** Blot reacted with rabbit polyclonal antibody against rMNDA. **Lane 1:** MNDA in U937 nuclear protein extract (20 μg) as a positive control; **lane 2,** no MNDA precipitated from 100 μg initial U937 nuclear protein extract reacted with control (no DNA) beads; **lane 3,** MNDA from 100 μg of U937 nuclear protein extract that bound to immobilized target DNA. **B:** Same as A, but blot was reacted with rabbit anti-YY1 antibody. **C:** The YY1 DNA beads incubated with HeLa nuclear extract with or without addition (as indicated) of exogenous rMNDA. A significant amount of rMNDA was bound when mixed with the nuclear protein extract that contains YY1 (**lanes 3 to 5**) compared to that in controls without YY1 (no extract) (**lanes 7 and 8**). MNDA incubated without beads (**lane 6**) or control beads (**lanes 9 and 10**) all produce similar levels of background signal.

to increase the association rate of YY1 DNA binding coupled with a reduction in the dissociation rate. Our evidence indicates that the direct interaction between YY1 and MNDA is the sole requirement for elevating YY1 DNA binding. However, since MNDA is a basic protein and binds a low level of DNA nonspecifically, it was possible that MNDA acted like HMG or YY1 to 'prime' or bend DNA thereby facilitating YY1 binding. An experiment to test this showed that pre-exposure of the DNA to MNDA did not enhance YY1 DNA binding and preincubation of YY1 with MNDA was required.

Regulation of DNA binding through protein-protein interactions has been documented in several other cases. The human heat shock transcription factor (HSF) is maintained in a non-DNA binding form under normal conditions and acquires the ability to bind specifically to the heat shock promoter element in response to heat shock and other physiological stress. The conversion of the nonDNA binding monomer to the active trimeric DNA-binding form of HSF is thought to be mediated by interaction with a heat shock inducible molecular chaperone hsp70 [Mosser et al., 1993]. Similarly, both the human T cell leukemia virus type-1 Tax transcriptional activator and HMG I(Y) proteins promote dimerization of members of the CREB/ATF family to enhance DNA binding [Du and Maniatis, 1994; Wagner and Green, 1993]. HMG I(Y) also stimulates NF-kB DNA binding apparently through binding A-T rich DNA and altering DNA structure [Nissen and

Reeves, 1995]. In an immunoprecipitation DNA binding assay, the tumor suppressor, p53 DNA binding activity was modulated by SV40 large T antigen and other cellular p53 conformational domain-binding proteins. These interactions implicated the involvment of p53 in the transcriptional control of the genes lacking known p53-binding elements in their promoter or enhancer sequences [Srinivasan and Maxwell, 1996]. Overall, the evidence emerging from several studies suggests that mediation of DNA binding through protein-protein interaction is an effective mechanism for a single viral or cellular protein to regulate the transcription of a wide spectrum of genes.

It has also been noted that the ability of the Tax protein to enhance DNA binding of other factors [Marriott et al., 1989] is target DNA specific, in that it enhances the binding of CREB1 to each 21-bp of the three HTLV-I repeats, but not to the palindromic CRE found in various eucaryotic gene promoters [Yin et al., 1995]. We do not know if the YY1 DNA binding activity stimulated by MNDA is also sequence specific, but it is possible that the magnitude of activated YY1 DNA binding resulting from the MNDA interaction is site dependent. MNDA might also exhibit negative regulation for YY1 DNA binding at different sites in the same promoter or different promoters. Additionally, posttranslational modifications, such as phosphorylation, dephosphorylation, reduction, oxidation as well as protease cleavage, are known mechanisms that alter transcription factors and can influence DNA binding activity. The AP1 complex is one of the well characterized examples where such modifications alter function [Abate et al., 1990; Angel and Karin, 1991; Xanthoudakis et al., 1992]. However, there are no indications in our experiments or in published reports to suggest that YY1 DNA binding and its effects on gene transcription is regulated by posttranslational modifications. The present findings document a direct physical interaction between MNDA and YY1 which is required for stimulation of YY1 DNA binding.

Recently, TFIIB was found to bind YY1, increasing its affinity for its target DNA element [Usheva and Shenk, 1996]. In that same report, YY1 was also shown to interact with the large subunit of polymerase II, directing it to the transcription initiation site [Usheva and Shenk, 1996]. As noted above, YY1 itself has been reported to facilitate DNA binding of other transcription factors, thus the interaction between MNDA and TFIIB with YY1 might also substantially impact the function of other transcription factors influenced by YY1 [Natesan and Gilman, 1995; Zhou et al., 1995]. MNDA stimulates YY1 DNA binding by altering both the on-rate and off-rate. MNDA would have an opportunity to alter the off-rate since it is present in the complex with YY1 and DNA. The participation of MNDA in a ternary complex is similar to that proposed for another YY1 binding protein, RPD3 [Yang et al., 1996]. The characterization of the RPD3 YY1 interaction suggested that YY1 negatively regulates transcription by tethering RPD3 to DNA as a cofactor. The functional consequences of the MNDA YY1 interaction remain to be elucidated, but clearly they will be restricted to specific hematopoietic cells [Miranda et al., submitted].

Relative to most transcription factors, YY1 binds heterogenous DNA sequences present in a variety of viral and cellular promoters. Many of the putative YY1 binding sites overlap sites of other known transcription factors; Therefore it has been suggested that the differences in the on-rates and off-rates of factors competing for sites overlapping YY1 sites are critical to the transcriptional regulation of target genes [Hyde-DeRuyscher et al., 1995]. In regard to the GM-CSF gene, regulation of transcription by core-binding factor was recently proposed to depend on competition for the same site with YY1 [Cockerill et al., 1996]. The ability of MNDA to enhance the affinity of YY1 for its target DNA could therefore, have a dramatic effect on the expression of many genes and provide specific regulation of transcriptional events during hematopoietic cell differentiation and end-stage cell function.

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