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Characterization of the Chromatin Binding Activity of Lamina-Associated Polypeptide (LAP) 2

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Previous studies have shown that the first and the second halves of the LAP2 N-terminal nucleoplasmic domain function independently in targeting LAP2 to the nuclear envelope, and that the second half is involved in association with the nuclear lamina. To further define the role of the nucleoplasmic domain, we have examined the targeting and chromatin binding functions of the first half of its N-terminus. Expressed polypeptides comprising residues 1–67 fused to the LAP2 transmembrane sequence were localized in perinuclear aggregates, while a residue within residues 244–296 was involved in the translocation of LAP2 to the nucleus as well as in DNA binding. Deletion of any of these domains resulted in a loss of the nuclear envelope targeting function. These data suggest that multimeric interactions of LAP2 with specific cellular components are required for correct targeting to the nuclear envelope and that the first N-terminus has function which is at least directly involved in chromatin association.

The nuclear envelope is composed of the inner and outer nuclear membranes, the nuclear pore complexes, and the nuclear lamina. The nuclear lamina is a filamentous protein meshwork lining the inner nuclear membrane, which contains mainly a polymer of nuclear lamins, members of the intermediate filament protein superfamily(reviewed in 1,2,3). A number of less abundant polypeptides are associated with the nuclear lamina as well, including certain integral membrane proteins. It is likely that integral membrane proteins are important for the association of the nuclear lamina with the inner nuclear membrane. (reviewed in 4,5). One is lamina-associated polypeptide (LAP) 2, a type II integral membrane protein with a single membrane-spanning segment.

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LAP2 is a ~53kDa polypeptide which is distributed in the inner nuclear membrane and is tightly associated with the nuclear lamina. cDNA cloning has shown that LAP2 contains a large hydrophilic amino-terminal domain exposed to the nucleoplasm(6,7). LAP2 specifically binds to B-type lamins and chromosomes in a mitotic phosphorylation dependent fashion. During late anaphase, it has been demonstrated to become localized at the surface of chromosomes prior to assembly of nuclear lamins at the nuclear envelope (6). These differential binding properties support a direct role for LAP2 in the assembly of the nuclear envelope at the end of mitosis. The large nucleoplasmic domain of LAP2 is important for targeting LAP2 to the nuclear envelope, and includes two non-overlapping nuclear envelope targeting signals. These functions, differential binding and targeting to the nuclear envelope, are present in the first half (residues 1-296) and the second half (residues 298-409), respectively, of the LAP2 N-terminus(7). The second half of the N-terminus is involved in association with the nuclear lamina, but the association property of the first half of the N-terminus remains unknown.

In the present study, in order to demonstrate that the first half of the N-terminal nucleoplasmic domain of LAP2 is involved in nuclear envelope targeting, we analyzed normal and mutated LAP2 nucleoplasmic domains using gene transfection and an in vitro binding assay. We report here that the first half of N-terminus of LAP2 directly interacts with chromatin.

MATERIALS AND METHODS

Construction of LAP2 deletion mutants and expression in eukaryotic vector. Clones 4b and 3a of LAP2 in pBluescript (7) were used for generating a set of cDNA fragments encoding deletion mutants. To generate fragments encoding the ΔC2 and ΔC2N1 fragments, pBluscript-4b and 3a, respectively, were digested with HincII, a BglII linker was added, and the construct was then digested with BamHI and BglII. For the ΔC2N2, the ΔC2 was digested by HindIII, a BamHI linker was added, and the plasmid was then digested with

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BamHI and BglII. For the ΔC2C2, pBluescript-4b was digested with XhoI, a BglII linker was added, and the DNA was then digested with BamHI and BglII. To generate two small fragments encoding residues 195-243 and 244-296, the PstI site of ΔC2N2 was replaced with a BamHI site, and the plasmid was then digested with BamHI and BglII. For the other fragments, the methods were as described elsewhere (7).

The LAP2 cDNA fragments obtained as described above were cloned into a eukaryotic expression vector driven by the cytomegalovirus enhancer and promoter. Fragments were inserted into the 3′ multicloning sites of the nucleotide sequence encoding the HA epitope(8). To construct the series of ΔI2N and ΔI2C, eukaryotic expression plasmids containing appropriate fragments were digested with BglII, and a fragment encoding residues 401-452 was inserted. For tPK and PK expression plasmids, truncated pyruvate kinase fragments with residues 1-418 deleted and full length PK, respectively, were inserted between the HA epitope and the LAP2 cDNA fragments. The epitope tagged expression vectors were transfected into MOP8 cells in 10 cm dishes containing coverglasses by the calcium phosphate coprecipitation method as described previously (9). The localization of chimeric proteins were detected by immunofluorescence using an anti HA peptide specific-monoclonal antibody (Boehringer Mannheim Co.) by described previously. (8).

DNA binding assay with single and double strand DNA cellulose. Purified glutathione S-transferase (GST) and GST fused LAP2 mutant proteins were incubated with 50 μl aliquots of single and double strand DNA cellulose in 500μl binding buffer [0.2 M KCl, 0.5 mM EGTA, 20 mM Hepes (pH7.6), 0.1% TritonX-100, 20% glycerol, 0.1mM PMSF, 1μg/ml aprotinin and 1 mg/ml BSA] for 6hr at 4° C with rotation. After incubation, DNA cellulose-protein complexes were washed 3 times with binding buffer and one times with binding buffer without BSA. The LAP2 mutant proteins were then directly eluted with 2% SDS solution. To generate GST fused LAP2 mutants, fragments of a series of LAP2 mutants (described in 7) were inserted into the 3′ multicloning sites of GST in the pGEX-2T vector.

Western blotting. Protein samples which were obtained by the in vitro binding assays were electrophoresed on SDS-polyacrylamide gels (10), and then analyzed by Western blotting with GST specific polyclonal antibodies (Pharmacia) as described in (11).

RESULTS

Characterization of the First Half of the N-Terminal Sequences Targeting LAP2 to the Nuclear Envelope

To study the mechanisms of targeting and association of the first half of the N-terminus of LAP2 with the nuclear envelope, we investigated a series mutant peptide fragments containing the first half of the N-terminus of LAP2 with or without the LAP2 transmembrane domain sequence. A series of HA-tagged deletion mutants of LAP2 were expressed in MOP8 cells by transient transfection, localization of the expressed constructs was followed by immunofluorescence.

Three deletions extending from the N-terminus of ΔI2, which contain the transmembrane sequence were analyzed (Fig.1A, ΔI2N1-ΔI2N3; Fig.1B). Like full length of LAP2, the first half of the N-terminus of LAP2 combined with the transmembrane sequence (e.g. Fig. 1B, ΔI2) efficiently localized to the nuclear envelope (as described previously) (7). This was not the case for the mutant lacking the first 137 residues of the N-terminus (ΔI2N1). Similar results were obtained with deletion mutants lacking residues 1-195(ΔI2N2) and 1-243 (ΔI2N3). All three mutants were localized to the cytoplasm in an ER-like reticular distribution. We next examined three deletions extending from the C-terminus at residue 296 of ΔI1 (Fig.1A, ΔI2C1-ΔI2C3; Fig.1B). These three mutants were found to be distributed in the cytoplasm around the nucleus in MOP8 cells, but unlike to reticular distribution observed for the series of ΔI2N mutants, ΔI2C mutants were accumulated in perinuclear aggregates in addition to the nuclear envelope.

In a second experiment, we investigated the localization of expressed mutants lacking parts of the first half of the N-terminus without the transmembrane sequence (Fig.2A). We earlier demonstrated that fragments containing the first half of the N-terminus (Fig.2A, ΔC2), comprising residues 1-296, but not residues 1-195 (ΔC2C1) or 298-400, exhibit a diffuse distribution throughout the nucleus (7). The amino acid sequence between residues 400 and 452 includes a hydrophobic transmembrane sequence but has no basic amino acid sequence similar to a nuclear localization signal. Thus residues 196-296 appear to target LAP2 to the nucleus. To test this, three deletion mutants extending from the N or C-termini were constructed (Fig.2A, ΔC2N1, ΔC2N2 and ΔC2C2; Fig.2B). The mutants lacking residues 1-137 (ΔC2N1) and 1-195 (ΔC2N2) specifically localized to the nucleus similar to the ΔC2. In contrast, the constructs comprising residues 1-67 (ΔC2C2) and 1-195 (ΔC2C1) were found to be distributed diffusely throughout both the nucleoplasm and the cytoplasm. These results indicate that the residues 196-296 are involved in translocation of LAP2 to nucleus. While the N-terminal amino acids between residues 1 and 67 combined with the transmembrane sequence (Figure 1B, ΔI2C1) aggregated in a perinuclear region, residues 1-67 themselves did not demonstrate a specific distribution (Fig.2B, ΔC2C2).

To further define the amino acid sequence responsible for nuclear localization, residues 196-243 and 244-296 were expressed. However, these two fragments could not be detected in MOP8 cells, possibly because of their short length (data not shown). These two fragments were therefore expressed as chimeras joined to the C-terminus of a truncated pyruvate kinase peptide (tPK; ~12kDa) with deleted residues 1-418. Their cellular localizations were then analyzed by immunofluorescence (Fig.3A, tPKΔS1 and tPKΔS2; Fig.3B). The chimeric tPKΔS1 protein was distributed diffusely throughout the nucloplasm and cytoplasm, whereas tPKΔS2 was specifically located at the nucleus. Therefore the sequence 244-296 of LAP2 is involved in targeting to the nucleus.

Entry into the nucleus may be through passive diffusion or active transport. Passive diffusion depends on

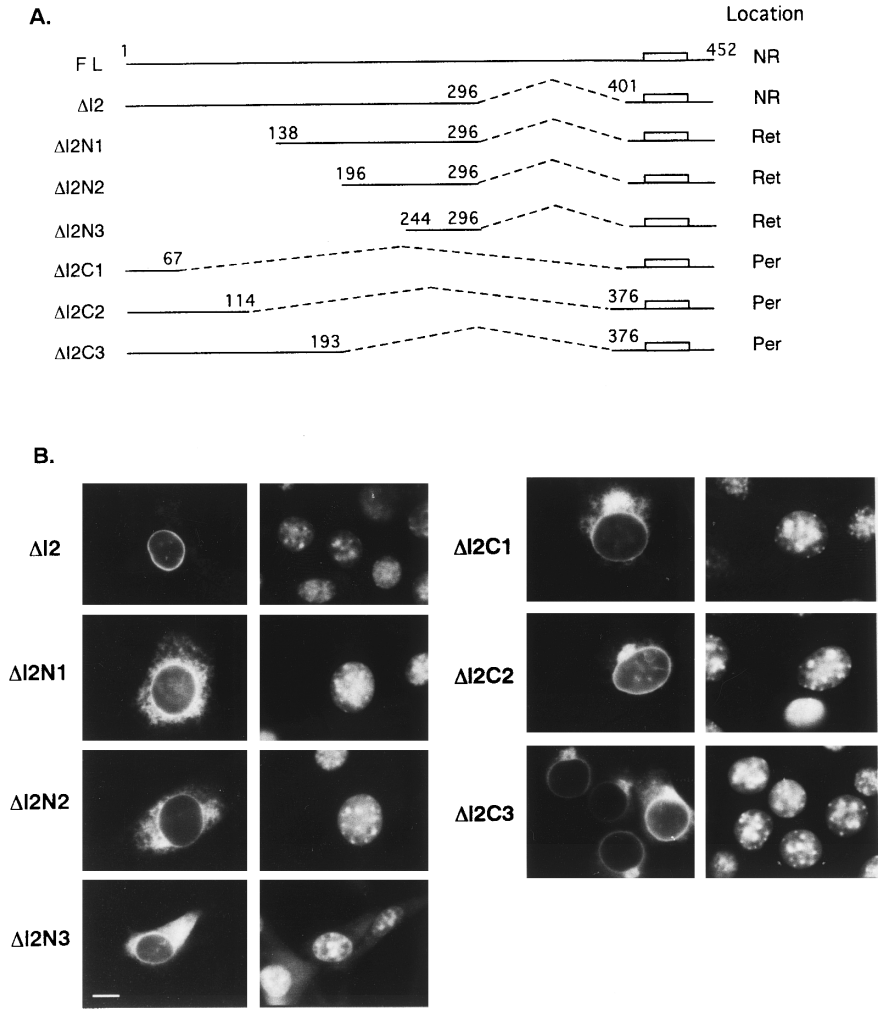

FIG. 1. Analysis of the targeting function of deletion mutants of LAP2 with the transmembrane sequence in transfected MOP8 cells. (A) The localization of a series of deletion mutants of HA-tagged LAP2 combined with the LAP2 transmembrane sequence was examined by transfection of constructs into MOP8 cells. Constructs analyzed were full length LAP2 (FL) and mutant LAP2 deleted at the N-terminal (ΔI2N) or C-terminal (ΔI2C). The transmembrane segment (residues 410–433 of LAP2) is indicated by an open bar in the diagram. The immunofluorescence localization is noted on the right. NR, nuclear rim; Ret, cytoplasmic reticular; Per, perinuclear aggregate. (B) Results from the transfected LAP2 deletion mutants described in (A). The localization of epitope-tagged protein products were analyzed by immunolabeling with an anti-HA monoclonal antibody. Staining with FITC-labeled secondary antibodies is shown on the left and the same fields labeled with Hoechst dye 33258 to identify nuclei on the right. The series of ΔI2C mutants were accumulated in perinuclear aggregates in addition to the nuclear envelope. Bar, 10 μm.

molecular size whereas active transport requires a specific nuclear localization sequence. The small amino acid sequence between residues 244 and 296 of LAP2 is highly basic: pI ~12.0 and contains the basic amino acid sequence; RGSRRTPRRR. To test whether this region is involved in active transport to the nucleus, hybrid genes encoding full length pyruvate kinase (PK) joined to the N-terminus of residues 244-296 and 1-296 were constructed and transfected into MOP8 cells (Fig.3A, PKΔS2 and PKΔC2; Fig.3C). The molecular weight of the HA-tagged full length PK is ~60kDa. Protein products from both constructs were found to be distributed diffusely throughout the cytoplasm with no nuclear staining detected. Since residues 244-296 did not direct PK-fusion proteins to the nucleus, we conclude that this region does not contain a sequence sufficient to direct a large peptide to the nucleus.

In these experiments, we established that deletion of a N- or C-region from the first half of the N-terminus of LAP2 (Fig.1A, ΔI2N and ΔI2C) resulted in loss of nuclear envelope targeting. However, the mutant lacking residues 68-400 (Fig.1B, ΔI2C1) was highly concentrated in perinuclear aggregates, and mutants containing residues 244-296 (See Fig.2B, ΔC2N2; Fig.3B, tPKΔS2) were localized to the nucleus. These data suggest that individual domains in the first half of the N-

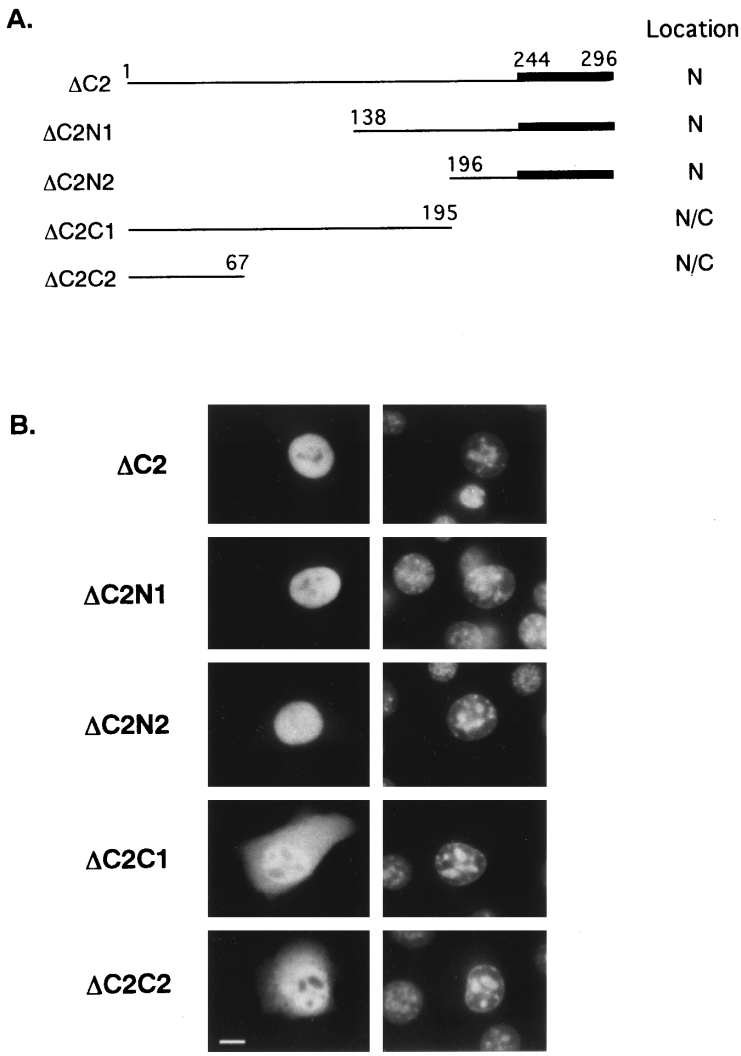

FIG. 2. Analysis of the targeting function of mutants with deletions of the first half of the N-terminus of LAP2 in transfected MOP8 cells. (A) A series of mutants of HA-tagged LAP2 without the transmembrane sequence was tested for localization by transfection of constructs into MOP8 cells. The first half of the N-terminus of LAP2 (ΔC2) was deleted at N-terminal (ΔC2N) or C-terminal (ΔC2C) sites. A region found particularly important for nuclear localization (residues 244–296) is indicated by the solid bar in diagram. N, diffuse intranuclear; C, diffuse cytoplasmic. (B) The localization of epitope-tagged protein products was analyzed 36–48 hr after transfection by immunolabeling with an anti-HA monoclonal antibody. Staining with FITC-labeled secondary antibodies is shown on the left, and the same fields labeled with Hoechst dye 33258 to identify nuclei are shown on the right. Bar, 10 μm.

terminus (residues 1-296) cooperate for targeting to the nuclear envelope.

Sequences Involved in Binding of the First Half of the N-Terminus to DNA

In addition to the results of the transfection analysis, the S/T-P-X-X and the basic amino acid sequence features of residues 244-296 suggest that LAP2 can bind to DNA. We therefore tested DNA binding activity in vitro using single or double stranded DNA cellulose and glutathione S-transferase (GST)-fused deletion mutants of LAP2 (Fig.4A). Purified GST and GST-fused LAP2 mutant proteins from bacterial lysates were incubated with single or double stranded DNA cellulose. Proteins were then eluted, and analyzed by Western blotting.

The GST-fused mutant protein which comprised amino acid residues 1-398 of LAP2 (G1) was efficiently retained on single and double stranded DNA cellulose (Fig.4B, lane 8). This protein-DNA interaction was resistant to elution with 0.2M KCl, but was abolished with 0.3MKCl as well as by DNase treatment (data not shown). To identify the amino acid sequences, that interact with the DNA, mutants with three C-terminal deletions of LAP2 were analyzed (Fig.4A, G2-G4). The mutant construct possessing 296 residues from the N-

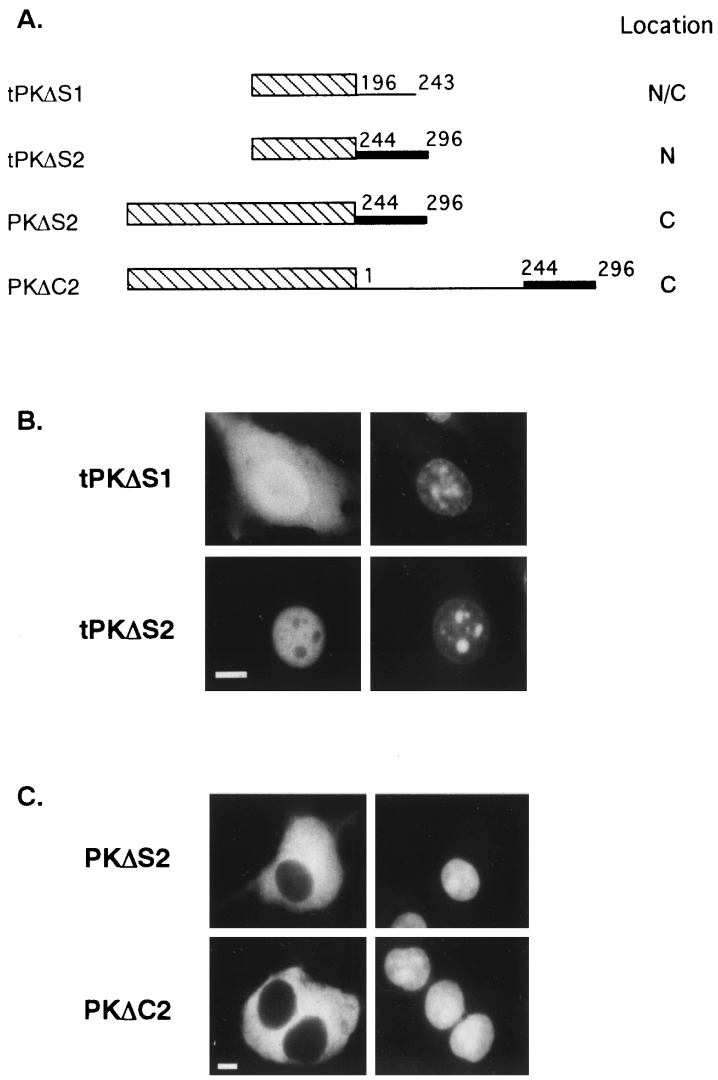

FIG. 3. Analysis of nuclear localization function of residues 196–296 of LAP2 in transfected MOP8 cells. (A) The residues 196–243, 244–296, and 1–296 of LAP2 were fused to HA-tagged truncated (tPK) and full length (PK) pyruvate kinase (hatched bar) at their N-termini and tested for localization by transfection of the constructs into MOP8 cells. Pyruvate kinase fragments were inserted into the mammalian expression vector between the HA-epitope and LAP2 cDNA. Residues 244–296 of LAP2 were found to be involved in nuclear localization and are indicated by the solid bar. (B) and (C) Intracellular distribution of tPKΔS1 and tPKΔS2 (B), and PKΔS2 and PKΔC2 (C) as determined by immunolabeling with an anti-HA monoclonal antibody after transfection into cells. Staining with FITC-labeled secondary antibodies is shown on the left, and the same fields labeled with Hoechst dye 33258 to identify nuclei on the right. Bar, 10 μm.

terminus (G2) was retained on both single and double stranded DNA cellulose (Fig.4B, lane 7), but the mutants (G3 & G4) which comprised amino acid residues 1-195 and 1-67 revealed no binding activity (Fig.4B, lanes 6 and 2). Thus the LAP2 sequence between residues 196 and 296 is involved in binding to DNA. Based on the transfection experiments described above, three additional mutants of LAP2 with deletions in both the N- and C-terminal regions were constructed and tested for DNA binding (Fig.4A, G5, G6 and G7). While the mutant comprising residues 138-373 (G5) was efficiently retained on both single and double strand DNA cellulose, the mutant comprising residues 298-398 (G6) was not (Fig.4B, lanes 5 and 4). The mutant G7 comprising residues 244-296, on the other hand, was still retained (Fig.4B, lane 3). These results are consistent with the transfection experiment findings (Fig.3), and suggest that the nuclear targeting activity of this region is due to direct binding to DNA.

DISCUSSION

In this study, we have characterized amino acid sequences in the first half of the LAP2 N-terminus involved in targeting it to the nuclear envelope.

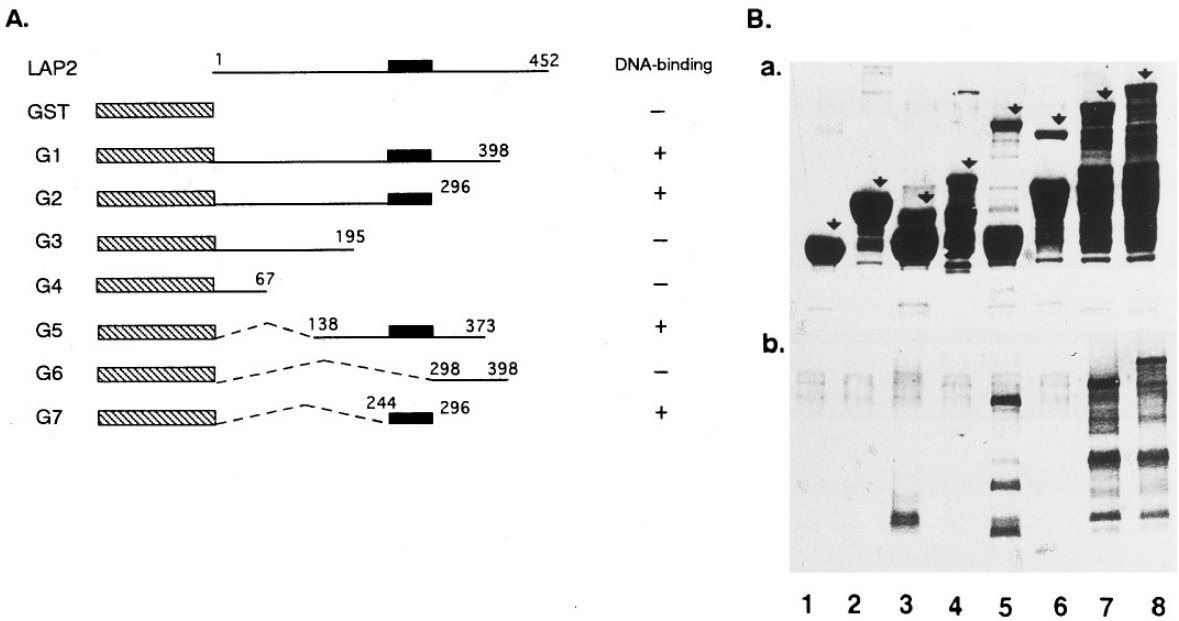

FIG. 4. Analysis of the DNA binding function of LAP2 in vitro. The DNA binding properties of a series of LAP2 deletion mutants were examined using double and single strand DNA cellulose in in vitro. (A) A series of deletion mutants of LAP2 were fused to glutathione S-transferase at their N-termini. LAP2 mutants were deleted at C-terminal or N/C-terminal sites. A region particularly important for the interaction of LAP2 with DNA cellulose (residues 244–296 of LAP2) is indicated by the solid bar. (B) DNA binding activity are indicated. Glutathione-agarose affinity purified fractions (shown in (a)) and DNA cellulose retained fractions (shown in (b)) were electrophoresed on 12% SDS-polyacrylamide gels and analyzed by Western blotting with anti-glutathione S-transferase specific antibodies. Lane 1 contains GST. Lanes 2, 3, 4 and 5 contain G4, G7, G6 and G5, respectively. Lanes 6, 7 and 8 contain G3, G2 and G1, respectively. The results with double strand DNA cellulose (not shown) were identical to those obtained with single strand DNA cellulose. The protein bands corresponding to the molecular weights of the GST fusion proteins are indicated by the arrows in (a).

Results obtained with a series of N- and C-terminal deletion mutants by an in vitro binding assay indicated that amino acids between 244-296 of LAP2 directly bind to DNA. A small lamin A tail fragment with the nuclear localization sequence deleted, has been reported to localize in the nucleus (12). This lamin tail domain has also been shown to directly interact with chromatin (13), so that its nuclear localization is thought to reflect the binding activity of the tail domain to chromatin. Similarly, the amino acid sequence between 244-296 of LAP2 does not contain a functional nuclear localization sequence, but, was apparently capable of translocating a small polypeptide to the nucleus. These results suggest that nuclear targeting by this sequence may also be associated with binding to chromatin. Thus, it supports that the idea that the DNA binding activity of LAP2 is involved in targeting LAP2 to the inner nuclear membrane as well as in providing anchoring sites for interphase chromosomes at the nuclear periphery. Further, LAP2 is known to bind to chromosomes in a mitotic phospholylation-regulated manner (6). The amino acid sequence between 244-296 of LAP2 contains two S/T-P motifs which are favorable amino acid substrate sequences for cdc2 kinase phosphorylation (14). These short sequences are also characteristic of a DNA binding motif (15). Therefore it is plausible that the LAP2 DNA binding activity of this region facilitates LAP2 binding to chromosomes at end of mitosis.

Recently another inner nuclear membrane protein, p58, has been reported to form multimeric complexes with the nuclear lamins, p58 kinase, p150, p34 and p18 polypeptides in phospholyration dependent manner (16, 17, 18, 19, 20). The N-terminal nucloplasmic domain of this protein also interacts with DNA and HP1 proteins (20, 21). Further, it has been shown to contain two non-overlapping nuclear membrane targeting signals in the N-terminal nucleoplasmic domain and the C-terminal hydrophobic domain (22, 23, 24). The signal that targets p58 to the nuclear envelope has been demonstrated to be present in the first transmembrane sequence of the C-terminal region (22), but it has not yet been specified in its N-terminal nucleoplasmic domain. Multimeric interactions in this region are thought to play important roles in targeting and retention of p58 to the nuclear envelope. We could not find a specific sequence in the first half of the N-terminus for targeting LAP2 to the nuclear envelope in this experiment. However, in addition to the DNA binding activity of residues 244-296, residues 1-67 combined with the

transmembrane sequence, accumulated specifically as perinuclear aggregates. The data thus suggest that each sequence of the first half of the N-terminus is associated with a specific component, so that multimeric interactions may also be involved in targeting of LAP2 to the nuclear envelope.

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