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cDNA Cloning of Nuclear Localization Signal Binding Protein NBP60, a Rat Homologue of Lamin B Receptor, and Identification of Binding Sites of Human Lamin B Receptor for Nuclear Localization Signals and Chromatin[1]

Shigeru Kawahire,*,† Masaki Takeuchi,* Takashi Gohshi,* Satoru Sasagawa,* Midori Shimada,* Minako Takahashi,* Takako K. Abe,†,‡ Takayuki Ueda,‡ Ryozo Kuwano,‡ Akihisa Hikawa,§ Tohru Ichimura,* Saburo Omata,* and Tsuneyoshi Horigome*,[2]

*Department of Biochemistry, Faculty of Science, and †Course of Biosystem Science, Graduate School of Science and Technology, Niigata University, 2-Igarashi, Niigata 950-21; ‡Research Laboratory for Molecular Genetics, Niigata University, 1-Asahimachi, Niigata 951; and §Immunochemistry System Department, Eiken Kagaku Ltd., Nogi-143, Nogi, Shimotsuga, Tochigi 329-01

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We previously purified and characterized a nuclear localization signal (NLS) binding protein, NBP60, in rat liver nuclear envelopes. In this study, we cloned and sequenced the cDNA of rat NBP60, and predicted an amino acid sequence comprising 620 amino acids. The sequence revealed that NBP60 is a rat homologue of lamin B receptor (LBR), and is 79 and 63% identical in amino acids to human and chicken LBR, respectively. Using three fusion proteins containing different parts of the amino-terminal domain of human LBR, it was shown that the stretch comprising amino acids 1 to 89, which contains a Ser-Arg rich region (RS region), binds to nucleoplasmin and that the binding was inhibited by a common NLS-peptide. These results suggested that the amino-terminal domain of LBR contains an NLS-binding site. Furthermore, it was shown that the stretch comprising amino acids 1 to 53, which does not contain the RS region or the predicted DNA-binding site, binds to *Xenopus laevis* sperm chromatin.

Key words: chromatin binding, heterochromatin, lamin B receptor, NBP60, nuclear membrane protein.

In eukaryotic cells, at least three domains are distinguishable in the nuclear envelope: the outer membrane domain, the inner membrane domain, and the nuclear pore membrane domain. Each of these domains is associated with specific macromolecular structures. The inner nuclear membrane is attached to the nuclear lamina and chromatin (*1*). Lamins, which comprise a filamentous protein meshwork that lines the nucleoplasmic surface of the inner nuclear membrane (*2-4*), may play a role in the attachment of chromatin to the nuclear membrane as well as in nuclear organization, because they interact with chromatin (*5-7*) or DNA (*8-10*). However, the nuclear lamina is a discontinuous structure that occupies only a fraction of the nuclear periphery (*11*), and at some points the lamina is disrupted. Recently, some integral proteins of the inner nuclear membrane were characterized (*12-22*), and it was found that one of these proteins, lamina-associated polypeptide 2 (LAP2), interacts directly with chromatin (*19*). However, if the inner membrane plays some role in the regulation of the chromatin functions, other membrane proteins probably also participate in the interaction.

A nuclear envelope inner membrane protein, LBR, was first identified in chicken (*12, 14*) and recently in man (*17*). It has been revealed that human LBR (hLBR) possesses a hydrophilic amino terminal domain, which is exposed to the nucleoplasm, followed by eight predicted transmembrane segments (*14, 17*). The amino-terminal domain of LBR contains consensus sequences for phosphorylation by $p34^{cdc2}$ protein kinase and protein kinase A (*23-25*). This domain is basic and also contains a stretch rich in arginine-serine (RS) motifs (*26*) and Ser/Thr-Pro-X-X sequences, which are frequently proposed to comprise a DNA-binding site (*14*). On the other hand, the hydrophobic regions and carboxyl-terminal domain of LBR are homologous to three yeast proteins (*27-29*). One of these, sterol C-14 reductase, is involved in sterol metabolism (*30*). Recent work has shown that RS motifs are found in a variety of splicing factors and mediate protein-protein interactions between components of the splicing machinery (*31, 32*). LBR also forms a multimeric complex which includes both A and B type lamins, a specific LBR kinase, and three other proteins

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[2] To whom correspondence should be addressed. Phone: +81-25-262-6160, Fax: +81-25-262-6165, E-mail: thori@sc.niigata-u.ac.jp

Abbreviations: GST, glutathione-S-transferase; hLBR, human lamin B receptor; HP1, heterochromatin-associated protein 1; LAPs, lamina-associated polypeptides; NBP60, NLS-binding protein with a relative molecular weight of 60,000; NLS, nuclear localization signal; PMSF, phenylmethanesulfonyl fluoride; RT-PCR, reverse transcription-PCR; SF2, splicing factor 2; T-peptide, peptide containing NLS of SV 40 large T-antigen.

(p18, p34, and p150) (*24, 33, 34*). Of these proteins, p34 has been found to be co-isolated with splicing factor 2 (SF2) (*26, 33, 35*). Furthermore, the amino-terminal domain of LBR interacts with human chromodomain proteins homologous to *Drosophila* HP1 (heterochromatin-associated protein 1), a heterochromatin protein involved in position-effect variegation (*36*).

In this study, we cloned cDNAs for nuclear localization signal binding protein (NBP60) of the rat liver nuclear envelope (*37, 38*), and established that the protein is a rat homologue of LBR. Using three fusion proteins containing different parts of the amino-terminal domain of hLBR, we demonstrated that the stretch comprising amino acids 1 to 89, which contains the RS region, can bind to an NLS-containing protein, and that the binding is inhibited by a synthetic NLS-peptide, T-peptide. Furthermore, we indicated that the stretch comprising amino acids 1 to 53 interacts directly with demembranated and decondensed sperm chromatin of *Xenopus laevis*.

MATERIALS AND METHODS

Buffer—PBS: 10 mM sodium phosphate, pH 7.4, containing 140 mM NaCl and 2.7 mM KCl; Incubation buffer: 50 mM triethanolamine-HCl, pH 7.5, containing 0.5% Triton X-100, 2 mM $MgCl_2$, 10% sucrose, 0.5 mM DTT, 0.2 mM PMSF, 10 μg/ml of leupeptin and chymostatin, 5 μg/ml of pepstatin A and antipain, and 150 mM NaCl; Buffer 1: 50 mM Hepes-KOH , pH 7.7, containing 0.25 M sucrose, 50 mM KCl, 2.5 mM EDTA, and 100 μg/ml of aprotinin and leupeptin.

Partial Amino Acid Sequencing of NBP60—NBP60 was purified from a Triton/high-salt extract of rat liver nuclear envelopes by nucleoplasmin-Sepharose affinity chromatography, followed by hydroxyapatite high-performance liquid chromatography as described previously (*37*). The purified NBP60 (30 μg) was electrophoresed in an 8% polyacrylamide gel containing 0.1% SDS (SDS-PAGE), and stained with Coomassie Brilliant Blue R-250, and then the protein-containing gel slices were subjected to BrCN cleavage, and lysylendopeptidase and trypsin digestion as follows. BrCN cleavage: the procedures were as described by Jahnen *et al.* (*39*). The gel slices were dried by lyophilization and then treated with BrCN in 70% (v/v) formic acid for 16 h at 25°C (NBP60/BrCN ratio = 1 : 100, w/w). At completion of the reaction, BrCN and formic acid were removed by centrifugal lyophilization in a speed-vac centrifuge. The materials in peptide fragment-containing gel slices were separated by Tricine-SDS-PAGE. The protein was electrotransferred onto a PVDF filter and visualized with Amido Black 10B. The materials in the thus separated five bands were subjected to amino acid sequencing. *In situ* lysylendopeptidase (Wako Chemicals, Osaka) and trypsin (Miles-Seravac, UK) digestion: the gel slices were equilibrated with 50% acetonitrile containing 200 mM ammonium hydrogen carbonate twice for 20 min at 30°C, and then brought to near-dryness in a vacuum desiccator. Proteases at an enzyme-to-substrate ratio of 1 : 25 (w/w) in 200 mM ammonium hydrogen carbonate containing 0.02% Tween 20 were added to the gel slices followed by incubation for 16 h at 37°C. The slices containing digested peptides were centrifuged after homogenization, and the supernatant was collected, dried by lyophilization, and then subjected to reverse-phase HPLC on a Silica-base C8 column (4.6×250 mm, Capcel Pak C8 column; Shiseido, Tokyo). Peptides were eluted with a linear gradient of 5-75% acetonitrile containing 0.1% trifluoroacetic acid at 0.5 ml/min. The sequences of the isolated peptides were determined with a Protein Sequencer 470A (Applied Biosystems, USA).

Analysis of cDNA Clones—A rat liver cDNA library prepared from Fisher 344 rats and constructed in lambda zap II (Stratagene, USA) was screened with human lamin B receptor cDNA as a probe (*17*). cDNA probes, prepared as below and termed N/C, were ^{32}P-labeled using a random-primed DNA labeling kit (Boehringer Mannheim Biochemica, Germany), and screening was carried out by standard procedures (*40*). The purified cDNA inserts were subcloned into a Bluescript II SK (−) plasmid vector, and their sequences were determined by the dideoxy chain termination method (*41*).

DNA Construction—A human lamin B receptor cDNA clone was prepared by reverse transcription-PCR (RT-PCR) using full-length cDNA sequence information previously described (*17*). Total RNA was isolated from human blood with ISOGEN (Wako) and a 7 μg aliquot was reverse-transcribed with Superscript II reverse transcriptase (Life Technologies, USA). The products were then used as PCR templates with synthetic oligonucleotide primers: N, 5′-TATGGATCCTAATGCCAGGNAGGAAATTTGCC-3′ as a 5′ primer; and C, 5′-TTAGAATTCAGTCGACAGTAGATGTATGGAAATATACGGTA-3′; K, 5′-TTAGAATTCAGTCGACTCCTCCAAACTCCAAGTCCTTTG-3′, and M, 5′-TTAGAATTCAGTCGACATCATTCTCTTTCAATTCAAGCTC-3′ as 3′ primers (C, K, and M are complementary). The sequences between the three primers; N/C, N/K, and N/M were amplified by PCR with *Ex Taq DNA polymerase* (Takara, Otsu), as follows: 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min for 35 cycles, and then 72°C for 7 min. The N/K thus obtained was digested with *Bam*HI and *Eco*RI, and then the DNA fragments were inserted into the *Bam*HI/*Eco*RI site of expression vector pGEX-3X (Pharmacia LKB Biotech, Sweden). A plasmid containing N/S was generated by digestion of a plasmid containing N/K with *Sal*I, followed by self-ligation.

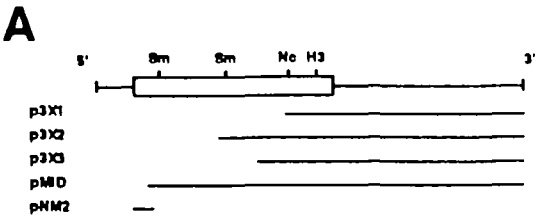
Expression and Purification of Human Lamin B Receptor—Expression plasmids, which encode various regions of hLBR (*17*) fused with glutathione S-transferase (GST), were transfected into *Escherichia coli* DE3 (BL21) cells made competent with RbCl. The *E. coli* cells were grown in LB-medium containing ampicillin at 37°C to an $A_{600} = 0.8$. Expression of fusion proteins was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (*42*), followed by incubation for 6 h at 30°C. The bacterial cells were collected by centrifugation and resuspended in PBS. Vigorous sonication was performed before centrifugation at 12,000×g for 20 min. The resulting supernatant was loaded onto a column (1.4×2 cm) packed with 2 ml of glutathione-Sepharose 4B (Pharmacia, Sweden). The column was washed extensively with PBS, and then bound GST-fused proteins were eluted with PBS containing 50 mM glutathione. The eluted proteins were stored at −80°C until use.

SDS-PAGE, Silver Staining, and Immunoblotting—Proteins were separated by SDS-PAGE according to the method of Laemmli (*43*). The acrylamide concentration of

the separation gel was 10% unless otherwise stated. Silver staining was carried out as previously described (*44*). When protein amounts were estimated by silver staining followed by densitometry (Figs. 5 and 6), bands corresponding to the full-length protein were used, and a calibration curve was made on every gel with a series of known amounts of bovine serum albumin. Immunoblotting was performed as previously described (*38*).

NLS Binding Assay with Nucleoplasmin-Sepharose—Purified nucleoplasmin was conjugated with CNBr-activated Sepharose 4B as previously described (*37*). GST-fused proteins were incubated with nucleoplasmin-Sepharose gel at 4°C for 12 h in a batchwise manner as previously described (*37*). Anti-rat NBP60 antiserum was raised by immunizing female rabbits with purified NBP60 (*38*). NBP60 was purified from rat liver nuclear envelopes according to the reported method (*37*).

Inhibition of the Binding of Recombinant Proteins and Nucleoplasmin-Sepharose by T-Peptide—Test solutions (20 μl) contained 400 ng of N/S-GST fusion protein, 0.4 mM T-peptide, and incubation buffer. After incubation for 30 min at 4°C, 10 μl of the nucleoplasmin-Sepharose gel suspended in the incubation buffer was added and the mixture was incubated for 16 h at 4°C. N/S-GST proteins in the bound and unbound fractions were analyzed by



B

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      10        20        30        40        50        60        70        80        90       100
ATGCCAGGAAGGAAATTTGCCGATGGTGAAGTGGTAAGAGGTCGATGGCCTGGGAGTTCACTTTATTATGAAGTAGAAATTCTGAGCCACGACAGCACC
M  P  G  R  K  F  A  D  G  E  V  V  R  G  R  W  P  G  S  S  L  Y  Y  E  V  E  I  L  S  H  D  S  T

     109       119       129       139       149       159       169       179       189       199
TCCCAGCTTTACACTGTGAAGTATAAAGATGGCACCGAGCTGGAGCTGAAGGAGAGTGACATCAAGCCTTTAAAGTCCTTTAAGCAGAGAAAGAGTGGC
S  Q  L  Y  T  V  K  Y  K  D  G  T  E  L  E  L  K  E  S  D  I  K  P  L  K  S  F  K  Q  R  K  S  G

     208       218       228       238       248       258       268       278       288       298
TCAACTTCCAGCTCTCCTTCAAGACGCCGCAGCAGCCGCAGCCGCTCCCGATCACGCTCCCGATCCCCGGGCCGGGCACCCAAAGGTTCCAGAAGATCA
S  T  S  S  S  P  S  R  R  R  S  S  R  S  R  S  R  S  R  S  P  G  R  A  P  K  G  S  R  R  S

     307       317       327       337       347       357       367       377       387       397
GTCTCTGCTTCCTACCAGGCTGACGCTAAGGAAAAGGAAATGAGGAGGGAGATTTTGCAAGTTAAATTGACTCCGCTCGTGCTGAAGCCGTTTGCAAAC
V  S  A  S  Y  Q  A  D  A  K  E  K  E  M  R  R  E  I  L  Q  V  K  L  T  P  L  V  L  K  P  F  A  N

     406       416       426       436       446       456       466       476       486       496
AGCGTCAGTGTGTACAACGGGGAGCCCGAACACATGGAAAAGAGCGCCACACCTCCTAAAAACAAGCAGGAAAGAGTCATTTTGTCAACAGAAGACAGT
S  V  S  V  Y  N  G  E  P  E  H  M  E  K  S  A  T  P  P  K  N  K  Q  E  R  V  I  L  S  T  E  D  S

     505       515       525       535       545       555       565       575       585       595
TATATAGCTACACAGTACAGCCTTCGCCCCAGAAGAGAAGAGGTCAAACCAAAACATAGAGTCCGAGGAACAAACCTTGTCACCAGAGGACCTGTACCT
Y  I  A  T  Q  Y  S  L  R  P  R  R  E  E  V  K  P  K  H  R  V  R  G  T  N  L  V  T  R  G  P  V  P

     604       614       624       634       644       654       664       674       684       694
TTGGGAACCTTTCAAGTGACCACTCCACAGAGGAGGGACTTGGAGTTCGGAGGAGTACCTGGTGCGCTCCTCATTATGCTTGGCCTGCCTGCCTGTGTC
L  G  T  F  Q  V  T  T  P  Q  R  R  D  L  E  F  G  G  V  P  G  A  L  L  I  M  L  G  L  P  A  C  V

     703       713       723       733       743       753       763       773       783       793
TTCCTGCTGCTGCTGCAGTGTGCGCAGAAGGACCCCGGGCTGCTCCAGTTCCCTCCACCTCTGCCAGCTCTCCGTGAGCTGTGGGAAGCCAGAGTGTGT
F  L  L  L  L  Q  C  A  Q  K  D  P  G  L  L  Q  F  P  P  P  L  P  A  L  R  E  L  W  E  A  R  V  C

     802       812       822       832       842       852       862       872       882       892
GGCGTTTACCTCCTCTGGTTCTTCCTTCAAGCTCTCTTCTCCCTTCTACCAGTTGGGAAGGTTGTAGAAGGAACACCTCTTGTTGATGGAAGAAGACTC
G  V  Y  L  L  W  F  F  L  Q  A  L  F  S  L  L  P  V  G  K  V  V  E  G  T  P  L  V  D  G  R  R  L

     901       911       921       931       941       951       961       971       981       991
AAGTACAGACTGAATGGACTGTATGCCTTCATCCTGACATCAGCAGCCGTGGGGACAGCTGTCTTCTGGGACATAGAACTATATTACCTGTACACTCAC
K  Y  R  L  N  G  L  Y  A  F  I  L  T  S  A  A  V  G  T  A  V  F  W  D  I  E  L  Y  Y  L  Y  T  H

    1000      1010      1020      1030      1040      1050      1060      1070      1080      1090
TTCCTGCAGTTTGCACTGGCAGCCATTGTGTTTTCAGTGGTCCTGAGTGTTTACCTCTATGCCCGCTCTTTGAAAGTCCCCAGGGATGAGCTGTCACCT
F  L  Q  F  A  L  A  A  I  V  F  S  V  V  L  S  V  Y  L  Y  A  R  S  L  K  V  P  R  D  E  L  S  P

    1099      1109      1119      1129      1139      1149      1159      1169      1179      1189
GCCAGTTCTGGAAATGCTGTCTATGACTTCTTCATTGGCCGTGAGTTAAACCCTCGAATTGGTGCTTTCGATCTCAAATTCTTCTGTGAATTGCGCCCT
A  S  S  G  N  A  V  Y  D  F  F  I  G  R  E  L  N  P  R  I  G  A  F  D  L  K  F  F  C  E  L  R  P

    1198      1208      1218      1228      1238      1248      1258      1268      1278      1288
GGATTGATCGGATGGGTGGTTATTAACTTGGTGATGCTTTTGGCTGAAATGAAAGTCCAGGAGCGTAGTGCTCCGTCCTTGGCGATGACCCTGGTCAAC
G  L  I  G  W  V  V  I  N  L  V  M  L  L  A  E  M  K  V  Q  E  R  S  A  P  S  L  A  M  T  L  V  N

    1297      1307      1317      1327      1337      1347      1357      1367      1377      1387
AGCTTCCAGCTCCTGTATGTGGTGGACGCCCTCTGGTTTGAGGAAGCATTGCTGACCACCATGGACATCATCCACGATGGCTTTGGCTTCATGCTGGCC
S  F  Q  L  L  Y  V  V  D  A  L  W  F  E  E  A  L  L  T  T  M  D  I  I  H  D  G  F  G  F  M  L  A

    1396      1406      1416      1426      1436      1446      1456      1466      1476      1486
TTTGGGGATTTAGTGTGGGTTCCATTTACCTACAGTCTCCAGGCCTTCTACCTGGTCAACCATCCCCAGGACTTGTCCTGGCCATTGACTTCTGTCATC
F  G  D  L  V  W  V  P  F  T  Y  S  L  Q  A  F  Y  L  V  N  H  P  Q  D  L  S  W  P  L  T  S  V  I

    1495      1505      1515      1525      1535      1545      1555      1565      1575      1585
ATCGCTCTGAAGCTGTGTGGATATGTAATATTCCGTTGTGCAAACTCTCAGAAAAATGCTTTTCGGAAGAATCCCACTGATCCAAAGCTTGCACATTTA
I  A  L  K  L  C  G  Y  V  I  F  R  C  A  N  S  Q  K  N  A  F  R  K  N  P  T  D  P  K  L  A  H  L

    1594      1604      1614      1624      1634      1644      1654      1664      1674      1684
AAAACCATCCCTACTTCGACGTGGAAGAGTCTGCTTGTGTCTGGATGGTGGGGCTTTGTTCGCCATCCCAATTACTTGGGTGATCTCATCATGGCCTCTG
K  T  I  P  T  S  T  W  K  S  L  L  V  S  G  W  W  G  F  V  R  H  P  M  Y  L  G  D  L  I  M  A  L

    1693      1703      1713      1723      1733      1743      1753      1763      1773      1783
GCGTGGTCCCTCCCATGTGGCTTCAACCACATCCTGCCTTACTTCTATGTCATCTACTTCACCGCATGGCTTATCCATCGAGAAGCCCGTGATGAACAC
A  W  S  L  P  C  G  F  N  H  I  L  P  Y  F  Y  V  I  Y  F  T  A  L  L  I  H  R  E  A  R  D  E  H

    1792      1802      1812      1822      1832      1842      1852      1862      1872
CAGTGCAGGAGGAAGTACGGCCTGGCCTGGGAGAAGTACTGTCAGCGCGTGCCCTACCGCATATTCCCCTACATTTACTGA
Q  C  R  R  K  Y  G  L  A  W  E  K  Y  C  Q  R  V  P  Y  R  I  F  P  Y  I  Y  *
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Fig. 1. **The nucleotide sequence of rat NBP60 cDNA and the deduced amino acid sequence.** A: Alignment of partial-length NBP60 cDNAs isolated by screening with radioactive oligonucleotide probes and PCR from a rat liver cDNA library constructed in lambda Zap II. The first line illustrates a putative cDNA derived from full-length mRNA of rat NBP60. Major restriction sites and a large open reading frame (open box) are indicated. The restriction enzyme sites are designated as follows: *Sma*I (Sm), *Nco*I(Nc), and *Hin*dIII(H3). B: The amino-terminal methionine residue is designated as +1 of the amino acid sequence and A of the initiator ATG codon as +1 of the cDNA nucleotide sequence of NBP60. The regions of the deduced amino acid sequence corresponding to the partial amino acid sequences determined in protein sequencing experiments are underlined. These sequence data are available from DDBJ/GenBank/EMBL under accession number AB002466.

SDS-PAGE. The amounts of N/S-GST protein were estimated by densitometry as described above.

Chromatin Binding Assay—Chromatin binding to immobilized GST-fused proteins was examined by phase contrast and fluorescence microscopy. The demembranated sperm chromatin consisted of *Xenopus* sperm treated with lysolecithin to remove the plasma and nuclear membranes without affecting the highly condensed chromatin. The chromatin was prepared as described (*45*). The cytosol fraction of *Xenopus* eggs (*45*) was boiled for 10 min, cooled in ice-water for 5 min, and then centrifuged at $10,000 \times g$ for 10 min to remove denatured proteins. The resulting supernatant, heated cytosol, was stored at −80°C until use. To determine chromatin binding to the immobilized GST-fused proteins, 1 μl of demembranated sperm chromatin (10,000/μl) in buffer 1 was incubated in 10 μl of heated cytosol at 23°C for 30 min for decondensation of the chromatin. After the incubation, 0.2 μg of purified GST was added, followed by incubation for 20 min to reduce non-specific binding, and then incubation with GST-fused proteins immobilized on glutathione-Sepharose 4B beads for an additional 60 min at 4°C. Chromatin bound to the beads was determined after staining with a DNA dye: Hoechst 33342 (Calbiochem-Novabiochem, USA) by phase-contrast and fluorescence microscopy.

RESULTS

Isolation and Sequencing of cDNA for NBP60—Partial amino acid sequences of the nine peptides derived on BrCN cleavage and proteolytic digestion of NBP60 were determined (Fig. 1B). On sequence analysis, it was revealed that two peptides completely matched parts of the amino acid sequence of hLBR, the others being 70–90% identical in terms of amino acids. These results suggested strongly that NBP60 is a rat homologue of LBR. Therefore, we decided to isolate a rat LBR cDNA clone, analyze the DNA sequence, deduce the amino acid sequence, and then compare the latter sequence with those of peptides obtained from NBP60. A pair of oligonucleotide primers for PCR was synthesized based on the amino acid sequence of hLBR. Human blood total RNA was subjected to RT-PCR, a product of the expected size being obtained. The product was sequenced and confirmed to encode hLBR. It was used

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NBP60    1:MPGRKFADGEVVRGRWPGSSLYYEVEILSHDSTSQLYTVKYKDGTELELKESDIK 55
hLBR     1:MPSRKFADGEVVRGRWPGSSLYYEVEILSHDSTSQLYTVKYKDGTELELKENDIK 55
cLBR     1:MPNRKYADGEVVMGRWPGSVLYYEVQVTSYDDASHLYTVKYKDGTELALKESDIR 55

NBP60   56:PLKSFKQRKSGSTSSSPSRRRSSRSRSRSRSRSPGRAPKGSRRSVSASYQADAKE 110
hLBR    56:PLTSFRQRKGGSTSSSPSRRR-G-SRSRSRSRSPGRPPKSARRSASASHQADIKE 108
cLBR    56:LQSSFKQRKSQSSSSSPS--R--RSRSRSRSRSPGRPAKGRRRSSSHS-R-EHKE 104

NBP60  111:KEMRREILQVKLTPLVLKPFANSVSVYNGEPEHMEKSATPPK-NKQERVILSTED 164
hLBR   109:-A-RREV-EVKLTPLILKPFGNSISRYNGEPEHIERNDAPHK-NTQEKFSLSQES 159
cLBR   105:-DKKKIIQETSLAP-P-KPSENNTRRYNGEPDSTERNDTSSKLLEQQKLKPDVEM 156

NBP60  165:SYIATQYSLRPRREEVKPKH-RVRGTNLVTRGPVPLGTFQVTTPQRRDLEFGGVP 218
hLBR   160:SYIATQYSLRPRREEVKLKEIDSKEEKYVAKE-LAVRTFEVTPIRAKDLEFGGVP 213
cLBR   157:ERVLDQYSLRSRREE-KKKE-EIYAEKKI-FEAIKTPE-K-PSSKTKELEFGGRF 206

NBP60  219:GALLIMLGLPACVFLLLLQCAQKDPGLQFPPPLPALRELWEARVCGVYLLWFFL 273
hLBR   214:GVFLIMFGLPVFLFLLLLMCKQKDPSLLNFPPPLPALYELWETRVFGVYLLWFLI 268
cLBR   207:GTFMLMFFLPATVLYLVLMCKQDDPSLMNF-PPLPALESLWETKVFGVFLLWFFF 260

NBP60  274:QALFSLLPVGKVVEGTPLVDGRRLKYRLNGLYAFILTSAAVGTAVFWDIELYYLY 328
hLBR   269:QVLFYLLPIGKVVEGTPLIDGRRLKYRLNGFYPFILTSAVIGTSLFQGVEFHYVY 323
cLBR   261:QALFYLLPIGKVVEGLPLSNPRKLQYRINGFYAFLLTAAAIGTLLYFQFELHYLY 315

NBP60  329:THFLQFALAAIVFSVVLSVYLYARSLKVPRDELSP-ASSGNAVYDFFIGRELNPR 382
hLBR   324:SHFLQFALAATVFCVVLSVYLYMRSLKAPRNDLSP-ASSGNAVYDFFIGRELNPR 377
cLBR   316:DHFVQFAVSAAAFSMALSIYLYIRSLKAPEEDLAPGGNSGYLVYDFFTGHELNPR 370

NBP60  383:IGAFDLKFFCELRPGLIGWVVINLVMLLAEMKVQERSAPSLAMTLVNSFQLLYVV 437
hLBR   378:IGTFDLKYFCELRPGLIGWVVINLVMLLAEMKIQDRAVPSLAMILVNSFQLLYVV 432
cLBR   371:IGSFDLKYFCELRPGLIGWVVINLAMLLAEMKIHNQSMPSLSMILVNSFQLLYVV 425

NBP60  438:DALWFEEALLTTMDIIHDGFGFMLAFGDLVWVPFIYSLQAFYLVNHPQDLSWPLT 492
hLBR   433:DALWNEEALLTTMDIIHDGFGFMLAFGDLVWVPFIYSFQAFYLVSHPNEVSWPMA 487
cLBR   426:DALWNEEAVLTTMDITHDGFGFMLAFGDLVWVPFVYSLQAFYLVGHPIAISWPVA 480

NBP60  493:SVIIALKLC-GYVIFRCANSQKNAFRKNPTDPKLAHLKTIPTSTWKSLLVSGWWG 546
hLBR   488:SLIIVLKLC-GYVIFRGANSQKNAFRKNPSDPKLAHLKTIHTSSGKNLLVSGWWG 541
cLBR   481:AAITILN-CIGYYIFRSANSQKNNFRRNPADPKLSYLKVIPTATGKGLLVTGWWG 534

NBP60  547:FVRHPNYLGDLIMALAWSLPCGFNHILPYFYVIYFTALLIHREARDEHQCRRKYG 601
hLBR   542:FVRHPNYLGDLIMALAWSLPCGFNHILPYFYIIYFTMLLVHREARDEYHCKKKYG 596
cLBR   535:FVRHPNYLGDIIMALAWSLPCGFNHILPYFYVIYFICLLVHREARDEHHCKKKYG 589

NBP60  602:LAWEKYCQRVPY-RI-FPYI-Y---------------------------  620
hLBR   597:VAWEKYCQRVPY-RI-FPYI-Y---------------------------  615
cLBR   590:LAWERYCQRVPYTHISLHLLEHSTYLICKLKYTSHLCTWSVCYLGFKH  637
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Fig. 2. **Amino acid sequence comparison of rat NBP60 with the human and chicken lamin B receptors.** Amino acids which are identical in rat NBP60, and human and chicken lamin B receptors are boxed. The human lamin B receptor amino acid sequence was taken from Ref. *17*, and the chicken lamin B receptor sequence from Ref. *14*.

for subsequent rat liver cDNA library screening to isolate rat LBR clones. Three cross-hybridizing clones were isolated from 3×10^6 plaques of a lambda Zap II rat liver cDNA library. All three clones contained a part of the lamin B receptor cDNA. The longest clone thus obtained, termed p3X2 (Fig. 1A), contained a cDNA insert of about 2.8 kbp, including a short stretch of a poly(A) tail. However, this clone did not contain a nucleotide sequence corresponding to the 5′-end of the open reading frame of hLBR (*17*).

We then rescreened the same cDNA library with an about 1 kbp fragment from the 5′ end of clone p3X2, and isolated another cDNA clone, termed pMID (Fig. 1A). The nucleotide sequence was identical over an about 2 kbp region with that of clone p3X2. The clone contained a 5′-extension of about 250 bp compared to p3X2. However, the nucleotide sequence determined has not yet reached the initial ATG codon. We therefore synthesized a pair of oligonucleotides, predicted from the amino acid sequence of a peptide derived from rat NBP60, for PCR. The amino acid sequence was the same as that of the amino-terminal region of hLBR in 6 out of 7 amino acids. An antisense nucleotide corresponding to the 5′-end region of the nucleotide encoded by pMID was synthesized. Then, PCR was carried out on the same cDNA library using these primers. A product of the expected size, a 150 bp fragment, was obtained. This product contained nucleotide sequences corresponding to five peptide sequences found in rat liver NBP60.

Amino Acid Sequence Deduced from cDNA—The 1,860-nucleotide-long open reading frame of the cDNA constructed from pMID and pNM2 encodes a protein of 620 amino acid residues with a calculated molecular weight of 70.6 K. This molecular weight is in close agreement with the relative molecular mass of NBP60, 60 K, estimated on SDS-PAGE. The amino acid sequences of all nine BrCN and proteolytic peptides of NBP60, determined by Edman degradation, completely matched those deduced from cDNA clones with the sole exception of Glu[8] (see below) (Fig. 1B). The amino acid sequence of rat NBP60 thus obtained showed 79 and 63% amino acid identities with human and chicken lamin B receptor, respectively (Fig. 2). The molecular weight of NBP60 calculated from the amino

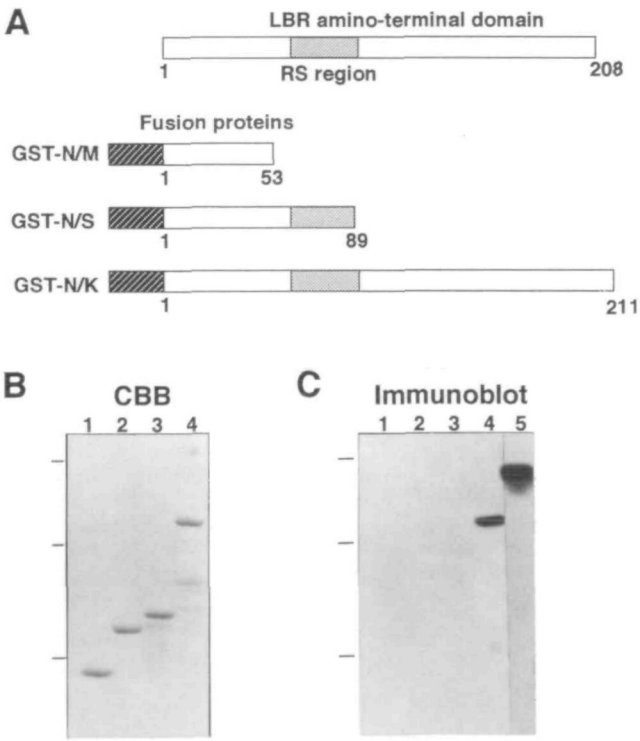

Fig. 3. **Expression of GST-fusion proteins of amino-terminal domain fragments of hLBR.** A: The diagram shows the nucleoplasmic amino-terminal domain of hLBR, and those of the three GST fusion proteins that were expressed in *E. coli* and contain different portions of the domain. GST is not drawn to scale. The numbers of the first and last amino acids of hLBR in each fusion protein are given. B: GST and GST fusion proteins were expressed in *E. coli* and purified by using glutathione-Sepharose beads. These proteins (1 μg each) were separated by SDS-PAGE, followed by CBB R-250 staining. Since the molecular weight of GST is 26 K (lane 1), the calculated sizes of the GST fusion proteins are: 32.1 K (lane 2), 36.1 K (lane 3), and 50.1 K (lane 4), respectively. C: Proteins shown in B (lanes 1 to 4) and purified NBP60 (lane 5) were transferred to a nitrocellulose filter, and then incubated with anti-NBP60 antiserum (1 : 500, w/w). The bars at the left of B and C indicate the positions of marker proteins of 66.3, 43, and 28.7 K, from top to bottom.

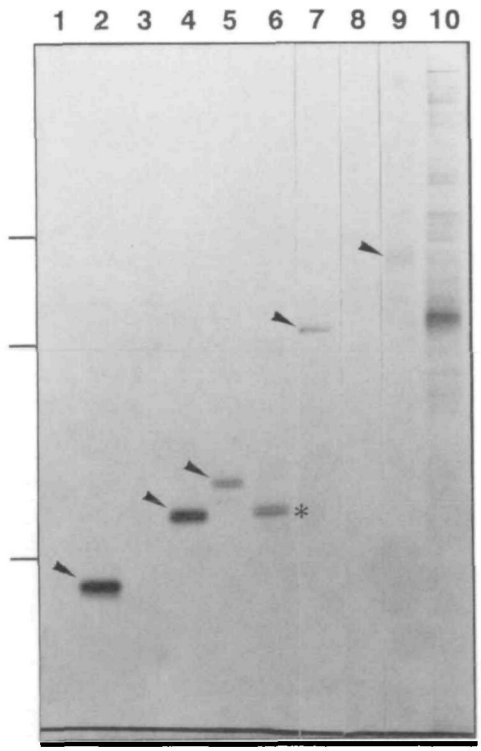

Fig. 4. **Binding of LBR amino-terminal fragments to nucleoplasmin-Sepharose.** The recombinant proteins and a rat liver nuclear envelope extract were incubated with nucleoplasmin-Sepharose gel beads, and then the bound (lanes 1, 3, 5, 7, and 9) and unbound (lanes 2, 4, 6, 8, and 10) fractions were subjected to SDS-PAGE and stained with silver. Lanes 1 and 2, GST alone (300 ng); lanes 3 and 4, GST-N/M (300 ng); lanes 5 and 6, GST-N/S (300 ng); lanes 7 and 8, GST-N/K (200 ng); lanes 9 and 10, the Triton/high-salt extract fraction of rat liver nuclear envelopes containing NBP60 (1.2 μg). The asterisk in the figure shows a band derived from GST-N/K, with the loss of the RS region, on proteolytic degradation. Arrowheads from left to right show bands of GST, GST-N/M, GST-N/S, GST-N/K, and rat NBP60, respectively. The bars at the left of the blot indicate the positions of the same marker proteins as in Fig. 3.

acid sequence was also close to those of these receptors. We concluded, therefore, that the sequence of the cloned cDNA represents that of NBP60, and the protein is a rat homologue of hLBR. The amino-terminal domain of NBP60 contains four Ser/Thr-Pro-X-X motifs (Ser^{71}, Ser^{88}, Thr^{123}, and Thr^{149}), and a Ser-Arg-rich stretch from Ser^{73} to Ala^{99} (RS region). Consensus sites for phosphorylation by $p34^{cdc2}$ kinase (Ser^{71}, Thr^{123}, and Thr^{204}), protein kinase A (Ser^{78}), and calmodulin-dependent kinase II (Thr^{45}, Thr^{137}, and Ser^{161}) were also present in the amino-terminal domain. Following the amino-terminal domain, there were eight stretches of hydrophobic amino acids, Phe^{214}-Cys^{235}, Leu^{258}-Gly^{283}, Arg^{301}-Ile^{322}, Phe^{331}-Ala^{351}, Phe^{390}-Ala^{411}, Val^{415}-Val^{437}, Val^{467}-Leu^{498}, and Leu^{557}-His^{587}, that could potentially serve as transmembrane segments as in other LBRs.

Expression of Human Lamin B Receptor—To characterize the nucleoplasmic amino-terminal domain of lamin B receptor, a set of peptides representing different parts of the protein were expressed in *E. coli* cells as GST fusion proteins (Fig. 3A). The N/M portion (residues 1-53) does not contain the RS region, which was shown to mediate protein-protein interactions (*31-33*). The N/S portion (residues 1-89) contains the N/M portion and the RS region. The N/K portion (residues 1-211) contains the whole amino-terminal domain of LBR. The expression of full-length hLBR of our clone in *E. coli* cells has not been successful so far. The expressed fusion proteins were purified on glutathione-Sepharose, and then analyzed by SDS-PAGE and immunoblotting (Fig. 3, B and C). The purified preparations of GST-N/S and GST-N/K, which have an arginine-serine (RS) region, contained some degradation products (Fig. 3B, lanes 3 and 4). Anti-rat NBP60 polyclonal antibodies reacted only with the fusion protein containing amino acids 1 through 211 of NBP60 (Fig. 3C, lane 4), *i.e.* not with smaller fusion proteins cotaining amino acids 1 through 53 and 1 through 89 (Fig. 3C, lanes 2 and 3). These results showed that the fusion protein of hLBR was recognized by anti-rat NBP60 antibodies, and that the epitope(s) was localized at amino acids 90 through 211 within the nucleoplasmic, amino-terminal domain.

NLS Binding Activity of Lamin B Receptor—We previously reported that NBP60 is an NLS-binding protein. To identify the NLS binding site within the amino-terminal domain of LBR, recombinant proteins were incubated with nucleoplasmin-Sepharose, and then the bound proteins were analyzed by SDS-PAGE (Fig. 4). GST and GST-N/M did not bind to nucleoplasmin-Sepharose (Fig. 4, lanes 1 and 3), whereas GST-N/S, GST-N/K, and NBP60 did (Fig. 4, lanes 5, 7, and 9). Interestingly, a protein probably derived from GST-N/K, with loss of the RS region, on proteolytic degradation lost the binding activity to nucleoplasmin too (Fig. 4, lane 6 asterisk). These results suggested that the region from Ile^{64} to Arg^{89}, which contains the RS region, is necessary for the binding of nucleoplasmin to NBP60. To determine whether the binding of GST-N/S to nucleoplasmin-Sepharose depends on NLS, as in the case of NBP60 (*37*), or not, we examined the inhibition of the binding by the NLS peptide of SV-40 large T-antigen, T-peptide (Fig. 5). We used 0.4 mM peptide, at which concentration the binding of NBP60 to nucleoplasmin-Sepharose is suppressed to about 20% with the T-peptide but not with the mutant T-peptide (*37*). The binding of GST-N/S to nucleoplasmin-Sepharose was suppressed to less than 20% under the conditions used (Fig. 5). This value

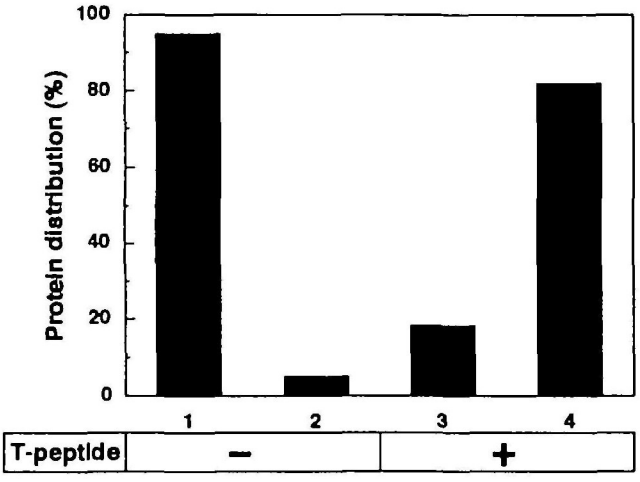

Fig. 5. **Inhibition of the binding of the LBR amino-terminal fragment to nucleoplasmin by T-peptide.** GST-N/S was incubated with nucleoplasmin-Sepharose in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of 0.4 mM T-peptide. The GST-N/S bound (lanes 1 and 3) or unbound fractions (lanes 2 and 4) were separated by SDS-PAGE and then stained with silver. The amounts of GST-N/S were estimated by densitometry.

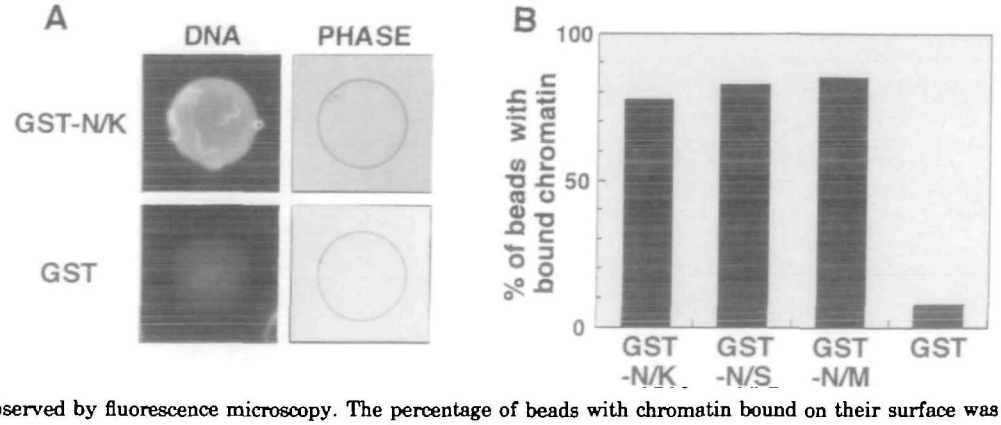

Fig. 6. **Chromatin binding of LBR amino-terminal fragments.** A: Demembranated and decondensed sperm chromatin was incubated with either GST-N/K (upper) or GST (lower) attached to glutathione-Sepharose beads at 4°C for 60 min. After staining of DNA with Hoechst 33342, the beads were observed by phase contrast (right) and fluorescence (left) microscopy. B: GST and the three kinds of GST fusion proteins of the amino-terminal domain of LBR bound to beads, and they were incubated with chromatin as in A and then observed by fluorescence microscopy. The percentage of beads with chromatin bound on their surface was determined.

was very close to that for NBP60 mentioned above. These results suggested that the binding of nucleoplasmin to the amino-terminal domain of NBP60 is dependent on the NLS of nucleoplasmin. In other words, residues 1 through 89, which contain the RS region of LBR, constitute a major NLS-binding site of NBP60.

Binding of the LBR Amino-Terminal Domain with Chromatin—One of the preliminary experiments for further characterization of the amino-terminal domain of LBR showed that the domain may have chromatin-binding activity. When the binding conditions were optimized, it was shown clearly that demembranated and decondensed *Xenopus* sperm chromatin binds to Sepharose beads bearing GST-N/K (Fig. 6A). Then the binding of chromatin to beads bearing three recombinant proteins consisting of parts of the amino-terminal domain of LBR was analyzed by fluorescence microscopy. When GST-N/K immobilized on glutathione-Sepharose 4B beads was incubated with chromatin, 82% of the beads bound chromatin on their surface. Similar binding was observed when GST-N/S and GST-N/M were used instead of GST-N/K. However, when GST itself was used instead of these fusion proteins, the beads that bound chromatin comprised less than 10% (Fig. 6B). The "% of beads with bound chromatin" of GST-Sepharose in Fig. 6B was increased to only 18% when the binding experiment was carried out in the absence of GST protein in the medium (data not shown). These data indicate that the amino-terminal N/M part of LBR contains the chromatin-binding site.

DISCUSSION

We have purified a nuclear localization signal binding protein, NBP60, from rat liver nuclear envelopes and characterized its properties (*37, 38*). In this study, we obtained partial amino acid sequences of the purified protein, determined the nucleotide sequence of NBP60 cDNA, and concluded that NBP60 is a rat homologue of LBR from the similarities of the nucleotide and amino acid sequences obtained in this study, subcellular distribution (*38*), and solubility (*37*). The amino acid sequence from Asp^8 to the carboxyl-terminal of NBP60 was deduced from the cDNA sequences of pMID and pNM2, and that from Pro^2 to Ala^7 was determined from a peptide sequence (Fig. 1). The presence of amino-terminal Met could not be confirmed directly. However, we considered that Met, which undergoes some post-translational modification, is present in the native NBP60 molecule in the rat liver nuclear envelope, because, although NBP60 purified from rat liver nuclear envelopes was resistant to Edman degradation (data not shown), a BrCN fragment exhibited the amino acid sequence from the second amino acid, Pro, of Pro-Gly-Arg-Lys-Phe-Ala. Although the initial Met could not be demonstrated, we concluded that Met^1 of NBP60 shown in Fig. 1 is the initial methionine because (1) the amino acid sequence of Met^1 to Glu^{51} of NBP60 completely matched to the sequence from the initial Met^1 to Glu^{51} of hLBR with the sole of a conservative change at position 3, and (2) the molecular mass values calculated from the full-length amino acid sequences of NBP60 (70.6 K) and hLBR (70.7 K) were very close to each other. On the other hand, the nucleotide sequence of C ($^+$22) to C ($^+$1860) in Fig. 1 was obtained for clones pMID and pNM2. The nucleotide sequence of A ($^+$1) to C ($^+$21), except for seven nucleotides ($^+$6, $^+$9, $^+$10, $^+$12, $^+$15, $^+$18, and $^+$21), was predicted from the amino acid sequences of peptides derived from rat liver NBP60. These seven nucleotides, which have no effect on the amino acid sequence, were presumed on the basis of hLBR. The nucleotide sequence thus obtained is shown in Fig. 2. The aspartic acid at position 8 deduced from the nucleotide sequence was different from the glutamic acid obtained on Edman degradation of a peptide derived from rat liver NBP60. The difference may reflect differences between rat strains: the cDNA library and NBP60 protein were prepared from Fisher 344 and Wistar rats, respectively.

Sequences in NBP60, hLBR, and chicken-LBR were highly conserved in the first 100 amino acids of the amino-terminal domain containing the RS region. This conservation suggested that the region participates in some important functions of LBR. It is known that the amino-terminal domain of LBR, protruding into the nucleoplasm, contains binding sites for lamin B, p32 and DNA (*17, 26*). p32 and DNA bind specifically to the RS region, and the p32-binding is inhibited on phosphorylation of the RS region (*33*). We showed in this study that the N/S part contains the NLS-binding site (Fig. 4, lane 5). Moreover, when Ile^{54} to Arg^{89} was removed from the N/S part, the NLS-binding activity was lost (Fig. 4, lanes 3 and 4). This means that the Ile^{54} to Arg^{89} region, of which most is occupied by the RS region, is necessary for the NLS-binding activity. Therefore, the RS region is probably necessary for the binding. Recent studies on the RS regions of other proteins have shown that the regions mediate protein-protein interactions between components of the splicing machinery (*31, 32*), probably in a phosphorylation-dependent manner (*46, 47*). The $U2AF^{65}$ RS domain of splicing factors plays a direct role in modulating spliceosomal RNA-RNA interactions (*48*). In the case of LBR, the RS region may not interact with basic NLS sequences directly, because the region contains many basic residues. Therefore, the overall structural features of residues 1-89 containing the RS region may be important for NLS recognition. On the other hand, it was shown recently that NLS proteins are translocated through nuclear pore complexes to the nuclear interior as complexes with importin α, and subsequently move to and accumulate at their destined intranuclear positions (*49*). If NBP60 participates in the protein nuclear localization mechanism, it may act as a competitor of importin α, and thus dissociate NLS-proteins from importin α in the cell nucleus. LBR may also act as a temporary anchoring site for nuclear proteins in the nucleus. Another possibility is that an unknown NBP60-binding protein interacts with NBP60 at the NLS-containing domain of the protein.

In *Xenopus* eggs, a trypsin-sensitive integral membrane protein(s) is responsible for targeting mitotic membrane vesicles to chromatin to initiate nuclear envelope assembly *in vitro* (*50*). In mammalian cells, a population of mitotic membrane vesicles, which contain LBR, are targeted to decondensed chromosomes in the anaphase and telophase of the cell cycle (*16*). In this study, we observed the binding of the N/M part of LBR to *Xenopus* sperm chromatin *in vitro* (Fig. 6). Therefore, LBR may be involved in the targeting of nuclear envelope precursor vesicles to chromatin *in vivo*. It is known that hLBR is phosphorylated *in vivo*

by $p34^{cdc2}$ protein kinase (*23*), which is involved in the initiation of mitosis (*51*). We showed that purified NBP60 is phosphorylated by $p34^{cdc2}$ protein kinase, protein kinase A, calmodulin-dependent kinase II, and casein kinase II *in vitro* (*38*). On the other hand, it was reported that partially purified protein kinases are involved in the membrane-releasing activity in *Xenopus* egg extracts (*52*). It was suggested that the kinase regulates the interaction of the nuclear membrane and chromatin with a phosphatase during nuclear envelope assembly and disassembly. Moreover, of the four membrane proteins phosphorylated, the molecular mass of the 60 kDa protein is very similar to that of LBR (*53*). Thus, because of these potential properties as well as its ability to bind to the lamina and chromatin, LBR may function in both dissociation and association of nuclear precursor vesicles and chromatin in the mitotic phase of the cell cycle.

It was reported previously that the amino-terminal domain of hLBR contains DNA-binding motifs (Ser/Thr-Pro-X-X), which occur four times (*14, 53*). It was indeed indicated that the stretch of residues 71-100 of the amino-terminal domain is necessary for DNA binding by a gel shift assay (*17*). On the other hand, our results showed that *Xenopus* sperm chromatin interacts with the amino-terminal domain of LBR (Fig. 6). Interestingly, the region responsible for the chromatin binding, residues 1-53 of the amino-terminal domain, contains neither the proposed DNA-binding motif nor the DNA-binding region. These findings suggested that the interaction of LBR and chromatin consists of a protein-protein interaction at the 1-53 site and a protein-DNA interaction at the 71-100 site. Our preliminary finding, *i.e.*, that the binding of chromatin and GST-N/M was suppressed by brief pretreatment of chromatin with trypsin, supported the above idea. Chromodomain protein HP1 is a candidate for the proteineous binding site on chromatin because the amino-terminal domain of LBR bound to the human homologue of the protein (*36*). Therefore, the N/M part of LBR may be important in localization of the transcriptionally inactive heterochromatin to a site adjacent to the inner nuclear membrane (*54, 55*).

LAP2 is the only previously known protein which connects the inner nuclear membrane and chromatin directly (*19, 20*). In this study, LBR was shown to have similar characteristics to LAP2, *i.e.*, the protein is an intrinsic nuclear membrane protein (*12-14*), and binds to chromatin (Fig. 6) and lamin B (*17*). Therefore, these two proteins probably act as chromatin-binding sites on the inner nuclear membrane in the interphase of the cell cycle. However, the binding domains on chromatin, such as chromodomains, telomeres and others, may differ in the two sites. LBR may function not only as a simple binding site, but also as a regulator of chromatin functions, because chromodomain and adjacent regions, which were suggested to bind to LBR, are transcriptionally inactivated. It is suggested that the amino-terminal domain of LBR forms a multimeric complex including p34 (*26*), a lamin B kinase (*24, 33*), lamin B (*17*), some NLS-proteins (*37*), chromatin (Fig. 6), and others. So, LBR can be considered to be the center of the perinuclear membrane network on the inner nuclear membrane and may regulate chromatin functions.

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