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The lamin B receptor (LBR) provides essential chromatin docking sites at the nuclear envelope

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Morphological studies have established that peripheral heterochromatin is closely associated with the nuclear envelope. The tight coupling of the two structures has been attributed to nuclear lamins and lamin-associated proteins; however, it remains to be determined which of these elements are essential and which play an auxiliary role in nuclear envelope–chromatin interactions. To address this question, we have used as a model system *in vitro* reconstituted vesicles assembled from octyl glucoside-solubilized nuclear envelopes. Comparing the chromosome binding properties of normal, immunodepleted and chemically extracted vesicles, we have arrived at the conclusion that the principal chromatin anchorage site at the nuclear envelope is the lamin B receptor (LBR), a ubiquitous integral protein of the inner nuclear membrane. Consistent with this interpretation, purified LBR binds directly to chromatin fragments and decorates the surface of chromosomes in a distinctive banding pattern.

Keywords: chromatin/immunodepletion/integral membrane proteins/nuclear lamina

Introduction

The nuclear envelope (NE) comprises three distinct regions: the outer nuclear membrane, the inner nuclear membrane and the pore membrane. The outer membrane faces the cytoplasm and represents an extension of the rough endoplasmic reticulum (RER). The inner membrane faces the nucleoplasm and is lined by the nuclear lamina meshwork. The pore membrane connects the two nuclear membranes and accommodates the nuclear pore complex (for recent reviews, see Georgatos, 1994; Gerace and Foisner, 1994).

In morphological terms, the inner nuclear membrane and the nuclear lamina are closely associated with peripheral heterochromatin (Paddy *et al.*, 1990; Belmont *et al.*, 1993; Marshall *et al.*, 1996). This has prompted several laboratories to search for factors interconnecting the neighboring structures. Recent studies have shown that the nuclear lamins bind directly to chromosomes (Burke, 1990; Glass and Gerace, 1990; Glass *et al.*, 1993), reconstituted chromatin (Hoeger *et al.*, 1991), polynucleosomes (Yuan *et al.*, 1991; Taniura *et al.*, 1995), matrix-associated DNA (Luderus *et al.*, 1992) and core histones (Taniura *et al.*, 1995). Apart from that, there are indications that chromatin anchorage to the NE involves integral membrane proteins (Foisner and Gerace, 1993; Ye and Worman, 1994, 1996).

Several integral membrane proteins of the NE have been characterized in higher eukaryotes. A 73 kDa protein, termed LBR (lamin B receptor, or p58), has been identified in a variety of species and seems to be a ubiquitous component of the inner nuclear membrane (Worman *et al.*, 1988, 1990; Courvalin *et al.*, 1990; Bailer *et al.*, 1991; Ye and Worman, 1994). Four other proteins, the lamina-associated polypeptides (LAPs) 1A, 1B, 1C and 2, have been originally detected in rat hepatocytes and certain mammalian cell lines (Senior and Gerace, 1988; Foisner and Gerace, 1993). It is now clear that the LAPs 1A, 1B and 1C are differentially spliced products of the same gene (Martin *et al.*, 1995), whereas LAP2 is identical to the widely expressed protein thymopoietin which comprises at least three isotypes (Harris *et al.*, 1994; Furukawa *et al.*, 1995). Finally, a 93 kDa Ca^{2+} binding protein related to calnexin has been identified in the inner nuclear membrane of rat hepatocytes (Gilchrist and Pierce, 1993), while a low molecular weight protein, p18, recently has been localized in the inner and outer nuclear membrane of avian erythrocytes (Simos *et al.*, 1996). Most of these polypeptides seem to associate with the nuclear lamins. LAP2 also binds to chromosomes (Foisner and Gerace, 1993), while the NH_2-terminal domain of LBR has been shown to bind to naked DNA and to the heterochromatin-specific protein HP1 (Ye and Worman, 1994, 1996).

The multiplicity of NE proteins makes it difficult to distinguish which of these elements are essential for the attachment of chromatin to the inner nuclear membrane and which play an auxiliary role. This is particularly evident when one considers the process of nuclear reassembly at the end of mitosis. *In vitro* assays with mammalian and insect cell homogenates have shown that post-mitotic NE reassembly is a lamin-dependent process (Burke and Gerace, 1986; Ulitzur *et al.*, 1992; Maison *et al.*, 1995), whereas other experiments with amphibian egg extracts have indicated that NE reformation around demembranated sperm chromatin proceeds normally when the major lamin form in this system (L_{III}) is removed by immunodepletion (Newport *et al.*, 1990; Meier *et al.*, 1991; Jenkins *et al.*, 1993). More recent findings provide a reasonable explanation for this discrepancy, revealing that amphibian eggs contain multiple lamin isotypes,

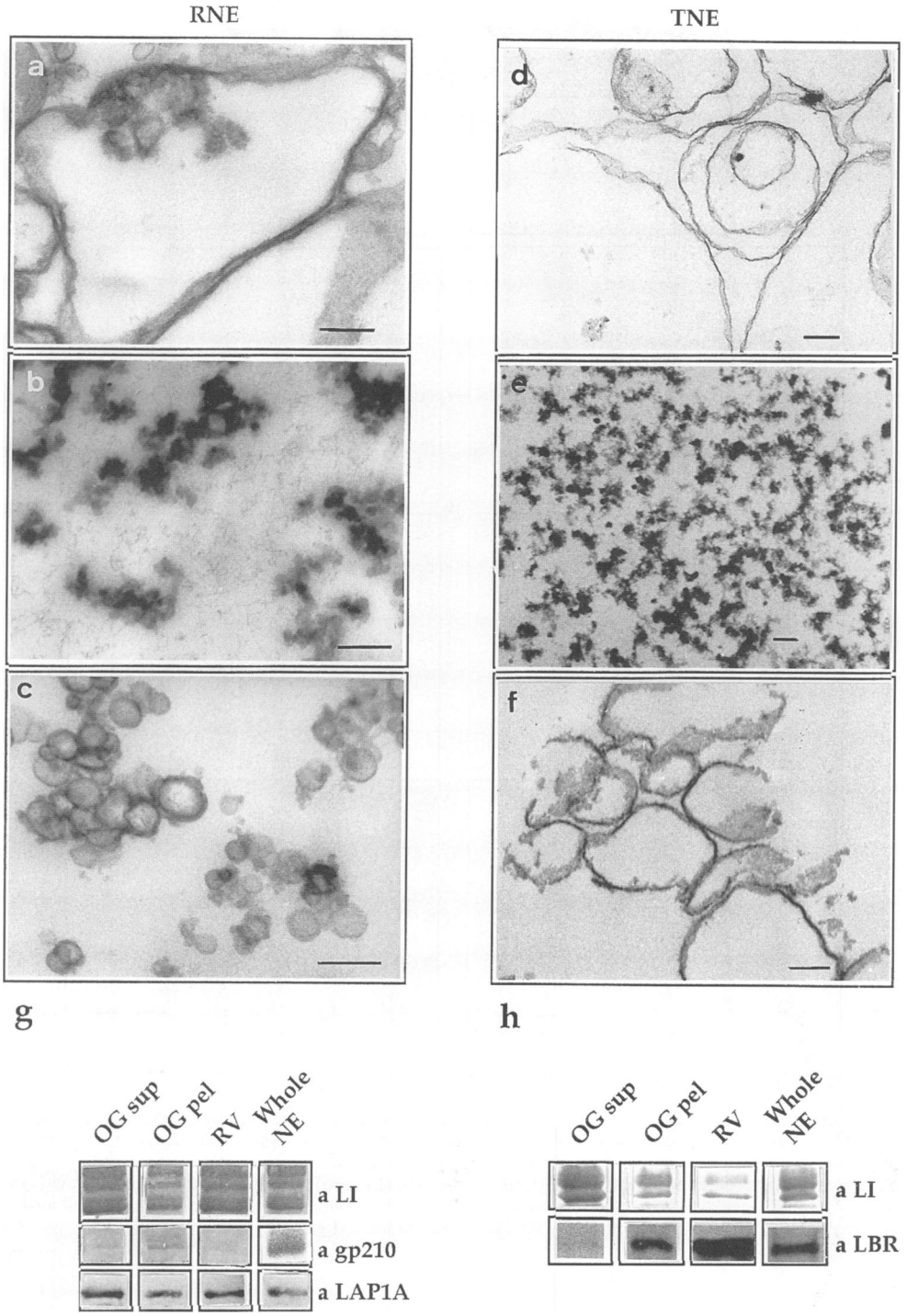

Fig. 1. Reconstitution of membrane vesicles from octyl glucoside-solubilized NEs. Samples taken at various steps during solubilization/reconstitution were fixed with glutaraldehyde and processed for thin-sectioning and transmission electron microscopy. (**a**) and (**d**) Whole NEs (starting material) revealing the presence of membranous sheets and 'nuclear ghosts'. (**b**) and (**e**) Clarified octyl glucoside extracts of NEs. Notice the absence of membranous structures and the presence of aggregates forming after glutaraldehyde fixation (for pertinent information, see Nicchitta and Blobel, 1990). (**c**) and (**f**) Reconstituted NE vesicles forming after removal of the detergent. Column 'RNE' (a, b and c) represents material obtained from rat liver NEs. Correspondingly, 'TNE' (d, e and f) shows material obtained from turkey erythrocyte NEs. Bar, 200 nm. (**g**) and (**h**) Analysis of rat hepatocyte (g) and turkey erythrocyte (h) fractions by Western blotting. Equal amounts (~25 μg of protein) from octyl glucoside-solubilized NEs (OG sup), non-solubilized material (OG pel), reconstituted NEs (RV) and whole NEs (whole NE) were electrophoresed, blotted to nitrocellulose filters and probed with anti-lamin (aLI), anti-LBR (aLBR), anti-gp210 (agp210) and anti-LAP1A (aLAP1A) antibodies. Only the relevant area of each blot is shown.

some of which remain behind after L_{III} immunodepletion (Lourim and Krohne, 1994; Goldberg and Allen, 1995; Lourim *et al.*, 1996). Yet, whether the lamins are sufficient for chromatin binding and NE reassembly, or whether other membrane proteins are the principal players in this process, remains to be examined.

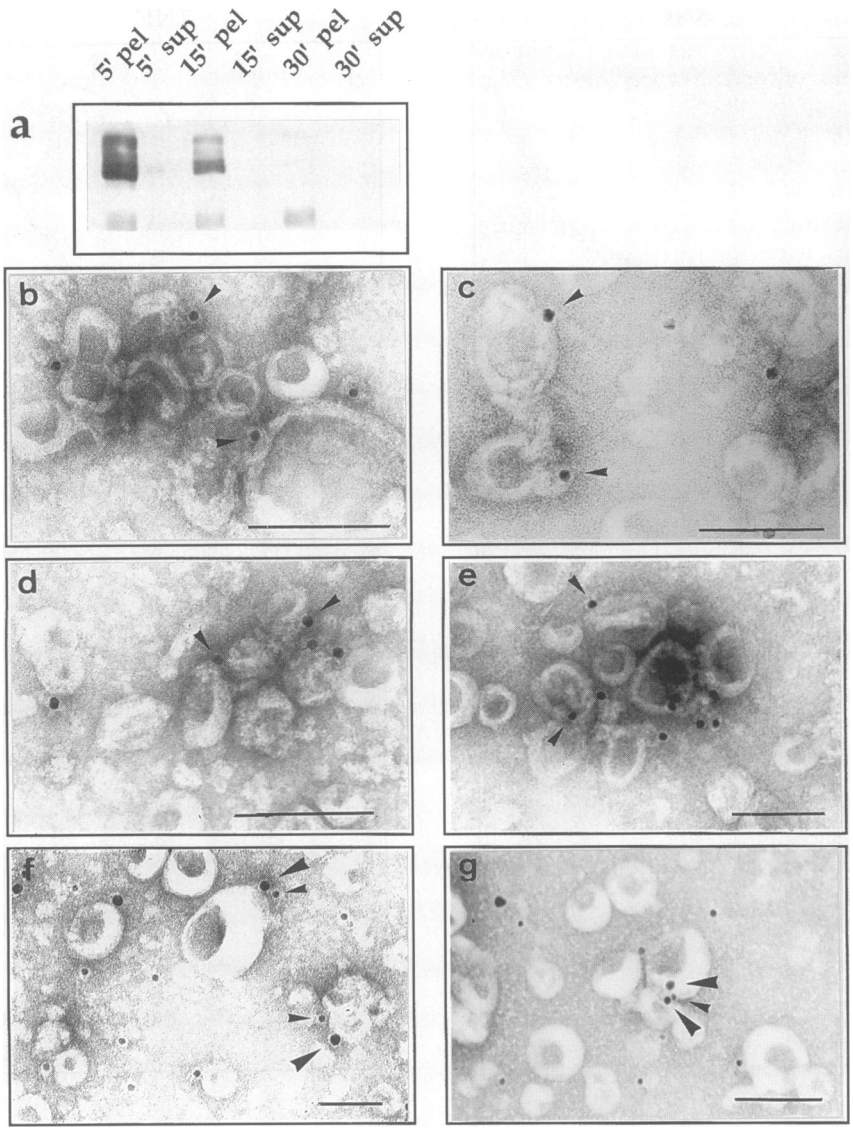

Fig. 2. Orientation of *in vitro* reconstituted vesicles. (**a**) RVs assembled from rat hepatocyte NE extracts were digested with trypsin (1:30 w/w) for 5, 15 and 30 min at room temperature and subjected to centrifugation at 13 000 *g* for 15 min. The corresponding pellets (pel.) and supernatants (sup.) were immunoblotted with the aLI antibody. The same results were obtained using trypsin immobilized on beads. (**b–g**) RVs prepared from rat (b, d and f) and turkey (c, e and g) material were incubated with affinity-purified antibodies and protein A–gold. The samples were counter-stained with 2% uranyl acetate and visualized in the electron microscope (whole mount immunoelectron microscopy). Immunolabeling is as follows: (b and c) aLI; (d), aLAP2; (e) aLBR; (f) aLI (small arrows) and aLAP2 (large arrows); (g) aLI (small arrows) and aLBR (large arrows). Incubation of the same samples with protein A–gold alone did not yield any staining (not shown). Bar, 100 nm. The relative sizes of rat and turkey RVs in this figure could be deceiving, as smaller vesicles adhere more efficiently than large ones to the EM grid.

To address these questions in a systematic manner, we have developed a novel assay system which involves reconstituted membrane vesicles assembled from detergent extracts of purified NEs. Furthermore, we have prepared vesicles from which lamins or integral NE proteins have been removed selectively by immunodepletion or chemical extraction. Comparing the chromosome binding properties of such 'mutant' vesicles with that of 'wild-type' NE vesicles, we obtained data suggesting that LBR represents the predominant chromatin binding site at the NE. This interpretation has been confirmed by showing that purified LBR binds directly to native chromatin fragments.

Results

Assembly of membrane vesicles from detergent-solubilized NEs

To identify proteins that are essential for anchorage of chromatin to the NE, we isolated nuclei and NEs from two different sources: rat hepatocytes and the turkey erythrocytes. These cells represent the most popular model systems for investigating nuclear architecture and many of their NE proteins have been molecularly characterized (see Introduction).

In agreement with previously published observations (Harris and Brown, 1971; Dwyer and Blobel, 1976), rat

hepatocyte NEs consisted of large membrane sheets and dilated cisternae (Figure 1a), while turkey erythrocyte NEs had the appearance of round 'nuclear ghosts' (Figure 1d). Upon solubilization of the NEs in octyl glucoside and ultracentrifugation at 400 000 *g*, lamellar structures were no longer detectable in the high speed supernatant (Figure 1b and e). However, when octyl glucoside was removed from the extract by dialysis, an abundance of membrane vesicles were formed (Figure 1c and f). Morphometric analysis showed that the majority of rat liver NE-derived vesicles were ~100 nm in diameter, but their actual sizes ranged from 50 to 160 nm. Turkey erythrocyte-derived vesicles had variable sizes and were generally larger than 400 nm.

In vitro reconstituted vesicles (referred to hereafter as RVs) assembled from rat hepatocyte NE extracts contained lamins and markers of the inner nuclear membrane (e.g. LAP1A), but were depleted of the major pore complex glycoprotein gp210 (Figure 1g). Similarly, RVs assembled from turkey erythrocyte NEs contained both lamins and the inner nuclear membrane protein LBR (Figure 1h). The relative amounts of these proteins in the final RV fractions were slightly different from that in the whole NEs because octyl glucoside released different proportions of each polypeptide, while the solubilized proteins exhibited different propensities to incorporate into RVs. For instance, ~10% of total LBR, but >50% of the lamins, were solubilized by the detergent (Figure 1h, compare lanes OG sup and OG pel). However, all of the solubilized LBR was incorporated into vesicles, whereas only a fraction of the solubilized lamins were reconstituted when the detergent was dialyzed out (Figure 1h, compare lanes OG sup and RV).

To assess the orientation of *in vitro* reconstituted membranes, we treated RVs with soluble or bead-immobilized trypsin, harvested the vesicles by centrifugation and examined the digests by Western blotting. As illustrated in Figure 2a, all membrane-bound lamins were degraded by the protease, suggesting that most of the vesicles had a 'nucleoplasmic-side out' orientation. This point was confirmed further by immunoelectron microscopy. As depicted in Figure 2b–g, the surface of rat and turkey RVs was decorated by anti-peptide antibodies recognizing the lamins and the nucleoplasmic NH_2-terminal regions of LBR or LAP2.

The lamins recovered by pelleting RVs after removal of the detergent were incorporated into the membranes and did not represent 'loose', co-sedimenting polymer. This could be shown by performing Western blotting analysis on vesicles isolated by flotation in sucrose gradients or examining such sucrose-purified vesicles by immunoelectron microscopy (data not shown).

Binding of reconstituted NE vesicles to chromosomes

To examine whether reconstituted vesicles bind to chromatin, we used as a substrate prometaphase chromosomes isolated from nocodazole-arrested Chinese hamster ovary (CHO) cells. The chromosome preparations were free of endogenous membranes, except for a few vesicles (Figure 3c), and did not contain detectable amounts of lamins (see Glass and Gerace, 1990; Maison *et al.*, 1995).

Upon co-incubation with RVs or whole NEs, the surface of chromosomes became covered by numerous membranous structures (Figure 3a and b). Membrane binding did not require cytosolic factors and nucleoside triphosphates, suggesting a direct and spontaneous association. However, RVs that had bound to chromosomes did not fuse with each other, indicating that soluble factors are indeed required for the formation of a complete envelope around chromatin.

In a permutation of this experiment, we used fluorescently labeled membranes to monitor chromosome binding at the level of the light microscope. Equivalent amounts

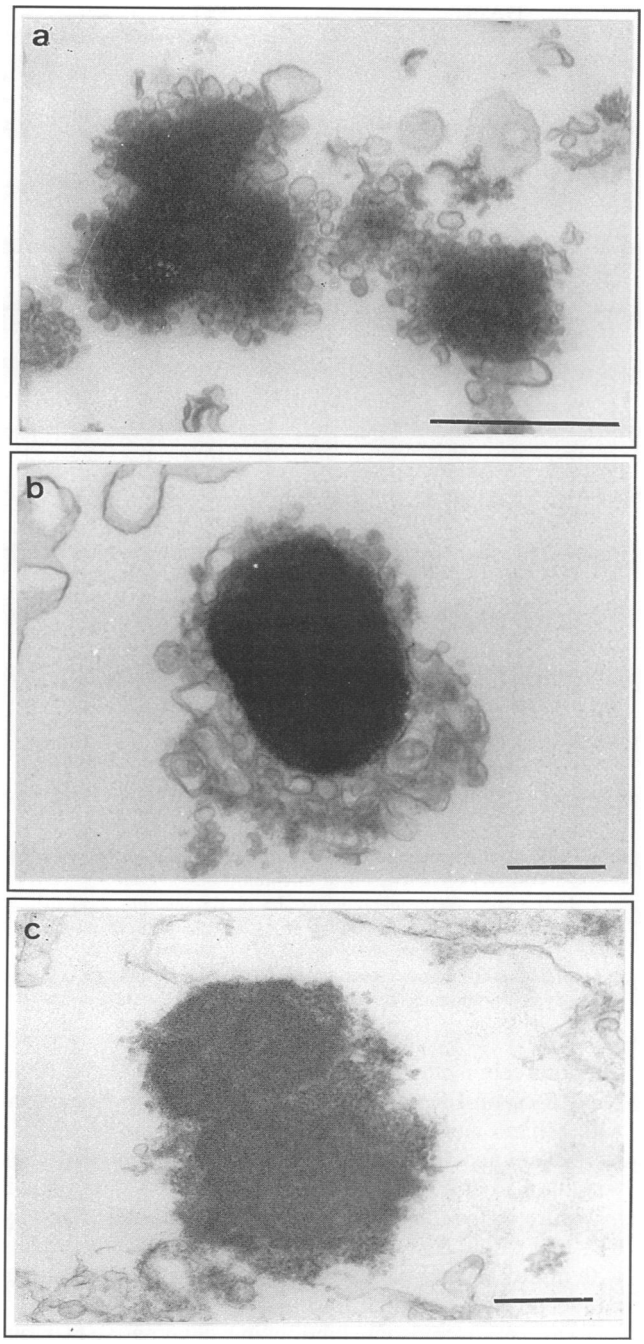

Fig. 3. Binding of reconstituted NE vesicles to CHO chromosomes. Rat hepatocyte RVs (**a**) or NEs (**b**) were co-incubated with CHO chromosomes and processed for electron microscopy as specified in Materials and methods. (**c**) A sample of chromosomes incubated with assay buffer alone (control). Bar, 200 nm.

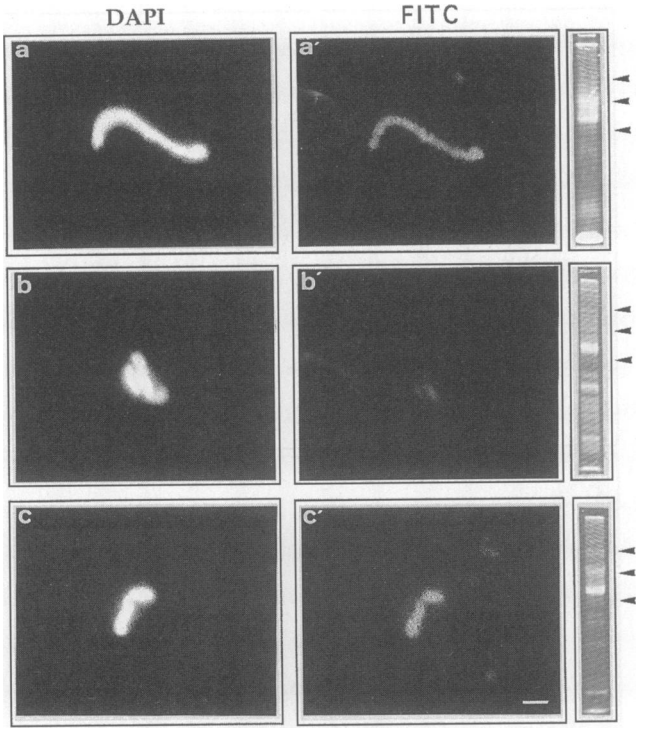

Fig. 4. Binding of FITC-labeled membranes to CHO chromosomes. Various membrane preparations were labeled with FITC and then assayed for binding to chromosomes as specified in Materials and methods. (**a–c**) Staining of DNA-containing particles with DAPI. (**a′–c′**) FITC fluorescence of the same specimens. Rat hepatocyte NEs (a and a′) and RVs (c and c′) bind to chromosomes, while ER vesicles (b and b′) do not. Electrophoretic profiles on the far right show the corresponding FITC-labeled proteins in each preparation as visualized under UV. Arrows indicate the position of 97, 66 and 45 kDa molecular weight markers. Assays with FITC-labeled turkey erythrocyte RVs gave the same results (not shown). Bar, 1 μm.

of fluorescein isothiocyanate (FITC)-tagged rat hepatocyte NEs, RVs or ER microsomes (control) were incubated with a fixed amount of chromosomes and the specimens were examined after staining all DNA-containing particles with 4′,6′-diamidino-2-phenylindole (DAPI). As shown in Figure 4, the chromosomes were heavily decorated by RVs and whole NEs, but not by ER vesicles. Experiments with turkey erythrocyte RVs yielded the same results (data not shown; for pertinent information see Table I).

To assess chromosome binding in a quantitative fashion, we performed the light microscopy assay under saturating conditions (for details see Materials and methods). Morphometric data summarized in Table I, show that 70–100% of the chromosomes were decorated by RVs, whereas only 14% of them had ER vesicles attached. From these results, it can be concluded that RVs bind specifically to chromosomes.

Chromosome binding properties of NE vesicles lacking specific components

Having at hand an assay system that measures NE vesicle binding to chromosomes, we attempted to study the involvement of the nuclear lamins in this process. To this end, we applied clarified octyl glucoside extracts of rat or turkey NEs to immunoaffinity columns containing anti-lamin IgG (aLI) cross-linked to protein A. In parallel, samples of the same extracts were passed through control

Table I. Binding of NE preparations to CHO chromosomes

Type of membrane	No. of experiments	% of chromosomes decorated	Net binding (total minus EmR)
ER	11	14 (range 6–20)	0
RL-rNEs	12	70 (range 54–81)	56
TE-rNEs	2	100	86
RL-uNEs	4	80	66
RL-uNE/mit cyt.	4	31	17
RL-uNEs/trypsin	2	7	0
RL-urNEs	2	79	65
TE-uNEs	4	64	50
TE-uNEs/trypsin	2	16	2
TE-urNEs	2	96	82
RL-rNEs/Lm(–)	6	40	26
RL-rNEs/LAP2(–)	5	60	46
RL-rNEs/mock depl.	3	74	60
TE-rNEs/Lm(–)	2	43	29
TE-rNEs/LBR(–)	3	26	12
TE-rNEs/mock depl.	3	78	64

FITC-labeled membranes were assayed for chromosome binding exactly as specified in Materials and methods. RL denotes fractions derived from rat liver, whereas TE denotes fractions prepared from turkey erythrocytes. Other abbreviations are as follows: ER, microsomes from rat liver; uNEs, urea-extracted NEs; uNEs/trypsin, trypsin-treated uNEs; uNE/mit cyt., uNEs treated with mitotic cytosol; rNEs, reconstituted NE vesicles; urNEs, urea-extracted rNEs; rNEs/Lm(–), lamin-depleted rNEs; rNEs/LAP2(–), LAP2-depleted rNEs; rNEs/LBR(–), LBR-depleted rNEs; rNEs/mock depl., mock-depleted rNEs. The relatively low chromosome-binding activity of urea-extracted turkey erythrocyte nuclear envelopes (TE-uNEs) in comparison to reconstituted vesicles (TE-rNEs) is due to the fact that the former, but not the latter, retain in part the 'ghost' configuration of the original NEs and, therefore, their nucleoplasmic side is not entirely exposed to the outside environment.

columns containing non-immune IgG coupled to protein A. Each flowthrough was dialyzed against isotonic buffer to remove the detergent, and the vesicles formed from immunodepleted or mock-depleted extracts were harvested by centrifugation.

As can be seen in Figure 5a (panels 'lamin depleted'), aLI immunoabsorption removed almost the entire lamin complement of the RVs. Quantitative assays with such lamin-depleted vesicles revealed that they had ~50% of the chromosome binding capacity of control membranes (Table I). However, at this stage, we could not exclude that the reduced binding of lamin-depleted vesicles was an indirect effect due to the co-depletion of some lamin and chromatin binding integral membrane protein.

To resolve this problem, we assayed side by side 'wild-type' RVs and RVs or NEs extracted with 8 M urea. Urea extraction is known to strip the membranes of all peripheral proteins (including the lamins) without affecting the integral membrane proteins (Worman *et al.*, 1988; Foisner and Gerace, 1993; Maison *et al.*, 1995). As shown in Table I, urea-extracted (i.e. lamin-free) RVs and NEs were as competent at binding to chromosomes as 'wild-type' RVs. In addition, chromosome binding of urea-extracted membranes could be abolished by prior treatment with trypsin and mitotic cytosol (Table I), suggesting an involvement of phosphoproteins in this interaction. When increasing quantities of urea-stripped NEs were assayed in the presence of a fixed amount of chromosomes, the percentage of decorated chromosomes increased in a linear

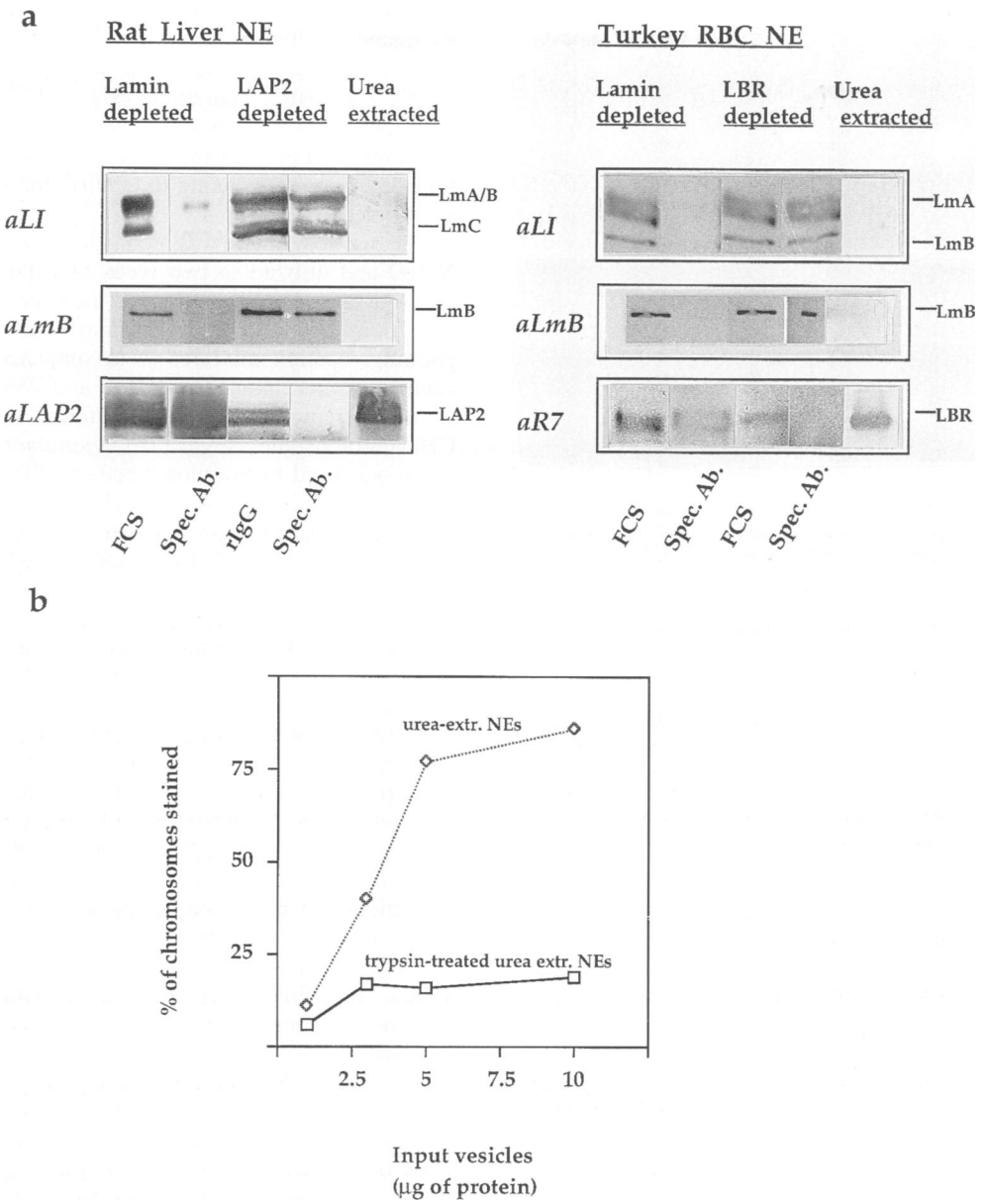

Fig. 5. Preparation of immunodepleted and urea-stripped vesicles. (**a**) Rat liver and turkey erythrocyte NEs were solubilized with octyl glucoside and the clarified detergent extracts were incubated with protein A–Sepharose beads coupled to aLI, aLAP2 or aLBR (aR_7) antibodies (denoted by the abbreviation 'Spec. Ab'). For control purposes, an equal portion of each extract was chromatographed through protein A–Sepharose coupled to total calf immunoglobulins (FSC), or non-immune rabbit IgG (rIgG). The non-bound material recovered from each column was analyzed by immunoblotting using aLI, a specific anti-lamin B antibody (aLmB), aLAP2 and aR_7. In parallel, samples of urea-extracted NEs (Urea extr.) were probed by the same antibodies. The positions of lamins A, B, C, LAP2 and LBR are indicated. (**b**) Binding of increasing quantities of untreated and trypsin-treated urea-stripped NEs to chromosomes. This dose-dependency assay was performed as specified in Materials and methods.

fashion and plateaued at ~80% (Figure 5b). In contrast, the binding of trypsin-treated, urea-stripped NEs was negligible at all concentrations tested (Figure 5b). This dose dependency and the loss of binding upon treatment with protease strongly suggested that NE vesicles associate with the chromosomes via integral membrane proteins.

To explore this idea further, we immunodepleted RVs from two well-characterized lamin-associated integral membrane proteins proposed to have chromatin binding properties: LAP2 and LBR. LAP2 immunodepletion experiments were done using solubilized rat liver NEs and anti-peptide antibodies developed against the NH_2-terminal nucleoplasmically exposed segment of LAP2 (for sequence information, see Harris *et al.*, 1994; Furukawa *et al.*, 1995). However, since antibodies against the mammalian form of LBR were not available, LBR immunodepletion experiments were done using solubilized turkey erythrocyte NEs and anti-peptide antibodies against the NH_2-terminal nucleoplasmic domain of avian LBR (Worman *et al.*, 1990; Simos and Georgatos, 1992; Meier and Georgatos, 1994).

Removal of LAP2 and LBR from octyl glucoside extracts was efficient (Figure 5a, panels 'LAP2 depleted' and 'LBR depleted'). Binding experiments with FITC-

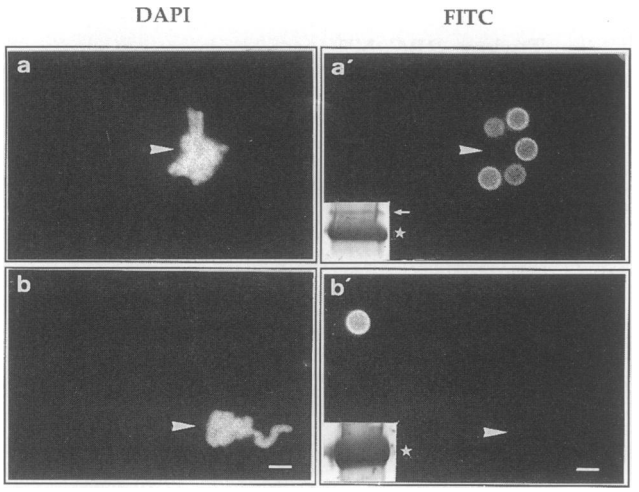

Fig. 6. Binding of CHO chromosomes to magnetic beads coated with LBR. Magnetic immunobeads carrying affinity-purified anti-LBR antibodies or normal rabbit IgG (control) were incubated with a Triton X-100 extract of urea-stripped turkey erythrocyte NEs. Following re-isolation and washing with buffer, a sample of each preparation was subjected to Western blotting, while another sample was incubated with chromosomes (for technical details, see Materials and methods). As shown in (a′) and (b′) (insets), beads containing anti-LBR antibodies captured Triton-solubilized LBR, whereas control beads did not. (The position of LBR is indicated by an arrow; asterisks denote the heavy chain of IgG.) (**a**) and (**a′**) Samples containing LBR–beads and chromosomes after staining with DAPI (to decorate the DNA) and FITC-labeled goat anti-rabbit IgG (to label the immunobeads). (**b**) and (**b′**) The corresponding experiment with control beads. Notice that LBR-containing beads gather around chromosomes, whereas control beads do not. Bar, 1 μm.

labeled membranes showed that removal of LAP2 had only a marginal effect, whereas removal of LBR abolished binding of RVs to the chromosomes (Table I). Consistent with the previous observations, the amount of LBR in lamin-depleted membranes was less than in mock-depleted vesicles, whereas this did not hold for the LAP2 protein (Figure 5a, panels 'lamin depleted' and 'LAP2 depleted'). These observations strongly suggested that binding of NE vesicles to chromosomes involves the LBR protein.

Binding experiments with bead-immobilized and soluble LBR

To confirm the immunodepletion data by a complementary approach, we solubilized urea-stripped turkey erythrocyte NEs with Triton X-100, which efficiently extracts the LBR protein (Worman *et al.*, 1988; Simos and Georgatos, 1992; Simos *et al.*, 1996). The Triton extract was then incubated with magnetic beads carrying specific anti-LBR antibodies or non-immune rabbit IgG (control). Immunoblotting experiments confirmed that the magnetic beads which carried specific antibodies had captured LBR, whereas control beads had not (Figure 6, insets). When the beads were incubated with CHO chromosomes and the samples examined by fluorescence microscopy, we observed that ~40% of the chromosomes were surrounded by LBR-carrying beads (Figure 6a and a′). This did not occur with control beads (Figure 6b and b′).

To distinguish whether LBR was involved directly or indirectly in interactions with chromatin, we purified this protein to homogeneity using SDS–PAGE and electro-elution (for details, see Materials and methods). Purification of LBR by this method has been shown not to affect its ligand binding properties and its phosphorylation by specific kinases (Nikolakaki *et al.*, 1996; Simos *et al.*, 1996). The LBR preparations (SDS–PAGE profile shown in Figure 7a) were routinely checked by Western blotting, while material eluted from SDS gels was analyzed several times by microsequencing to confirm the identity and the purity of the isolates.

The purified protein was reconstituted in 0.75–1% Triton X-100 and utilized in two types of experiments. In one set of assays, soluble LBR was incubated under isotonic conditions with or without chromosomes and the corresponding reaction mixtures were subjected to low-speed centrifugation. Upon SDS–PAGE and Western blotting, a significant amount of LBR was found to co-pellet with CHO chromosomes (Figure 7b). A similar type of pelleting assay was used to examine whether LBR binds to turkey erythrocyte polynucleosomes which are isolated free of endogenous lamins (for relevant information, see Yuan *et al.*, 1991). Again, LBR co-sedimented with the chromatin particles, but did not self-pellet to an appreciable extent (Figure 7c), even though this assay involved ultracentrifugation. Performing the assays at different temperatures did not reveal a difference in the binding at normal temperature (25–30°C) and at low temperature (4°C).

To ensure that the co-pelleting of LBR and chromosomes reflects a physiological association and not aggregation, we repeated the previous experiments and examined the samples *in situ*. As illustrated in Figure 8a and a′, purified LBR heavily decorated the surface of chromosomes, yielding a striking banding pattern. Inspection of numerous specimens did not reveal a preferential staining of telomeres or centromeres, but the overall pattern of decoration was highly reminiscent of G- or Q-banding (Holmquist, 1992). Matching experiments done with an equivalent amount of column-purified or electroeluted rat liver lamins A/C showed binding around the periphery of the chromosomes (Figure 8c and c′), as previously reported (Glass and Gerace, 1990). Chromosomes were not decorated after incubation with assay buffer alone and staining with anti-LBR or anti-lamin antibodies (Figure 8b, b′, d and d′). These data rule out the possibility that chromosome decoration by LBR is an artefact due to technique.

Discussion

A new assay system for studying NE–chromatin interactions

A problem encountered when one uses whole cell homogenates to study nuclear reassembly is the low abundance of NE precursor vesicles which constitute, at the most, 1% of the total membranes. The scarcity of NE vesicles makes the isolation of components mediating NE–chromatin interactions rather cumbersome. To circumvent this problem, we have developed an assay which involves membrane vesicles reconstituted from octyl glucoside extracts of purified NEs. The vesicles retain the major protein constituents of the inner nuclear membrane, but apparently lack components of the pore membrane and contain only a small proportion of the outer nuclear membrane (which ruptures during NE isolation and breaks away together with the bulk of the RER). They have the form of closed vesicles and orient themselves with the

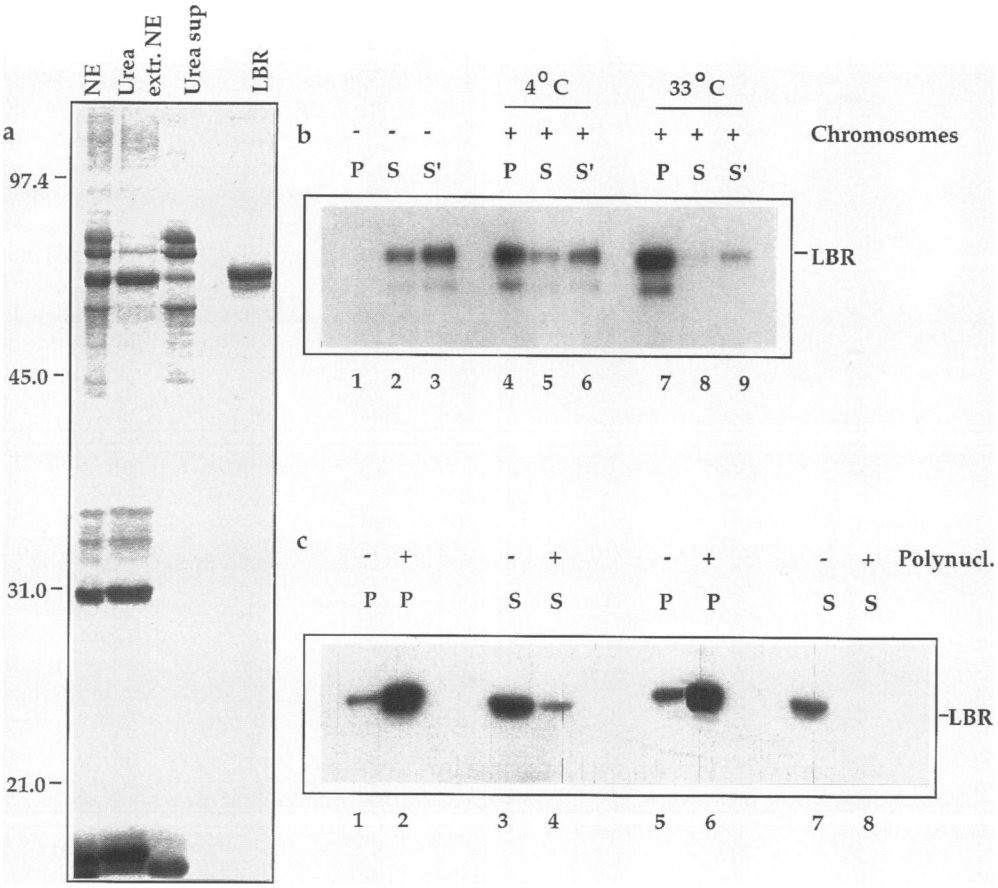

Fig. 7. Binding of purified LBR to CHO chromosomes and turkey erythrocyte polynucleosomes as detected by a pelleting assay. (**a**) Electrophoretic profiles of turkey erythrocyte NEs (NE), urea-extracted NEs (Urea extr. NE), urea extract of NEs (Urea sup) and purified LBR (LBR). Molecular weight markers are indicated on the left of the panel. (**b**) Purified LBR was incubated with (+) or without (–) CHO chromosomes in assay buffer and the mixtures were subjected to low-speed centrifugation. Pellets and supernatants were run on SDS–polyacrylamide gels and blotted with anti-LBR antibodies. P, S and S′ correspond to the entire pellet fraction, 1/10 of the supernatant and 1/6 of the supernatant of each assay mixture, respectively. Lanes 1–6, samples incubated at 4°C; lanes 7, 8 and 9, samples incubated at 30°C. (**c**) Purified LBR was incubated with turkey erythrocyte polynucleosomes (+) or assay buffer alone (–) and the samples were subsequently ultracentrifuged. The pellets (P) and the entire trichloroacetic acid-precipitated supernatants (S) were blotted with anti-LBR antibodies. Lanes 1–4, samples incubated at 25°C; lanes 5–8, samples incubated at 4°C.

nucleoplasmic side out. The system can be manipulated *in vitro* and be depleted of specific components by antibody absorption. These features render the *in vitro* reconstituted vesicles an ideal model for studying NE–chromatin binding under a defined set of conditions.

However, it should be stressed that while the quantitative fluorescence microscopy assay employed in this study provides a convenient tool to compare bulk properties of different membrane isolates, it is not suitable for measuring 'exact' parameters such as chemical affinity and number of binding sites. There are several reasons for this. By nature, the chromosomes and chromosome fragments used as a substrate in these and in relevant studies are not of uniform size and probably differ in condensation state and nucleoprotein composition. Furthermore, there is a certain limit in fluorescence microscopy below which weakly decorated particles would be scored as 'negative'. Thus, binding experiments should be performed near saturation using different chromosome and membrane isolates before extracting any conclusions.

Key findings and comparisons with earlier studies

We have shown that removal of LBR from the NE vesicles reduces binding to chromosomes significantly. Furthermore, we have provided evidence that purified LBR binds directly to chromosomes and polynucleosomes. Taken together, these data demonstrate that the basic machinery involved in NE–chromatin interactions resides in the intrinsic domain of the inner nuclear membrane.

The idea that LBR represents a major chromatin binding site at the NE is consistent with other observations. For instance, it has been reported recently that the NH_2-terminal domain of LBR binds to phage DNA and the heterochromatin-specific protein HP1 (Worman *et al.*, 1990; Ye and Worman, 1994, 1996). Although we have reasons to believe that binding of the NH_2-terminal domain of LBR to naked DNA may not be physiologically significant (intact LBR does not bind to DNA in our hands; E.Nikolakaki, T.Giannakouros and S.D.Georgatos, unpublished observations), the binding to HP1 is very relevant to the interactions reported here. Proteins of the HP1 family have been identified in many organisms and are characterized by the existence of a conserved region termed 'the chromodomain'. In *Drosophila melanogaster*, HP1 localizes in heterochromatin and is involved in position-effect variegation (James and Elgin, 1986; Eissenberg *et al.*, 1990; Singh *et al.*, 1991; Clark and Elgin, 1992; Saunders *et al.*, 1993).

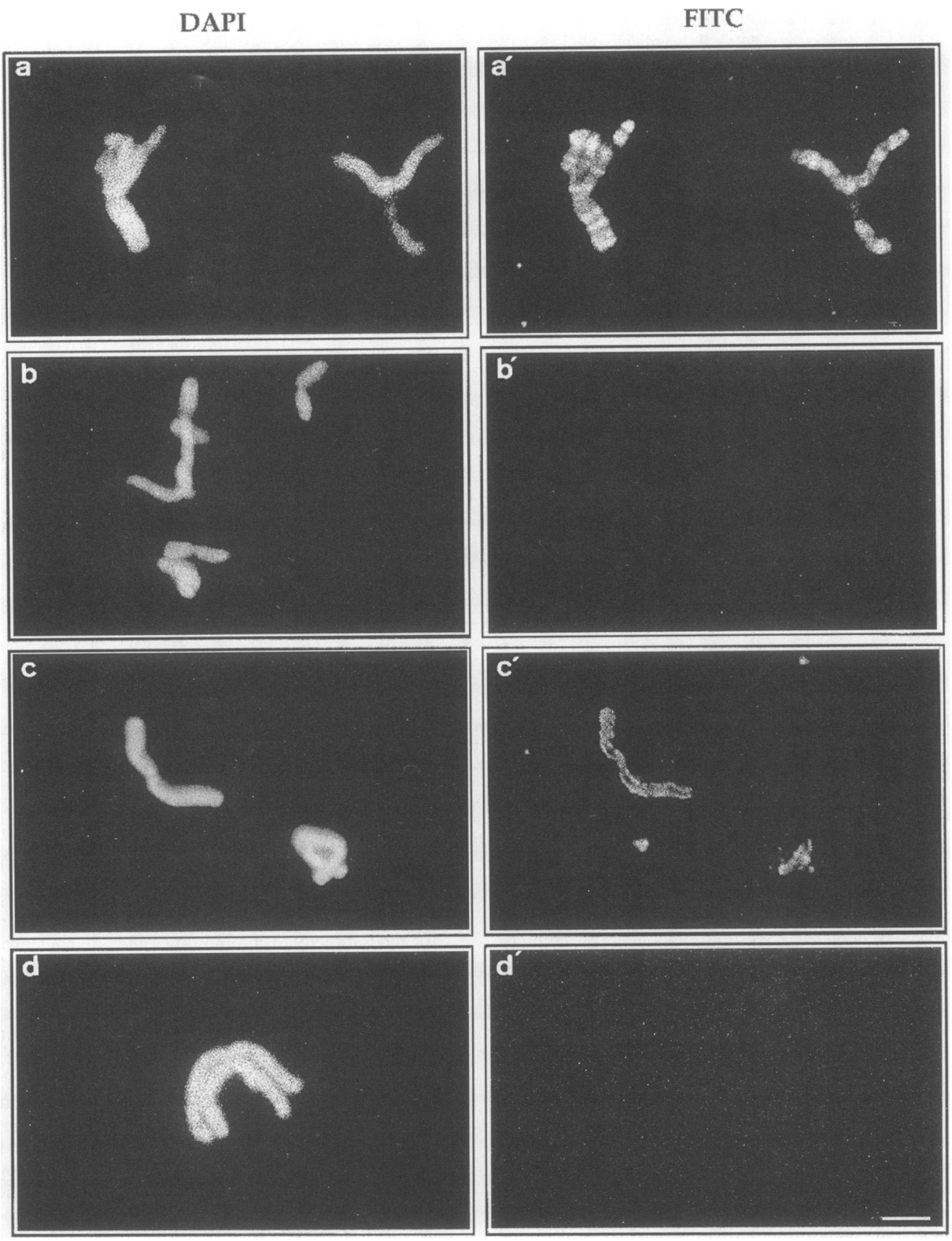

Fig. 8. Binding of purified LBR and lamins A/C to CHO chromosomes as detected by indirect immunofluorescence. CHO chromosomes were incubated with purified LBR (**a** and **a′**), KHM buffer alone (**b**, **b′** and **d**, **d′**) or rat liver lamins A/C (**c** and **c′**) (for details see Materials and methods). The specimens were then fixed and stained with affinity-purified anti-LBR IgG/anti-rabbit FITC (a′ and b′), anti-lamin A IgG/anti-rabbit FITC (c′ and d′) and DAPI (a–d). Bar, 1 μm.

Purified LBR binds to distinct zones along the chromosome arms, suggesting the existence of specialized sites which contain clustered 'LBR receptors'. This is in excellent agreement with recently published results concerning the arrangement of NE–chromatin contact sites in embryonic nuclei of *D.melanogaster* (Marshall *et al.*,

1996). Using *in situ* hybridization and three-dimensional fluorescence microscopy, these authors have identified ~15 foci of close NE–chromosome contact per chromosome arm. Binding of LBR to a limited number of chromosomal domains is not inconsistent *a priori* with binding to turkey erythrocyte polynucleosomes (which lack a high order structure). For instance, if LBR interacted with heterochromatin-specific proteins, one would also expect binding to bulk chromatin fragments prepared from highly heterochromatic nuclei.

The banding pattern observed when LBR is co-incubated with chromosomes differs significantly from the perichromosomal staining obtained with purified lamins and is reminiscent of G- or Q-banding. This type of banding or 'flavor' (obtained by Giemsa or quinacrine staining) is thought to be characteristic of regions which are generally gene-poor and late-replicating (Holmquist, 1992). Obviously, more refined experiments have to be done in order to characterize the sites on chromosomes which contain LBR binding elements.

Our observations are in line with other results (Foisner and Gerace, 1993) showing that chromosome decondensation is not a prerequisite for NE vesicle binding in somatic cells. That chromosomes need not be decondensed in order to bind NE vesicles makes physiological sense because NE reassembly *in vivo* is initiated in anaphase when most chromosomes are still condensed (Robbins and Gonatas, 1964; Foisner and Gerace, 1993; Meier and Georgatos, 1994). However, these data would contradict the fact that sperm chromatin must be decondensed in order to bind NE precursor vesicles isolated from frog egg extracts (Newport and Dunphy, 1992). In our view, these differences should be attributed to the fact that the physical state and protein composition of sperm chromatin is markedly different from that of somatic cell chromatin.

Under the conditions employed, we did not find the integral membrane protein LAP2 to be as essential as LBR for NE–chromatin interactions. This is probably due to the fact that LAP2 is merely one of several chromatin binding proteins present in the rat hepatocyte NE. Therefore, its absence in immunodepleted vesicles may be compensated for by the presence of other integral membrane proteins, primarily LBR (for relevant data, see Courvalin *et al.*, 1990). Although we could not prove this point directly because antibodies against mammalian LBR were not available, such an interpretation would be consistent with the fact that LAP2-depleted rat liver NE vesicles show a slightly lower chromosome binding than do mock-depleted membranes. An alternative explanation could be that LAP2 binding to chromatin is of low affinity and does not contribute significantly to the overall interaction. Future studies will address these problems in detail.

Finally, we need to note that the direct involvement of LBR in chromatin binding does not disqualify peripheral NE proteins, such as the lamins, as potential modulators of this interaction. Indeed, several studies support the hypothesis that B-type lamins are required for efficient binding of NE precursor vesicles to chromosomes (Burke and Gerace, 1986; Ulitzur *et al.*, 1992; Maison *et al.*, 1995). In view of the currently available information, it would seem plausible to suppose that the lamins play an auxiliary role in NE reassembly, perhaps enhancing the binding of NE precursor vesicles to the chromosomes, or preventing the 'coating' of NE fragments with reassembly-inhibiting factors.

Materials and methods

Cell fractionation

Cell culturing and synchronization was according to Maison *et al.* (1995). Chromosomes were prepared from mitotic CHO cells according to Glass and Gerace (1990). Polynucleosomes from turkey erythrocytes were prepared after Zentgraf and Franke (1984) and Yuan *et al.* (1991) with some modifications. Briefly, 40 ml of blood was drawn from turkeys and the red blood cells washed three times in 10 volumes of ice-cold 140 mM NaCl, 10 mM Tris–HCl pH 7.4 and 15 mM sodium citrate. The washed red blood cells were resuspended in 280 ml of buffer A [100 mM NaCl, 10 mM Tris–HCl pH 7.4, 0.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and divided into six aliquots. Nonidet-P40 was added to these aliquots to a final concentration of 0.5% with stirring. The erythrocytes were allowed to lyse for 5 min on ice and the lysate was spun at 17 000 *g* for 10 min at 4°C. The supernatant was discarded and the pellet was washed twice with buffer A and once with buffer B (100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Tris–HCl pH 7.4, 0.5 mM PMSF). The membrane-free nuclear pellet was resuspended in 5 ml of buffer B and the optical density (OD) at 254 nm was measured in a spectrophotometer. The suspension was then diluted to 50 OD/ml and split into 0.5 ml aliquots. For digesting the DNA, 100 IU of micrococcal nuclease (Boehringer, Mannheim) were added to each aliquot and the samples were incubated for 2 min at 37°C. To stop the reaction, 6 µl of 0.5 mM EGTA were added. The mixture was then loaded on linear 50–10% sucrose gradients (in 100 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EDTA and 0.5 mM PMSF) and spun for 90 min at 200 000 *g* at 4°C. One ml fractions were collected from the top to the bottom of the gradient and analyzed by SDS–PAGE to identify the peak of the polynucleosomes. Pooled fractions were kept at –80°C or used immediately in binding assays.

Rat liver nuclei and NEs were prepared as specified by Dwyer and Blobel (1976) and Blobel and Potter (1966). Turkey erythrocyte NEs were prepared after Georgatos and Blobel (1987). ER microsomes were isolated from rat liver as described by Blobel and Dobberstein (1975). To remove peripheral membrane proteins, whole NEs or reconstituted NE vesicles were resuspended in 8 M urea, 10 mM Tris–HCl, pH 7.3, 1 mM EDTA, 1 mM DTT and 1 mM PMSF, sonicated and centrifuged at 400 000 *g* for 30 min at 20°C. Pellets were washed with urea-free buffer and used in assays.

Reconstitution of membrane vesicles, immunodepletion and Western blotting

NEs were solubilized at 50 OD/ml with 60 mM *n*-octyl-D-glucopyranoside in 400 mM sucrose, 400 mM KCl, 20 mM Tris–HCl pH 7.6, 1.5 mM $MgCl_2$ and 1 mM EDTA, for 20–30 min at 4°C (see Nicchitta and Blobel, 1990). The extract was then spun in a Beckman tabletop ultracentrifuge for 1 h at 400 000 *g* to remove non-solubilized material. NE vesicles were reconstituted after dialysis of the clarified detergent extract for 12–18 h at 4°C against KHM buffer (Burke and Gerace, 1986). Samples of FITC-labeled membranes were used in assays or solubilized in electrophoresis sample buffer (Laemmli, 1970) and run on 10% SDS mini gels. In the latter case, the protein bands were visualized under UV. For immunodepletion experiments, NE fractions from rat hepatocytes or turkey erythrocytes were solubilized as above and the clarified extracts were incubated for 1–2 h at 4°C with protein A–Sepharose cross-linked to the 'all-anti-lamin' antibody aLI (Simos and Georgatos, 1992), a rabbit anti-LAP2 antibody developed against the peptide QALTRESTRGSRRTPRRRVEK (for sequence information, see Fukuwama *et al.*, 1995) or the anti-LBR antibody aR_7 (Meier and Georgatos, 1994). The unbound material was collected in each case and the detergent was dialyzed out to allow formation of proteoliposomes. Membrane preparations were labeled with FITC as specified in Maison *et al.* (1995).

Western blotting experiments were done using the antibodies described above (aLI and aR_7), the anti-peptide antibody No.16 which reacts specifically with B-type lamins (Maison *et al.*, 1993, 1995), a polyclonal antibody against gp210 (a gift from R.Wozniak and G.Blobel, The Rockefeller University, NY) and a monoclonal antibody recognizing LAP1A/C. The characterization of the anti-LAP1A/C antibodies will be described elsewhere.

Isolation of LBR

LBR was purified either electrophoretically or by immunoabsorption. In one series of experiments, turkey erythrocyte NEs were extracted with 8 M urea and run on 10% SDS gels. The gels subsequently were stained with copper as specified by Lee *et al.* (1987) and the appropriate bands excised with a blade. To remove the copper stain completely, the gel pieces were washed three times for 5 min with 250 mM EDTA and 250 mM Tris–HCl pH 9.0. After complete destaining, the LBR-containing bands were equilibrated in 192 mM glycine, 250 mM Tris–HCl pH 7.3, 25 mM SDS (twice for 10 min) and electroeluted for 3 h at 100 V. Samples of the electroeluted material were routinely checked by SDS–PAGE and immunoblotting, while some of the preparations were subjected to NH_2-terminal sequencing. The rest of the material was diluted in and dialyzed against 1% Triton X-100, 20 mM HEPES–KOH pH 7.3, 150 mM KCl, 1 mM DTT and 1 mM PMSF for 12 h at 4°C. Before using it in binding studies, the protein solution was spun for 20 min at 12 000 *g* at 4°C to remove aggregates.

For immunoisolating LBR, urea-extracted turkey erythrocyte NEs were resuspended and sonicated in 8 M urea, 2 mM EGTA, 20 mM Tris–HCl pH 7.3, 150 mM KCl, 1 mM PMSF. Urea was removed by dialysis and LBR was extracted from the membranes with 1% Triton X-100. The solubilized protein was immobilized on magnetic immunobeads (Dynabeads, Dynal, Norway) carrying specific anti-LBR antibodies (for methods, see Maison *et al.*, 1993, 1995).

Assays

Quantitative light microscopy binding assays were performed as follows. FITC-labeled membranes (10–20 μg) were combined with chromosomes (2.5–5 μl of 5 OD_{260}/ml) in KHM-gelatin buffer (78 mM KCl, 50 mM HEPES–KOH pH 7.0, 4 mM $MgCl_2$, 8.37 mM $CaCl_2$, 10 mM EGTA, 1 mM DTT, 20 μM cytochalasin B, 1 mM PMSF and 1–2 mg/ml boiled fish gelatin) in a final volume of 50 μl. The reaction mixture was incubated in a test tube for 45 min at room temperature and then deposited onto untreated glass coverslips or onto coverslips layered with 0.1% Alcian blue (a cationic dye). Particles were allowed to adhere to the solid support for 15 min at room temperature, stained with the DNA binding dye DAPI, washed once with assay buffer, air-dried and mounted onto slides. Occasionally, the specimens were post-fixed with 4% formaldehyde for 10 min at room temperature before visualization, but this did not appear to be necessary.

To quantify binding with each membrane isolate, the number of DAPI-stained particles (chromosomes) surrounded by FITC-bearing vesicles (see Figure 4) was recorded and this was divided by the total number of DAPI-stained particles (both decorated and undecorated by FITC vesicles). To ensure that the binding experiments were done near saturation, dose-dependency studies were performed using RVs isolated by flotation in sucrose gradients, urea-extracted NEs and trypsinized urea-extracted NEs. In these experiments, a standard amount of chromosomes (5 μl of 5 OD_{260}/ml) was incubated with increasing amounts of FITC-labeled membranes and the samples processed as above. The percentage of DAPI-stained particles that had bound FITC-labeled vesicles in each case was assessed by fluorescence microscopy. At the same time, the specimens were inspected to ensure that FITC-labeled membranes in the background exceeded the number of DAPI-stained particles. Saturation was generally achieved by using 10 μg of FITC-labeled vesicles and 5 μl of 5 OD_{260}/ml of chromosomes.

When needed as controls, urea-extracted NEs were treated with trypsin (1:30 w/w) for 1 h at room temperature or pre-incubated in KHM buffer, 1 mM ATP/GTP, 10 μM microcystin and mitotic cytosol (0.4 mg/ml) for 30 min at room temperature. After treatment, the membranes were washed in assay buffer containing PMSF and trypsin inhibitor and used in assays.

To monitor binding of purified LBR to chromosomes, LBR-loaded immunobeads (see above) were incubated with 10 μl of chromosomes (5 OD_{260}/ml) for 45 min at room temperature. Binding of the beads to the chromosomes was then assessed by light and fluorescence microscopy.

To examine chromosome binding of LBR, 20–30 μl from the electroeluted preparation (~25–40 μg of protein/ml) was dialyzed extensively and incubated with 50 μl of chromosomes (3 OD_{260}/ml) in KHM buffer containing 0.75–1% Triton X-100. Boiled skin gelatin was added to a final concentration of 2 mg/ml to block non-specific binding. The mixture was incubated for 1 h at 4 or 30°C and spun in an Eppendorf centrifuge through a 75 μl cushion containing 30% sucrose at 6000 r.p.m. (20 min, 4°C). The pellets and the supernatants were analyzed by Western blotting.

Binding studies with polynucleosomes were performed as described in Yuan *et al.* (1991) with some modifications. In brief, polynucleosomes were incubated with purified LBR in 0.5% Triton X-100 in 100 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EDTA, 0.5 mM PMSF, 5 mg/ml fish skin gelatin, for 1 h at 4 or 25°C. The assay mixture was then pelleted through a 30% sucrose cushion for 60 min at 200 000 *g*, at 4°C. The pellets and the supernatants were analyzed by Western blotting.

To examine binding of LBR to chromosomes *in situ*, we employed indirect immunofluorescence microscopy. In this case, the same amounts of LBR and chromosomes were used as in the pelleting assay described above, but the specimens were usually diluted to 1/10 the usual volume with assay buffer before application to a glass coverslip. In some experiments, latex beads (Seradyn, Indianapolis, USA) were used as a negative control instead of chromosomes. Chromosome binding of rat liver lamins A/C and turkey lamin A (isolated by column chromatography or by electroelution) was assessed in exactly the same way.

Microscopy

For electron microscopy, samples were fixed with 1–1.5% glutaraldehyde on ice for 1 h. The specimens were then processed for thin-section electron microscopy. Sections were examined in a Philips 400 transmission electron microscope operated at 80 keV. Indirect immunofluorescence microscopy was done using the aR_7 and aLI anti-peptide antibodies (Meier and Georgatos, 1994).

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