Direct membrane protein–DNA interactions required early in nuclear envelope assembly

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Among the earliest events in postmitotic nuclear envelope (NE) assembly are the interactions between chromatin and the membranes that will fuse to form the NE.

It has been proposed that interactions between integral NE proteins and chromatin proteins mediate initial membrane recruitment to chromatin. We show that several transmembrane NE proteins bind to DNA directly and that NE membrane proteins as a class are enriched in long, basic domains that potentially bind DNA. Membrane fractions that are essential for NE formation are shown to bind directly to protein-free DNA, and our data suggest that these interactions are critical for early steps in NE assembly.

Introduction

The nuclear envelope (NE) of eukaryotic cells consists of the inner nuclear membrane (INM) and outer nuclear membrane, as well as nuclear pore complexes (NPCs), which span both membranes and mediate transport processes. In metazoa, the NE breaks down before mitosis and is reformed after chromosome segregation. This reassembly of the NE starts in late anaphase with a rapid accumulation of membranes around chromatin. In living cells, this membrane recruitment happens within minutes, whereas the subsequent expansion and maturation of the NE takes at least 1 h (Ellenberg et al., 1997).

In cell-free extract systems, such as the *Xenopus laevis* egg extract, NE assembly can be reconstituted in vitro (Lohka and Masui, 1983). Similar to the situation in vivo, membrane vesicles attach to *X. laevis* sperm chromatin within minutes, followed by a much longer phase of NE maturation (for reviews see Gant and Wilson, 1997; Hetzer et al., 2005). Binding of membranes is independent of energy or cytosol and is not restricted to defined regions on chromatin. However, one important prerequisite for membrane recruitment in this system is the decondensation of chromatin. This is mediated by nucleoplasmin, which is a protein that removes basic proteins and protamines from sperm chromatin and allows the deposition of histones (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200512078/DC1; Philpott and Leno, 1992). Decondensation probably exposes binding sites for membrane vesicles, which in turn efficiently accumulate on chromatin. Hence, at the onset of NE assembly, the accessibility of chromatin-binding sites and a dramatic change in the affinity of membranes for chromatin are critical. However, it is unclear what mediates this initial interaction between membranes and chromatin.

In vitro studies demonstrated that specific populations of membrane vesicles exist that bind to chromatin and function in NE assembly (Vigers and Lohka, 1991; Antonin et al., 2005). The affinity of membranes for chromatin is thought to depend on transmembrane proteins and is modulated by mitotic phosphorylation (Wilson and Newport, 1988; Foisner and Gerace, 1993). Two nuclear transmembrane proteins that directly bind chromatin in vitro, lamin B receptor (LBR) and lamina-associated polypeptide 2β (Lap2β), have been identified. There is also evidence that, at least in some systems, LBR can target membranes to chromatin (Collas et al., 1996; Pyrpasopoulou et al., 1996), but there is no evidence that the depletion of either protein would affect NE assembly.

In contrast, much less is known about the nature of the binding sites on chromatin. Both LBR and Lap2β interact with chromatin proteins (HP1 and BAF, respectively), but they also bind to naked DNA (Ye et al., 1997; Dechat et al., 2000). LBR has a higher affinity for DNA than for chromatin proteins (Duband-Goulet and Courvalin, 2000). BAF interacts with other integral membrane proteins of the NE, including emerin and MAN1, which contain the so-called LEM domain (for review see Gruenbaum et al., 2005). However, there is no evidence that HP1, BAF, or histones are directly involved in membrane recruitment during NE assembly. On the other hand, a direct test for the involvement of DNA is difficult to perform, as chromatin templates are destroyed upon the removal of DNA

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(Imai et al., 1997; unpublished data). In previous NE assembly studies that used protein-free DNA, membrane binding was only investigated after the DNA was converted into chromatin (Forbes et al., 1983; Newport, 1987). In this analysis, we address directly whether NE precursor membranes interact with DNA and provide evidence that membrane–DNA interactions are critical during NE assembly.

Results and discussion

In the first experiment, we tested whether DNA could compete with chromatin for binding of membranes during NE assembly. *X. laevis* sperm chromatin was incubated with boiled cytosol to allow initial decondensation and then transferred to cytosol containing membranes and plasmid DNA as a competitor. We found that at early time points (after 10 min) almost no vesicles were recruited to chromatin in the presence of competitor DNA (Fig. 1 A, row 3) and that this effect on vesicle recruitment was not dependent on the presence of cytosol (Fig. 1 A, rows 5 and 6). After 2 h, control reactions showed normally shaped nuclei with fully decondensed chromatin and a smooth membrane staining

(Fig. 1 B, row 1). No such structures were detectable in samples containing competitor DNA (Fig. 1 B, row 2). Although membrane vesicles were attached to chromatin, they did not form a smooth NE, and the chromatin did not fully decondense. To test whether plasmid DNA, indeed, competed with chromatin for membranes, we added more membranes, cytosol, or buffer to the reactions. Only additional membranes could rescue the inhibition by competitor DNA to allow normal closed nuclear formation (Fig. 1 A, row 4, and B, rows 3–5).

To investigate whether plasmid DNA has an unspecific, inhibitory effect on membranes, we added the competitor DNA at different time points after the initiation of nuclear assembly. The inhibitory effect of DNA depended on its presence early on in assembly (Fig. 1 C). The number of nuclei formed returned to control levels when DNA was added 30 min after the initiation of assembly (Fig. 1 C), at a time when the NE still has to expand substantially. This indicates that DNA does not generally affect NE assembly. Instead, it seems to interfere with specific membranes that attach to chromatin early in the assembly process. The data suggest that DNA competes with chromatin for the binding of these membranes.
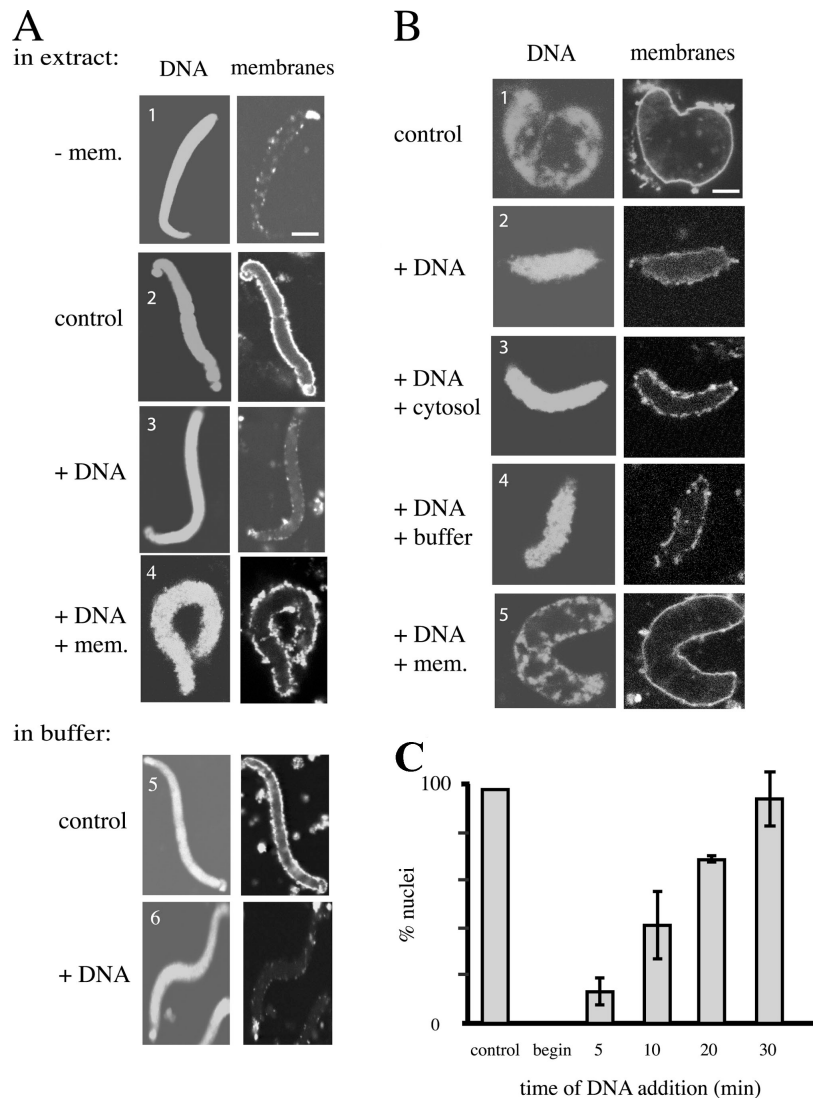

Figure 1. **Plasmid DNA interferes with vesicle recruitment to chromatin and NE assembly.** (A) In vitro NE assembly reactions. Decondensed sperm chromatin was added to cytosol containing membranes (row 2) and plasmid DNA (rows 3 and 4) or to buffer containing membranes (row 5) or membranes and plasmid DNA (row 6). Additional membranes were added in row 4. The negative control had no membranes (row 1). After 10 min, the reactions were stained for DNA and membranes. (B) NE assembly reactions after 120 min. Control (row 1) and reactions in the presence of competitor DNA, either alone (row 2) or in reactions containing equal volumes of extra cytosol (row 3), buffer (row 4), or membranes (row 5). (C) Percentage of normally shaped nuclei (with decondensed chromatin, expanded NE, and smooth membrane staining) in reactions to which competitor DNA was added at different time points after assembly had been initiated. Mean values of three experiments. Error bars represent the SD. Bars, 10 μm.

To investigate the basis for inhibition in more detail, we incubated membrane vesicles purified from *X. laevis* egg extracts with protein-free DNA that was immobilized on magnetic beads. Note that because all known chromatin proteins and assembly factors are soluble, this treatment should detect direct DNA interactions, rather than those that depend on chromatin assembly. The beads were removed, and the remaining vesicles were transferred to *X. laevis* cytosol to analyze their ability to form a NE around sperm chromatin. The quantity of membranes added from the control and DNA-depleted samples was normalized by protein content. In control reactions, membranes were efficiently targeted to chromatin after 10 min, and after 120 min normal nuclei had formed (Fig. 2 A, middle row). The chromatin was fully decondensed and NPCs had assembled. In contrast, membranes that were passed over the DNA column only rarely formed normal nuclei (Fig. 2 A, top row; Fig. 2 B for quantitation). After depletion over DNA beads, almost no membranes were detectable on chromatin after 10 min. After 120 min, the chromatin was associated with membrane vesicles and still condensed. In addition, very little NPC immunofluorescence signal was detectable. A punctate staining was observed, which was only marginally stronger than the background in control reactions without membranes (Fig. 2 A, bottom row).

Hence, although membranes eventually accumulated on chromatin, this recruitment was significantly delayed and did not yield a functional NE.

These effects were not observed when membranes were passed over a column to which the negatively charged polymer heparin sulfate was attached (unpublished data), suggesting that the DNA column was not just acting as a nonspecific negatively charged ion exchanger.

The fusion capacity of the membranes not removed by the DNA column was not significantly affected, as they still efficiently formed an ER-like network (Dreier and Rapoport, 2000) on coverslips (Fig. 2 C). This indicates that the negative effects shown in Fig. 2 A are specific for NE formation. To test whether transmembrane proteins with a known affinity for DNA were depleted in the inactive supernatants, we analyzed the membranes from three separate depletion experiments by Western blot (Fig. 2 D). *X. laevis* eggs contain a specific isoform of Lap2β, which is named Lap2ω (Schoft et al., 2003), and a variable amount of this protein and of LBR were removed. However, in most cases there was still a considerable quantity of both proteins left in the unbound fraction. This suggests either that a modest reduction in these transmembrane proteins is sufficient to block NE assembly or that unknown membrane
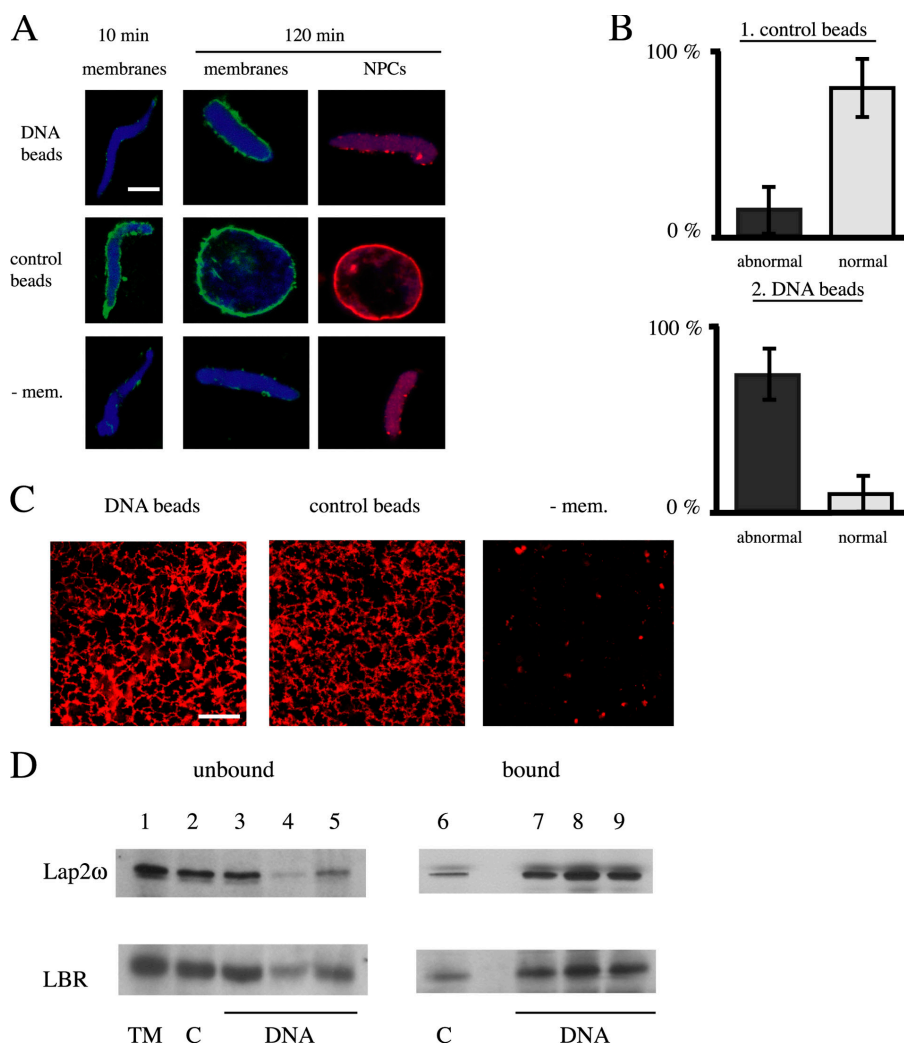

Figure 2. **Vesicles passed over a DNA column lose their ability to form NE.** (A) In vitro NE assembly. *X. laevis* membranes were passed over plasmid DNA immobilized on beads. The supernatant was transferred to cytosol containing sperm chromatin. After 10 and 120 min, the reactions were fixed and stained for membranes (top row, green). The 120-min sample was also stained for NPCs (right column, red). Supernatant from empty beads was used as a positive control (middle row). No membranes were added in the negative control (bottom row). DNA was stained with DAPI (blue). (B) Quantitation of the results shown in A (10 experiments each). Between 50 and 100 nuclei were counted per sample, and the percentages of normal nuclei (light gray bar) and condensed structures (dark gray bar) were calculated. (C) In vitro ER fusion assay. *X. laevis* membranes were passed over DNA (left) or control (middle) beads and transferred to cytosol on a microscope slide. After 90 min, the samples were analyzed. No membranes were added in the negative control (right). (D) Western blot showing the level of depletion of two DNA-binding transmembrane proteins of the NE in three independent depletions. Lane 1, total membranes; lane 2, control depletion; lanes 3–5, depletions using three different preparations of DNA beads. Equal amounts of total proteins were loaded. Lanes 6–9, bead-bound material; lane 6, empty beads; lanes 7–9, three different preparations of DNA beads. Bars, 10 μm.

proteins required for NE formation are efficiently depleted by the DNA column (see the following paragraph).

We conclude that the DNA column removed vesicles with an affinity for DNA and that these membranes are among those normally targeted to chromatin and required during NE assembly. Depletion of these vesicles blocks NE formation. The remaining membranes show a much lower affinity for chromatin and are unable to form a functional NE.

The affinity of vesicles for DNA is probably mediated by proteins associated with them. Indeed, pure liposomes neither bind to chromatin nor to DNA beads (unpublished data). Potential mediators of membrane binding to DNA are transmembrane proteins. Given the topology of the double NE membrane, these transmembrane proteins should be localized to the INM once the NE is assembled. In addition, the only parts of the proteins that can directly contact chromatin are their cytosolic (and later nucleoplasmic) domains.

To test whether cytosolic regions of nuclear transmembrane proteins bind to chromatin in our in vitro system, we expressed four such domains recombinantly: the *X. laevis* LBR NH$_2$ terminus, the human Lap2β NH$_2$ terminus, the human MAN1 COOH terminus, and the NH$_2$ terminus of BC08, a novel potential *X. laevis* NE transmembrane protein (unpublished data). As these protein fragments are all very basic (all have an isoelectric point [pI] of approximately nine), we included control proteins with different pI values in our analysis. The proteins were incubated with chromatin and analyzed by immunofluorescence. All transmembrane protein domains showed chromatin binding, although with different affinities (Fig. 3 A). Interestingly, we observed a correlation between the pI value of a protein and its ability to bind to chromatin; two neutral control proteins, maltose-binding protein and the nucleoporin Nup43, showed no binding, whereas two basic (ribosomal) proteins, RS10 and RS7, accumulated on chromatin to different extents. The binding properties of the proteins to chromatin correlated well with their affinity for DNA, as they showed identical binding patterns to DNA beads (Fig. 3 A). We conclude that the positive charge of these proteins confers affinity for chromatin, presumably via interactions with DNA. However, the different signals observed among the tested proteins suggest that basic charge may not be the only critical binding determinant.

Based on these results we investigated whether INM proteins are enriched in positively charged cytosolic domains.
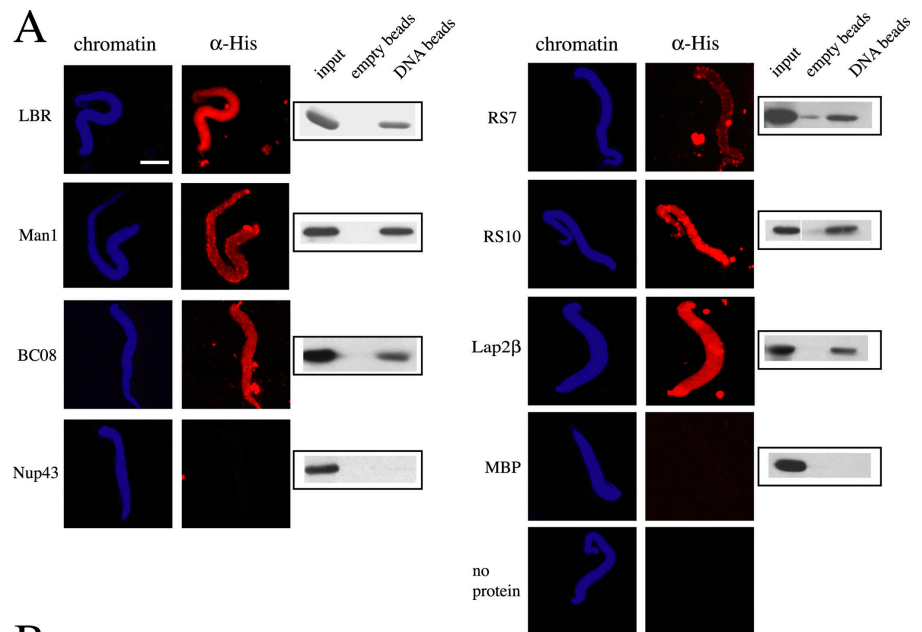

Figure 3. **INM proteins bind chromatin and are enriched in basic cytosolic domains.** (A) Chromatin-binding assay of recombinant proteins. His-tagged cytosolic domains of INM and control proteins were incubated with decondensed *X. laevis* sperm chromatin. After 10 min, the samples were stained with an anti-His antibody (red) and DAPI. Bar, 10 μm. The fragments were also analyzed for their ability to bind to DNA beads (rightmost images). (B) Graphic representation of cytosolic domains of mouse transmembrane proteins. Transmembrane proteins of the ER and Golgi were plotted for the length (in amino acids; cutoff 100) and pI value of their cytosolic domains. Known mouse NE proteins were added (red).

To date, 14 mammalian transmembrane proteins that localize to the INM (Table I) are known, and at least nine are conserved in *X. laevis*. Using computer programs we predicted their cytosolic domains and calculated the corresponding pI value.

For nine human proteins the membrane topology was either known or could be predicted unambiguously. Seven of these proteins were found to contain a basic cytosolic domain (pI > 8.5) larger than 100 amino acids, and this result was conserved in mouse and *X. laevis* (Table I). Hence, at least half of the known INM transmembrane proteins show this characteristic, including the proteins that were detectable early on chromatin during NE assembly, such as LBR, Lap2β, and pom121 (Burke and Ellenberg, 2002).

As 14 proteins are a rather limited dataset, we investigated a list of 67 potential NE transmembrane proteins that was published by Schirmer et al. (2003). By analyzing these proteins (that should at least be enriched in INM proteins) we found that 46% of the proteins contain a basic, cytosolic domain that is longer than 100 amino acids.

To test whether having such a domain is common to transmembrane proteins in general or whether these domains are enriched in the NE, we analyzed 150 transmembrane proteins that were not localizing to the NE, but to the ER or Golgi. A large basic domain was present in only 4% of these proteins.

We conclude that proteins of the INM are enriched in long, basic, cytosolic domains, which is a characteristic that is not prevalent in the proteins of other endomembrane systems (Fig. 3 B). As a large and basic domain confers affinity for chromatin (Fig. 3 A), the data suggest that interaction with DNA could be a general mechanism by which transmembrane NE proteins mediate membrane recruitment on chromatin. However, these results do not mean that cytosolic domains have to be basic to bind DNA. The cytosolic domain of the *X. laevis* egg-specific Lap2ω is not basic (Table I), yet the protein shows a high affinity for DNA (see the following paragraph).

The cytosolic domains of several INM proteins directly bind DNA (Fig. 3 A). To test the involvement of these domains in membrane binding to DNA, we performed a competition experiment. DNA beads were preincubated with recombinant proteins before they were added to membranes. The DNA-bound INM proteins were analyzed by Western blot. The NH_2 termini of both human Lap2β and *X. laevis* LBR efficiently inhibited vesicle recruitment to the beads (Fig. 4 A), as almost no bound Lap2ω or pom121 were detected. This efficient competition by the INM protein fragments suggests that these proteins are directly involved in recruiting vesicles to DNA. Interestingly, membranes bound normally to DNA that was preincubated with core histones (Fig. 4 A), suggesting that INM proteins might have a different mode of binding to DNA than histones.

As an alternative approach to test direct binding of INM proteins to DNA, we incubated DNA beads with membranes and UV cross-linked the samples. After several rounds of stringent washes, the cross-linked proteins were analyzed (Fig. 4 B, lane 1). In the cross-linked sample, Lap2ω and the transmembrane nucleoporin NDC1 (Mansfeld et al., 2006) were detected, whereas the late-recruited transmembrane nucleoporin gp210 was absent. The efficiency of UV cross-linking is very low. Using histone cross-linking, we calculated that only roughly 1% of the proteins were cross-linked to the beads under these conditions (unpublished data). Given the sensitivity of detection of our antibodies, this meant that we could not extend our analysis to additional INM proteins, as the signal in the cross-linked samples was, as predicted, under the detection limit.

Nevertheless, the data strongly support the hypothesis that several INM proteins directly mediate binding of membrane vesicles to DNA. To identify more DNA-binding INM proteins, we solubilized *X. laevis* membranes with a mild detergent and passed the mixture of proteins over the DNA column. The bead-bound material was analyzed by SDS-PAGE. The general protein pattern showed that several bands were specifically enriched in the DNA-bound fraction (Fig. 4 C). Analysis by Western blot revealed that several NE proteins bound to the DNA beads (Fig. 4 D); LBR, Lap2ω, NDC1, and pom121 were strongly enriched in the bound fraction. In contrast, neither gp210 nor Sec61a, which is an ER control protein, showed a comparable enrichment in the bound fraction.

Collectively, our data indicate that DNA can act as a binding site for membranes that are essential for NE formation.

Table I. **Vertebrate transmembrane proteins of the NE and their nucleoplasmic domains**

Protein	Domain length: human	pI of domain: human	pI of domain: mouse	pI of domain: *X. laevis*
LBR	208	9.85	9.96	9.99
Lap2β	411	9.36	9.43	8.81
Lap2ω				6.6
Lap1(B)	216	9.43	7.88	
MAN1	261 (ct)	9.33	9.3	8.65
	470 (nt)	6.0	7.88	8.68
pom121	1,171	10.31	10.13	9.7
gp210	56	9.78	9.78	10.42
Emerin	224	5.43	5.06	(1) 6.85
				(2) 8.95
LEM2	207 (nt)	11.28	11.3	
	109 (ct)	6.36	6.36	
NDC1	387	9.43	9.5	9.45

The membrane topology of Sun1, Sun2, LUMA, Nurim, and ring finger–binding protein is ambiguous. *X. laevis* has two emerin isoforms.
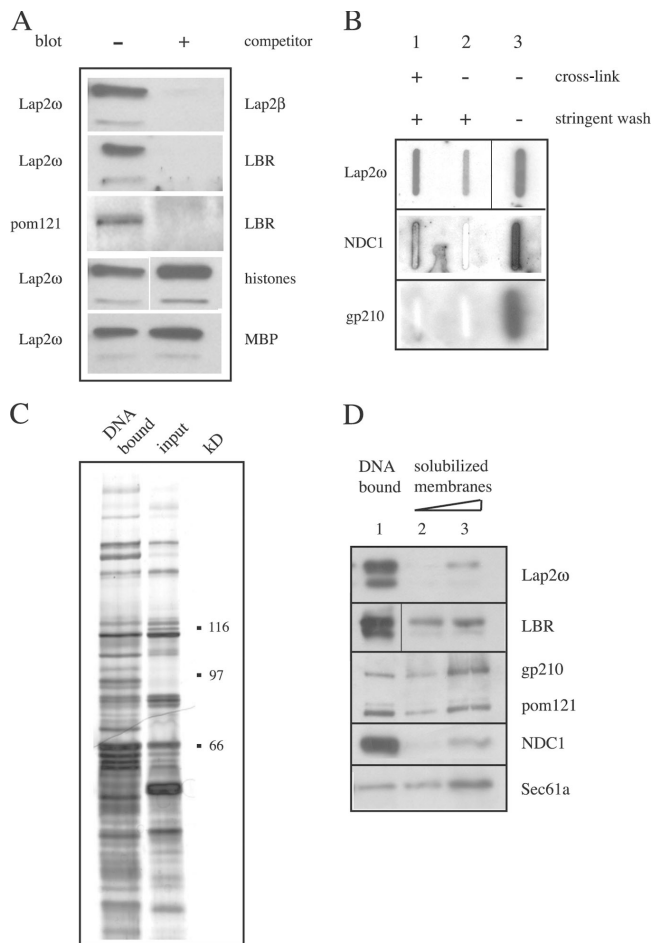
Figure 4. **INM proteins directly bind to DNA.** (A) Competition experiment. DNA beads were incubated with (+) or without (−) 4 μg of recombinant competitor proteins and added to *X. laevis* membranes. The presence of vesicles on the beads was determined by Western blot for INM proteins. (B) DNA beads were incubated with membranes and the samples were UV cross-linked, and then washed repeatedly with high salt and detergent (lane 1) or washed without cross-linking (lane 2), spotted on a membrane, and processed with antibodies. Lane 3 shows approximately 20% bead-bound material without stringent washing. (C) Membranes were solubilized with detergent and passed over DNA beads. (right) Silver-stained SDS-PAGE of the DNA-bound protein fraction compared with a fraction of the input material. (D) Western blot analysis of the DNA-bound fraction of C using a set of antibodies against NE proteins and sec61a as an ER protein. Lane 1 (DNA bound) had the same amounts of protein as lane 2, showing solubilized membranes (as judged by Ponceau staining).

This binding can be mediated by transmembrane proteins integrated in NE-precursor membranes. We suggest that binding of transmembrane proteins to DNA is at least part of the mechanism for the rapid and highly efficient recruitment of membranes to chromatin in late anaphase, which is when NE assembly begins.

As nuclear transmembrane proteins disperse throughout the ER during mitosis (Ellenberg et al., 1997), the redundancy in chromatin binding of multiple INM proteins that is suggested by our data could help collect transmembrane NE proteins at the right place when the mitotic spindle disassembles and chromatin needs to be rapidly enclosed. The redundancy of this mechanism could also account for the finding that in vivo knockdown studies with single nuclear transmembrane proteins

that contact specific chromatin proteins did not result in an inhibition of NE assembly in a variety of systems (Harborth et al., 2001; Wagner et al., 2004).

We did not analyze nontransmembrane proteins such as lamins. The role of lamins in the early steps of NE assembly is not clarified (for reviews see Gant and Wilson, 1997; D'Angelo and Hetzer, 2006), and we cannot exclude that lamins contribute to the initial contacts of membranes to chromatin via binding to DNA.

As a major chromatin component, DNA is present everywhere in chromatin, and is not limiting. Accordingly, the INM proteins LBR, Lap2ω, and pom121 are all uniformly distributed on chromatin during the first minutes of in vitro NE assembly (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200512078/DC1; Antonin et al., 2005). Although some proteins of the INM bind to specific regions of chromatin during NE assembly in somatic cells (Haraguchi et al., 2000), the molecular basis for their nonuniform distribution is currently unknown. In a living cell, the presence of the disassembling spindle may affect the membrane-targeting process because of membrane–spindle interaction and differential accessibility of chromatin.

As the NE matures, these first membrane–chromatin contacts could be the basis from which more specific interactions between INM proteins and chromatin are formed. These involve chromatin proteins that might compete with free DNA for binding to nuclear transmembrane proteins, such as the LEM domain proteins and the nuclear lamins.

Materials and methods

Nuclear assembly reactions
X. laevis egg cytosol, membranes, and demembranated sperm heads were prepared as previously described (Hetzer et al. 2001), except that the cytosol was centrifuged for an additional 12 min at 16,000 *g* to remove residual membranes. For nuclear assembly reactions, 10 μl cytosol was mixed with 0.3 μl sperm chromatin (3,000 sperm heads/μl) and incubated for 10 min at 20°C to allow chromatin decondensation. Subsequently, 2 μl of the mixture was added to 10 μl cytosol containing 0.3 μl of membranes, 20 mg/ml glycogen, an ATP regenerating system, and, where indicated, DNA (a 5-kb pBluescript-based plasmid) at 15 μg/ml. The negative effects of DNA on NE assembly were overcome by adding 1 μl of membranes to the reaction. At the time points indicated, the membranes were stained with the lipid dye DilC$_6$ (Invitrogen) and the reactions were fixed with 2% formaldehyde/0.5% glutaraldehyde. DNA was stained with DAPI, and the samples were spun through a 30% sucrose cushion onto coverslips.

For the ER fusion assay (Dreier and Rapoport, 2000), 10 μl cytosol containing an ATP generating system was mixed with DNA-depleted or control membranes and small amounts of fluorescently labeled (DiOC$_{18}$; Invitrogen) membranes. 1 μl of the reactions was transferred to a microscope slide, incubated for 90 min at 20°C, and analyzed by confocal microscopy.

Fluorescence images were analyzed at room temperature on a confocal microscope (TCS SP2; Leica) using a 40× objective (HCX PL APO CS; Leica), NA 1.25, immersion oil, and confocal software (version 2.5; all Leica). Photoshop 7.0 software (Adobe) was used for minor adjustments to contrast and to overlay channels.

Experiments with immobilized DNA
DNA (an 11-kb pBR-based plasmid) was linearized, biotinylated, and coupled to streptavidin-coated magnetic beads (Dynal) in 50 mM Tris-Cl, pH 8.0, 1 M NaCl, 2.5% polyvinylalcohol, and 2 mM EDTA overnight at 4°C.

For the membrane-depletion experiments, 1 μl of *X. laevis* membranes was incubated with 3–9 μl of DNA (or empty) beads in 8 μl S250 buffer (10 mM Hepes, pH 7.5, 50 mM KCl, 2.5 mM Mg$_2$Cl, and

250 mM sucrose) for 15 min at 20°C. The beads were removed with a magnet, washed, and processed for SDS-PAGE. The supernatants were equalized for protein concentration and volume and, subsequently, added to NE assembly reactions or ER fusion assays, as described in the previous section.

For the competition experiments, 4 µl DNA beads were incubated with 4 µg of recombinant proteins (see next section) or core histones (Roche) and 100 µg BSA in 10 µl S250 buffer. After 10 min, 2 µl of *X. laevis* membranes were added in 20 µl of buffer, and the binding was stopped after 15 min by washing the beads in buffer and processing them for SDS-PAGE.

For the DNA cross-linking assay, membranes were first floated in a sucrose gradient, in accordance with the study by Wilson and Newport (1988). 100 µl of the two lightest membrane fractions were incubated with 15 µl DNA beads. After binding, the samples were irradiated on ice in a UV Stratalinker (Stratagene) at 0.6 J/cm^2. The beads were washed repeatedly with 2 M NaCl and 1% Triton X-100 and spotted on a membrane. The dot blot was then processed with antibodies specifically recognizing the proteins of interest. After UV cross-linking and washing, empty beads did not yield signals. The efficiency of UV cross-linking was determined using core histones.

Floated membranes were solubilized in 500 µl PBS with 1% octyl-glucopyranoside (Calbiochem) and 0.5 M NaCl for 10 min at 4°C. Insoluble material was removed by centrifugation for 10 min at 280,000 g. The supernatant was incubated with 20 µl DNA beads for 15 min at 20°C. The beads were removed, washed (so empty beads did not detectably bind proteins), and analyzed by SDS-PAGE.

Experiments with recombinant proteins
Proteins were expressed from pQE plasmids (QIAGEN). The proteins had an NH$_2$-terminal His tag and were purified using Ni-NTA agarose by standard protocols. Maltose-binding protein from *E. coli* was Alexa Fluor 488–labeled and used in this form (a gift from K. Ribbeck, European Molecular Biology Laboratory, Heidelberg, Germany).

For chromatin-binding assays, 2 µg of recombinant proteins were added to decondensed sperm chromatin either in 10 µl cytosol or S250 buffer supplemented with 10 mg/ml BSA. After 20 min at 20°C, the reaction was fixed in 4% formaldehyde, spun on a coverslip, and processed for immunofluorescence using the monoclonal RGS-His antibody (QIAGEN). Alternatively, the proteins were incubated with DNA beads in 40 µl S250 buffer containing 100 µg BSA. The proteins used were LBR nt (amino acids 4–210), the novel protein B08 (amino acids 1–77, available from Gen-Bank/EMBL/DDBJ under accession no. BC082226), RS10, and RS7 (both full length), which were all from *X. laevis*; and human MAN1 (amino acids 672–911), Lap2β (amino acids 1–410), and Nup 43 (full length).

Antibodies
For the generation of a polyclonal antiserum against *X. laevis* LBR, we used the NH$_2$-terminal fragment corresponding to amino acids 4–210. Antibodies against *X. laevis* pom121, gp210, and NDC1 were previously described (Antonin et al., 2005; Mansfeld et al., 2006). Antibodies against *X. laevis* Lap2β (also recognizing Lap2ω) were a gift of G. Krohne (University of Würzburg, Würzburg, Germany). Antibodies against canine Sec61a (also recognizing the *X. laevis* homologue) were a gift of B. Dobberstein (University of Heidelberg, Heidelberg, Germany). NPCs were visualized by mAb 414 (BAbCO). An anti–mouse antibody labeled with Alexa Fluor 546 (Invitrogen) was used for immunofluorescence.

Computational analyses
Where unknown, the membrane topology of transmembrane proteins was determined using the TMHMM server at http://www.cbs.dtu.dk/services/TMHMM and PSORT II at http://psort.ims.u-tokyo.ac.jp/form2.html. The pI values of cytosolic domains were calculated at http://ca.expasy.org/tools/protparam.html.

Sequences of 150 transmembrane proteins not localizing to the NE were obtained from the mouse subcellular localization database at http://membrane.imb.uq.edu.au/.

Only cytosolic domains larger than 100 amino acids were included in the analysis, as caused by the "positive outside rule" (Hartmann et al., 1989) there are generally short cytosolic sequences in transmembrane proteins that are more positively charged than their lumenal counterparts.

Online supplemental material
Fig. S1 shows the chromatin structure of decondensed *X. laevis* sperm chromatin and assembled nuclei, which were analyzed by micrococcal nuclease digests. Fig. S2 shows the localization of LBR and Lap2ω on chromatin after 10 min of in vitro nuclear assembly. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200512078/DC1.

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