MNDA dimerizes through a complex motif involving an N-terminal basic region

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Abstract Human myeloid cell nuclear differentiation antigen (MNDA) is a myelomonocytic lineage-specific protein that influences gene expression through interactions with other nuclear proteins and transcription factors. MNDA also self-associates and chemical cross-linking was used to demonstrate that MNDA forms a dimer. C-terminal and internal deletion mutants were used to identify two regions in the N-terminal half of MNDA essential for self-association. One region contains an imperfect leucine zipper and the second is highly enriched in basic residues. The sequences that are essential for dimerization are separated by a highly basic amphipathic α-helical region which was not required for dimerization.

Key words: MNDA; Cross-linking; Dimerization; Mutation

1. Introduction

The human myeloid cell nuclear differentiation antigen (MNDA) was discovered by immunoblot-screening of nuclear antigens in human leukemia cell lines (K562, KG-1, U937 and HL 60) [1,2]. MNDA is expressed only in late-stage myeloid cell lines (U937, HL-60 and THP-1) that undergo monocytic differentiation in response to vitamin D3 induction and not in earlier-stage lines (K562) or lines originating from other lineages [2]. Leukemic cell lines expressing MNDA also differentiate in response to retinoid treatment whereas MNDA null lines do not. An analysis of >40 cell lines, peripheral blood, bone marrow and cases of leukemia by immunohistochemical staining as well as Northern and Western blotting confirmed the lineage- and stage-specific expression of MNDA [3,4]. MNDA is up-regulated specifically by interferon α in nearly all cells that exhibit constitutive expression. The MNDA promoter [5] contains several consensus regulatory elements, including c-myb [6–9], PU.1 [10–18], ISRE [19] and Sp1 [20–23], consistent with MNDA's myelomonocytic lineage-specific expression and regulation by interferon α. Analysis of MNDA-associated proteins and assessment of the effects of MNDA expression on promoter activities of different genes has led to the proposal that MNDA plays a role in transcription regulation by influencing the actions of other transcription factors [24,25]. A related mouse protein, p202, also interacts with other nuclear proteins and modulates transcription [26–29]. To further characterize the structural features of MNDA, we now identify regions of MNDA essential for self-association. Chemical cross-linking demonstrated that MNDA forms a dimer. C-terminal, N-terminal and internal deletion mutants were used in protein–protein blotting assays (Far Western) to localize regions required for self-association. Regions essential for MNDA dimerization were mapped to an imperfect leucine zipper and a basic region separated by an uninvolved basic amphipathic α-helical region in the N-terminal half of MNDA. MNDA is a member of the *gene 200 cluster* of interferon-regulated genes [3]. Each gene contains one or two copies of a highly conserved 200-amino-acid region. The 200-amino-acid conserved region is located in the C-terminal half of MNDA. Therefore, the regions of MNDA required for dimerization are not conserved in other members of the gene family consistent with the unique lineage-specific pattern of MNDA expression and diverse functional roles for members within this gene family.

2. Materials and methods

2.1. Labeling of MNDA

Recombinant MNDA was prepared using the pQE30 expression system as described previously [24]. To promote ^{125}I-labeling primarily in the His-tag region, the purified rMNDA was first dialyzed at 4°C with three changes against 20 mM Tris-HCl pH 8.2, 500 mM NaCl, 10% glycerol, 0.1% NP-40, 1 mM PMSF to remove imidazole. The final 3 ml reaction mixture was 20 mM Tris-HCl pH 8.2, 500 mM NaCl, 10% glycerol, 0.1% NP-40, 1 mM PMSF, 500 μg rMNDA, 5 μCi Na^{125}I. Six Iodo-Beads (Pierce Rockford, IL) were added to the mixture and incubated at room temperature for 3.5 min. Iodinated protein was separated from free ^{125}I with Ni^{2+}-NTA resin. A higher pH (8.2 vs. 7.0) used in the iodination reaction favored incorporation of ^{125}I into the imidazole ring-containing histidine residues [30].

The entire MNDA-coding sequence, including the initiation codon, was inserted into pSG5 (Stratagene), designated pSG5-MNDA and was used as a template for in vitro transcription-coupled translation of MNDA. For each 50 μl reaction, 1 μg of supercoiled vector was used in the T_NT T7 RNA polymerase-coupled rabbit reticulocyte lysate system (Promega) with incorporation of ^{35}S-methionine.

2.2. Cross-linking

In chemical cross-linking experiments, purified rMNDA was dialyzed extensively against 20 mM HEPES pH 7.9, 10% glycerol, 150 mM NaCl to remove imidazole. After dialysis, the sample was cleared by centrifugation and 10 μg/ml rMNDA was treated with DSG (disuccinimidyl glutarate) (Pierce), ranging from 10 μM to 1 mM for various times as indicated. The samples were quenched with 50 mM Tris pH 8.0 and analyzed by SDS-PAGE (7.5%). Cross-linked protein patterns were visualized by Coomassie blue staining or immunoblotting using anti-MNDA antibodies. To control for multimerization caused by cross-linking polyhistidine residues, the procedure was also performed with tag-less rMNDA produced by digesting with Factor Xa. In vitro translated, ^{35}S-met-labeled MNDA was prepared by diluting 5 μl of the in vitro reaction mixture to 480 μl of 20 mM HEPES pH 7.9, 10% glycerol and 150 mM NaCl. Fifteen μl of DSG in DMSO was then added to give a final concentration range of 0.01 to 1.00 mM. After 15 min at room temperature, reactions were terminated with 50 mM Tris-HCl pH 7.5. The proteins were then TCA-

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precipitated, acetone-washed and dried by vacuum centrifugation. MNDA bands in SDS-PAGE were detected by autoradiography.

2.3. Generation of MNDA deletion mutants

The pQE30-MNDA, described previously [24], was used to prepare His-tagged recombinant MNDA. C-terminal deletion mutations were prepared from the pQE30-MNDA construct 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-3X (Pharmacia, Piscataway, NJ) and designated pGEX-MNDA. pGEX-MNDA was 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 ≈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) that allowed continued in-frame translation of the MNDA after blunt ligation.

A construct with selective deletion of 82 N-terminal amino acids of the MNDA was obtained by removal of *Kpn*I and *Bst*BI fragment from pQE30-MNDA [24], followed by gel purification, fill in and blunt ligation. *E. coli* strain of M15 was used as the host cell for protein expression. Another construct, with deletion of aa 84–103 (core sequence for LDL receptor-binding motif) was prepared by 'loop-out' mutagenesis based on a method developed by Hutchison et al. [31] and modified by Kunkel [32]. Briefly, the complete cDNA-coding sequence for MNDA was excised from pQE30-MNDA and subcloned into M13. A 31-mer synthetic oligonucleotide containing sequence complementary to both 5′ and 3′ sequence flanking the aa 84–103 region was annealed to single-stranded M13-MNDA template prepared from *E. coli* strain CJ216 (ung^-, dut^-) and extended with DNA polymerase. The double-stranded DNA was then ligated and used to transform the normal *E. coli* host UT481 (ung^+, dut^+). Plaques containing the oligonucleotide-directed loop-out mutation were selected and subsequently subcloned into pQE30 for protein expression. Except where indicated, all the His-tagged MNDA mutants were induced and purified with the procedure described for the full-length MNDA [24]. Correct in-frame expression of deletion mutation constructs was verified by immunoblotting and specified site of deletion confirmed by sequencing.

2.4. SDS-PAGE gels, Western and Far Western blots

All procedures and antibodies used have been described in detail previously [2,24]. Three rat monoclonal antibodies against MNDA [33] determined to bind different epitopes were mixed for Western blot detection of deletion mutants of MNDA.

3. Results and discussion

3.1. MNDA is a dimer

The ability of MNDA to self-associate was reported earlier [24]. To establish the stoichiometry of self-association, chemical cross-linking experiments were performed. To maintain the native structure of the protein complex, a short length of cross-bridge bifunctional linker, disuccinimidyl glutarate (DSG), was selected. Cross-linking experiments were performed with U937 nuclear extracts, His-tagged rMNDA and factor Xa-treated tag-less rMNDA. Coomassie blue staining of SDS-PAGE analysis of purified recombinant proteins showed a faint band at molecular weight ≈110 kDa (suggesting dimerization of the 55 kDa monomeric MNDA). However, the results could not be confirmed using native MNDA in U937 nuclear protein extracts due to excessive cross-linking of MNDA to other nuclear proteins (data not shown). Results using in vitro translated ^{35}S-met-labeled MNDA confirmed dimerization. With increasing cross-linker DSG concentration, a band with M_r of 110 kDa equivalent to a dimer of MNDA was observed (Fig. 1). Agreement of results from both recombinant and in vitro translated MNDA indicates that dimerization accounts for the self-association of MNDA.

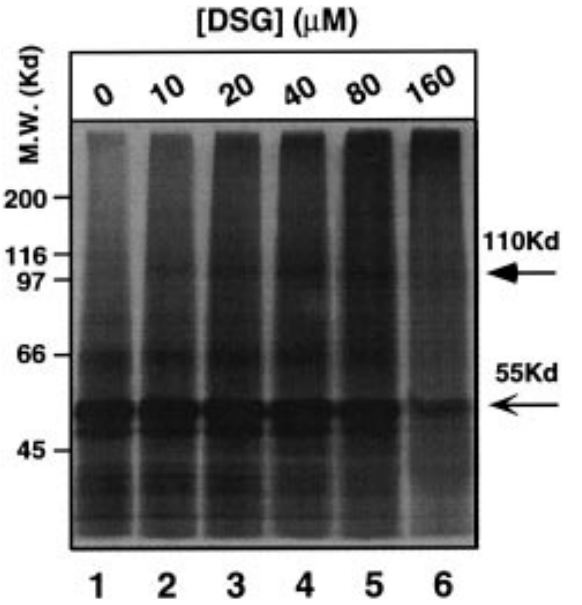

Fig. 1. Chemical cross-linking of in vitro translated MNDA. In vitro translated, ^{35}S-met-labeled MNDA shows a cross-linked pattern with increasing DSG concentrations (indicated at the top of each lane). With increasing cross-linker DSG concentration, a specific band at ≈110 kDa (top arrow) equivalent to dimers of MNDA appeared. The lower arrow identifies the monomeric form of MNDA (55 kDa).

3.2. Mapping the region for MNDA self-association

To map the region(s) involved in MNDA self-association, a series of terminal and internal deletion mutants of MNDA were tested for binding. Bacterial lysates containing roughly equal molar amount of MNDA and its derivatives, as assessed by Western blotting, were separated on SDS-PAGE and tested for self-association by Far Western blotting. In Fig. 2, wild-type and mutant recombinant forms of MNDA are defined schematically in panel A and assessed by Western blot in panel B. Samples transferred to a membrane and probed with ^{125}I-labeled rMNDA (Far Western) are shown in panel C. The results indicated that sequences within the C-terminal half of MNDA are not required for self-association. On the other hand, deleting residues within the N-terminal half of MNDA is deleterious to self-association. Deleting the specific region between aa 83 and 104 (a highly basic amphipathic α-helical region suspected of being a protein-binding site in the N-terminal half of MNDA) did not interfere with self-association.

To confirm these results and attempt to localize the sites of interaction more precisely, eight sequential small block deletion mutants of MNDA were generated (Fig. 3, panel A) and tested to determine ability to bind in vitro translated MNDA (Fig. 3, panel D). The purified internal GST-MNDA deletion mutants and GST alone were separated on 8% SDS-PAGE and detected by Coomassie blue staining (Fig. 3, panel B). Duplicate samples were separated by SDS-PAGE and after being transferred and blocked with 5% milk proteins in HYB-100 (20 mM HEPES pH 7.7, 100 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% NP-40, 1 mM DTT) the membrane was blotted with 25 μl of in vitro translated ^{35}S-met-labeled MNDA (Fig. 3, panel D) in a final 15 ml hybridization buffer (HYB-100 and 1% milk). The autoradiogram (Fig.

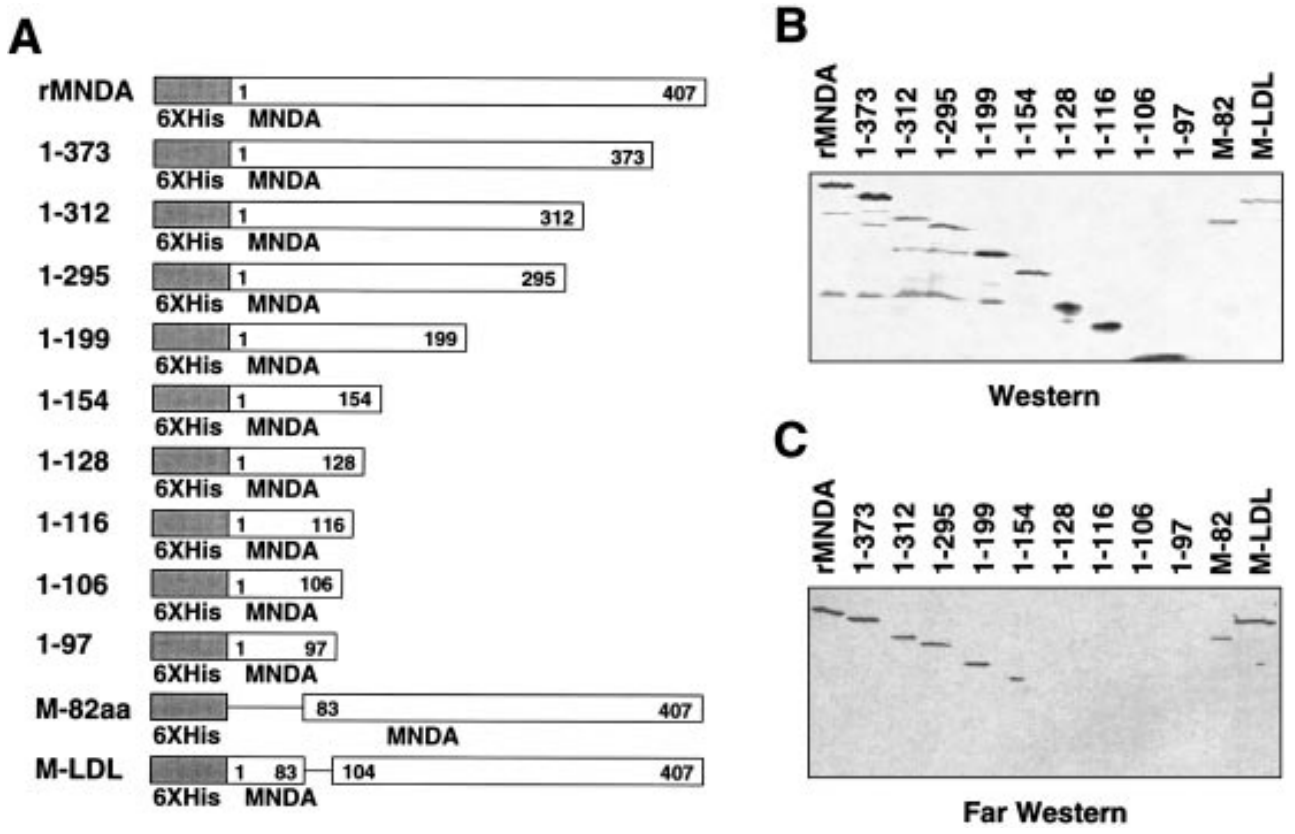

Fig. 2. Localization of regions essential for self-association by blotting C-terminal deletion mutants with radiolabeled rMNDA. (A) Schematic representation of His-tagged MNDA, C-terminal deletions, N-terminal and internal MNDA deletion constructs. (B) Bacterial lysates with expressed recombinant and mutated proteins separated on a 12.5% SDS-PAGE and analyzed by Western blotting with antibodies against MNDA. The slowest migrating band in each lane represents the appropriate size of the recombinant mutant protein determined by sequencing. (C) The expressed recombinant products were tested for ^{125}I-rMNDA binding by Far Western assay. MNDA mutants missing residues from the N-terminal half of MNDA bind weakly or not at all.

3, panel D) clearly demonstrated that MNDA binds to itself (GST-MNDA) (Fig. 3, panel D, lane 1) but not to GST (Fig. 3, panel D, lane 2). The highly basic MNDA region between aa 53 and 150 is essential for self-association since deletion of residues 53—100 and 101–150 inhibits self-association (Fig. 3, panel D, lanes 4 and 5). Results of co-precipitation with glutathione-immobilized GST-MNDA beads showed that the same regions were required for self-association (data not shown). These results, along with data shown in Fig. 2, demonstrated that sequence within the N-terminal half of MNDA is essential for self-association.

MNDA is a 407-amino-acid nuclear protein and relevant structural characteristics are summarized in Fig. 4. The C-terminal 200 aa sequence is conserved between MNDA and another related human gene (IF16) as well as a cluster of mouse genes (202, 204, D3) located on chromosome 1q [3,34,35]. The MNDA N-terminal region is enriched with positive charged amino acids [36]. Amino acids 87–109 form a basic amphipathic α-helical region [37]. This α-helical sequence exhibits >50% identity with a region in the human interferon regulatory factor 2 (IRF2) gene (aa 119–141), a known transcription repressor that binds the interferon-stimulated response element (ISRE) [19]. However, the putative DNA-binding regions of the IRF2 and the related IRF1 and interferon consensus sequence binding protein (ICSBP) are confined to the N-terminal 120 amino acids which show no similarity to MNDA sequence. Within the basic amphipathic α-helical region (aa 84–103) and also in the C-terminal region (aa 388–400) of MNDA, there are sequences matching the consensus LDL receptor-binding sequence of apolipoprotein B-100 [38]. While the N-terminal LDL receptor-binding motif (aa 84–103) is within the region required for dimerization (Fig. 3), the deletion of this region (aa 83–104) had no effect on self-association (Fig. 2). While deleting aa 3–52 did not alter self-association, deleting residues 1–82 reduced binding which implicates residues 52–82 in self-association. This region would most likely account for inhibiting self-association when residues 53–100 were deleted since residues 83–104 are not important for binding. The sequence between 52 and 82 contains an imperfect leucine zipper motif (MEKKFQGVACLDKLIELAKDMPSLKNLVNNL). The C-terminal deletion results clearly implicate residues 128–154 in self-association and this region could account for the effect of deleting residues 101–150. The region between 128 and 150 is highly basic (GRIPVAQKRKTPNKEKTEAKRNK). Recent results also demonstrated that the regions essential for dimerization (deletions of aa 53–100 and 101–150) were also essential for MNDA binding to YY1 zinc finger transcription factor [25]. Therefore, the dimerization of MNDA could be essential for its ability to interact with other nuclear proteins and transcription factors and the imperfect leucine zipper sequence in conjunction with a highly basic region flanking an N-terminal basic amphipathic α-helical region (aa 83–104) defines a novel protein–protein interaction motif.

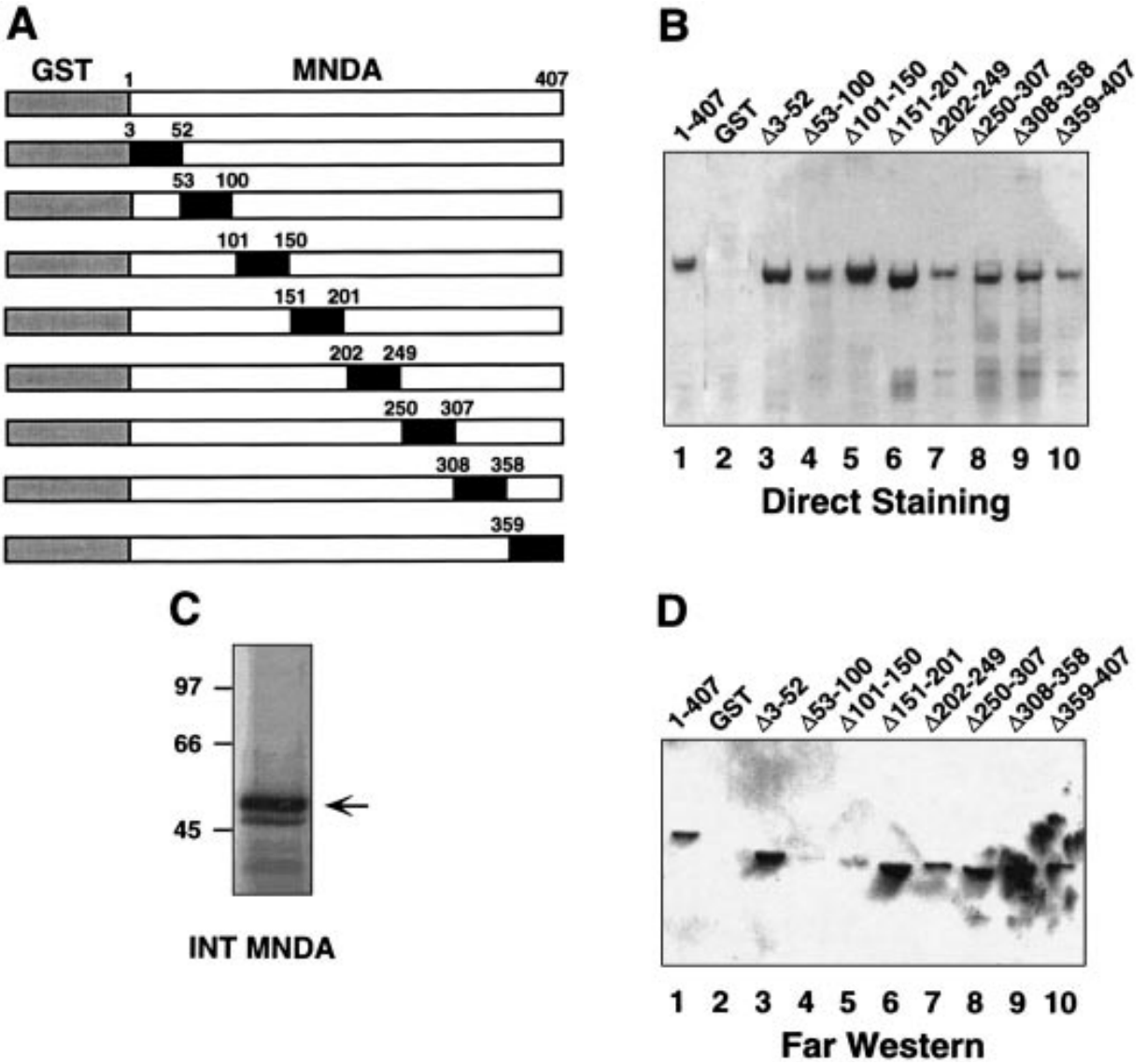

Fig. 3. Mapping MNDA dimerization region by blotting GST-MNDA internal deletion mutants with in vitro translated MNDA. (A) Schematic illustration of GST-MNDA and sequential internal deletion mutants. Using pGEX-MNDA as templates, PCR-based mutagenesis was performed to generate eight consecutive deletion mutants of MNDA spanning the entire coding region with each lacking an ≈50 amino acids. (B) Coomassie blue staining of 8% SDS-PAGE of purified GST-MNDA and its deletion mutants. Each lane was loaded with 1 μl of glutathione bead associated protein (containing 50–200 ng protein). (C) Autoradiogram of SDS-PAGE separated in vitro translated MNDA. (D) Same samples as in panel B separated on SDS-PAGE, transferred to membrane and blotted with in vitro translated, ^{35}S-met-labeled MNDA. Bands in the autoradiogram indicate positive binding to the labeled rMNDA. Lanes 4 and 5, corresponding to deletions of amino acids 53–100 and 101–150 bind very weakly, indicating that these mutants contain sequence essential for self-association.

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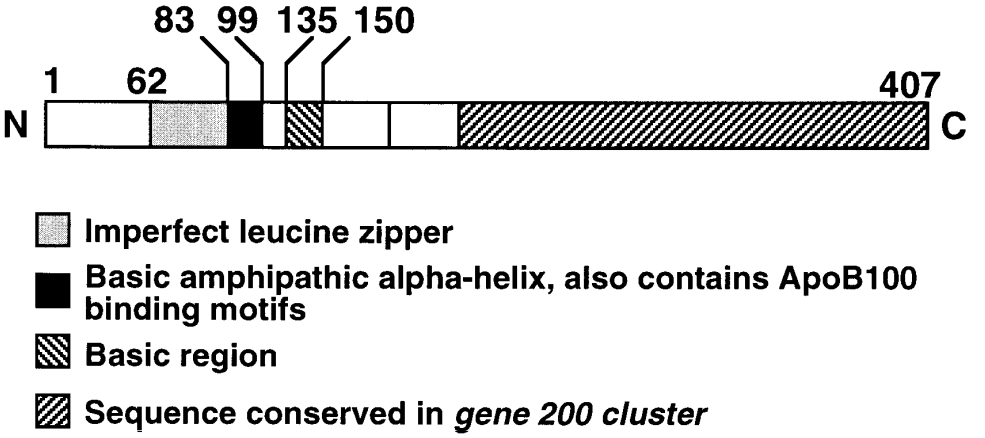

Fig. 4. Summary of the structural characteristics of primary MNDA sequence. Locations of major structural features of the MNDA relevant to this report (not to scale).

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