Lamins A and C Bind and Assemble at the Surface of Mitotic Chromosomes

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Abstract. To study a possible interaction of nuclear lamins with chromatin, we examined assembly of lamins A and C at mitotic chromosome surfaces in vitro. When a postmicrosomal supernatant of metaphase CHO cells containing disassembled lamins A and C is incubated with chromosomes isolated from mitotic Chinese hamster ovary cells, lamins A and C undergo dephosphorylation and uniformly coat the chromosome surfaces. Furthermore, when purified rat liver lamins A and C are dialyzed with mitotic chromosomes into a buffer of physiological ionic strength and pH, lamins A and C coat chromosomes in a similar fashion. In both cases a lamin-containing supramolecular structure is formed that remains intact when the chromatin is removed by digestion with micrococcal nuclease and extraction with 0.5 M KCl. Lamins associate with chromosomes at concentrations approximately eightfold lower than the critical concentration at which they self-assemble into insoluble structures in the absence of chromosomes, indicating that chromosome surfaces contain binding sites that promote lamin assembly. These binding sites are destroyed by brief treatment of chromosomes with trypsin or micrococcal nuclease. Together, these data suggest the existence of a specific lamin–chromatin interaction in cells that may be important for nuclear envelope reassembly and interphase chromosome structure.

THE nuclear lamina is a fibrillar protein meshwork that lines the nucleoplasmic surface of the nuclear envelope (Franke et al., 1981; Gerace et al., 1984). It is thought to provide an architectural framework for attachment of nuclear membranes and pore complexes. In addition, the lamina is postulated to anchor interphase chromatin to the nuclear envelope and thereby to influence higher order chromosome structure (Gerace et al., 1978; Lebkowski and Laemmli, 1982).

The proposed association of the lamina with chromatin is based largely on circumstantial evidence. Both light and electron microscopic studies have indicated that chromatin (often heterochromatin) is closely apposed to the inner surface of the nuclear envelope (reviewed by Franke, 1974). Because the lamina is physically interposed between the inner nuclear membrane and chromatin and forms a structure as much as 25–100 nm thick between these components (Fawcett, 1966), it is a plausible site for attachment of chromatin to the nuclear envelope. Supporting this possibility, when nuclei are treated with Triton X-100 to solubilize nuclear membranes, the lamina remains closely associated with peripheral heterochromatin (Aaronson and Blobel, 1974). However, no direct association between chromatin and proteins of the lamina has been demonstrated by biochemical procedures.

In higher eukaryotic cells the lamina consists mainly of one or several related proteins called lamins (Gerace and Burke, 1988). Many mammalian cells contain three major lamins termed lamins A, B, and C (Gerace and Blobel, 1980).

In addition, minor lamin isotypes have recently been identified in both rat and chicken cells (Lehner et al., 1986; Kaufmann, 1989). Molecular cloning and sequencing of cDNAs for lamins from a wide variety of species has revealed that lamins are members of the intermediate filament protein superfamily (McKeon et al., 1986; Fisher et al., 1986; Franke, 1987). Consistent with this sequence information, purified rat liver lamins A and C spontaneously assemble into intermediate filaments and related structures in vitro, and the lamina of *Xenopus* oocytes is an orthogonal array of 10 nm filaments as visualized by EM (Aebi et al., 1986).

Mitosis in higher eukaryotes provides a useful opportunity to investigate the nature and functions of the putative lamina–chromatin interaction, since the nuclear envelope and lamina are disassembled and subsequently reformed during this period (Gerace et al., 1978; Krohne et al., 1978). Lamina disassembly occurs during mitotic prophase, when lamins become dispersed throughout the cell. In mammalian cells, disassembly of the lamina appears to be controlled by hyperphosphorylation of all three lamins (Ottaviano and Gerace, 1985) and possibly by demethylation of lamin B (Chelsky et al., 1987). Disassembled lamins A and C occur as soluble 4–5S protomers, whereas disassembled lamin B remains associated with membranes that are presumed to derive from fragmented nuclear envelopes (Gerace and Blobel, 1980). Reassembly of the nuclear membranes and lamina takes place around the daughter chromosomes during telophase, and is accompanied by lamin dephosphorylation and lamin B meth-

ylation. Since lamina reassembly occurs in juxtaposition to chromosome surfaces, any interaction of the lamina with chromatin most probably would be reestablished at this time.

Recently, cell-free systems have been developed that reproduce major events of nuclear envelope disassembly and reformation during mitosis (Lohka and Masui, 1983; Miake-Lye and Kirschner, 1985; Burke and Gerace, 1986; Suprynowicz and Gerace, 1986; Newport, 1987). A nuclear assembly system based on homogenates of metaphase CHO cells was used to evaluate the role of lamins in nuclear envelope reformation (Burke and Gerace, 1986). Immunological depletion of lamins from this system resulted in substantial inhibition of subsequent nuclear membrane assembly around chromosomes, suggesting that lamins are directly involved in nuclear envelope assembly. However, precise roles of lamins in this process remain to be determined.

To study the putative lamina–chromosome interaction and its role in nuclear reassembly, we developed in vitro assays to analyze directly an interaction of lamins with mitotic chromosomes. We focused this work on lamins A and C, which are present in soluble extracts of mitotic cells and can be highly purified from rat liver nuclear envelopes. We found that lamins A and C specifically bind to mitotic chromosome surfaces and assemble into a supramolecular structure, and that lamin assembly is strongly promoted by chromosome surfaces. These results support the hypothesis that the lamina directly influences interphase chromosome structure and nuclear reassembly.

Materials and Methods

Cell Culture and Synchrony

CHO cells were maintained in Joklik's medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT) and 100 U/ml penicillin-streptomycin (Gibco Laboratories) at 37°C in 95% air/5% CO_2. The cells have a doubling time of ∼16–18 h under these conditions and were routinely passaged after reaching a maximum density of 6–8 × 10^5/cm². For mitotic synchrony, cells were seeded at an initial density of 5 × 10^3/cm² in 1,750-cm² roller bottles containing 500 ml Joklik's medium. After three days the medium was removed and replaced with 200 ml medium containing 2 mM thymidine (Sigma Chemical Co., St. Louis, MO). 11 h later the thymidine-containing medium was removed, the cells were washed once with PBS (10 mM sodium phosphate pH 7.4, 140 mM NaCl), and 150 ml of fresh medium was added to the bottles. After 4 h nocodozale (Calbiochem-Behring Corp., La Jolla, CA) was added to a final concentration of 0.1 μg/ml, and metaphase cells were collected by mechanical shake-off 3–5 h later. This procedure yields a >95% mitotic index and ∼6 × 10^7 mitotic cells per roller bottle.

Preparation of Metaphase Cell Homogenate (S140)

The mitotic cells were washed twice in ice-cold PBS, resuspended in an equal volume of isotonic homogenization buffer (KHM) (50 mM Hepes-KOH pH 7.0, 70 mM KCl, 3 mM $MgCl_2$, 1 mM DTT) and disrupted by Dounce homogenization with a tight-fitting pestle at 4°C. The homogenization was monitored by microscopy and, after complete disruption of cells, the suspension was spun at 2,000 rpm in a centrifuge (model J-6B; Beckman Instruments, Fullerton, CA). The supernatant was then centrifuged at 140,000 g for 20 min in a rotor (model 70 Ti; Beckman Instruments) yielding a postmembrane supernatant (S140). The S140 was used directly or frozen in liquid nitrogen and stored at −80°C until use. The protein concentration was 25–30 mg/ml as determined by the method of Bradford (1976) using BSA as a standard. The concentration of lamins in the homogenate was estimated by quantitatively immunoadsorbing with affinity-purified rabbit antilamin antibodies. The immunoadsorbed proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue. The amount of absorbed protein in lamin bands was determined by laser-scanning den-sitometry using BSA, phosphorylase B and ovalbumin as standards (Bio-Rad Laboratories, Richmond, CA). Similar results were obtained by quantitative immunoblot analysis of the S140, using purified rat liver lamins A and C as standards, the concentration of which was determined by the method of Bradford (1976).

Chromosome Isolation

Chromosomes were isolated from metaphase CHO cells by a modification of the procedure described by Mitchison and Kirschner (1984). Cells were swollen in 50 ml hypotonic buffer (10 mM Pipes-KOH pH 7.0, 5 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA) for 10 minutes at 4°C, pelleted at 1,000 rpm in a centrifuge (model J-6B; Beckman Instruments) and resuspended in 7 ml lysis buffer (10 mM Pipes-KOH pH 7.0, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 0.1% digitonin). Cells were then disrupted by Dounce homogenization with a loose-fitting pestle until most chromosomes were released from broken cells (typically involving 30 strokes). The homogenate was then spun for 1 min at 600 g to remove clumps and the supernatant was layered over a 20-ml 20–60% sucrose step gradient in 10 mM Pipes-KOH pH 7.0, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA. The gradients were spun at 1,200 g for 15 min, whereupon most chromosomes had sedimented to the 50–60% interface. The chromosomes were removed from this zone using a Pasteur pipette, the concentration was determined by absorbance at 260 nm and adjusted to 2.0–6.0 OD_{260} U/ml by dilution with KHM buffer. They were stored at −80°C after freezing in liquid nitrogen. Chromosomes were also isolated in homogenization buffers where the $MgCl_2$ had been replaced by 0.3 mM spermine and 0.75 mM spermidine, without noticeable differences in yield.

Lamin Purification

Rat liver lamins were purified by a modification of the procedure described by Aebi et al. (1986). All chromatography steps were performed at room temperature. The lamina-enriched pellet generated by 2% Triton X-100, 0.5 M KCl extraction of rat liver nuclear envelopes was resuspended at ∼2.5 mg/ml in buffer A (20 mM Tris-HCl pH 8.8, 8 M urea, 1 mM DTT, 1 mM PMSF, and 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin), sonicated briefly, and then agitated for 1 h at 4°C. The suspension was centrifuged at 10,000 g for 10 min and the supernatant passed over a 1.0-ml DEAE-cellulose column (DE-52; Whatman, Inc., Clifton, NJ) followed directly by a 1.0-ml phosphocellulose column (P-11; Whatman, Inc.) at 0.5 ml/min. Lamins A and C were eluted from the phosphocellulose column using a linear 0–400 mM KCl gradient in buffer A. The purified lamins A and C, eluting at ∼200 mM NaCl, were stored at a concentration of 0.2–1.0 mg/ml in buffer A at 4°C, and dialyzed into 20 mM Tris-HCl pH 8.8, 250 mM KCl, 1 mM DTT before use for assembly.

In Vitro Lamin Assembly

Lamin assembly using metaphase S140 was carried out by diluting the S140 to various concentrations in KHM buffer in the presence or absence of chromosomes and incubating samples at 33°C for up to 2 h. Chromosome concentration was usually 1.0–3.0 OD_{260}/ml and the reaction volumes were 50–200 μl. Samples were taken at appropriate times and processed either for immunofluorescence or SDS-PAGE for lamin quantitation (see below). Assembly reactions performed with 3 μg/ml lamins A and C and 2.5 OD_{260}/ml chromosomes resulted in a maximum of ∼30% of the total lamins bound to chromosomes after 2 h of incubation at 33°C, when the total amount of pelletable lamins had reached a plateau. Progressively increasing the chromosome concentration >2.5 OD_{260}/ml at 3 μg/ml lamins results in a corresponding increase in the percentage of lamins that enter a chromosomal pellet. At least 70% of lamins appear to be assembly competent by this assay. At 3 μg/ml lamins, under standard assay conditions, ∼1 μg of lamin protein was chromosome bound per OD_{260} unit of metaphase chromosomes.

Assembly of purified rat liver lamins A and C was carried out by initially suspending lamins and chromosomes at appropriate concentrations in 20 mM Tris-HCl pH 8.8, 1 mM DTT, 5 mM $MgCl_2$, 70 mM KCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 5 mg/ml BSA (fraction V; ICN Biochemicals, Cleveland, OH) in a volume of 50–150 μl. The samples were then placed in individual wells of a 12-well microdialyzer (Pierce Chemical Co., Rockford, IL) and dialyzed against buffer consisting of 50 mM Hepes-KOH pH 7.4, 1 mM DTT, 5 mM $MgCl_2$, 70 mM KCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin, at 37°C. We chose this

dialysis method because initial experiments showed that dilution of the lamins, at concentrations of >5 μg/ml, from dissociating conditions (20 mM Tris-HCl pH 8.8, 250 mM KCl; Aebi et al., 1986) directly into assay buffer conditions resulted in the rapid formation of paracrystalline structures that are incapable of forming structures associated with chromosomes.

3-μm-diam carboxylate and amino derivatized microspheres (Polysciences, Inc., Warrington, PA) as well as histone and DNA-histone-coupled microspheres were used to analyze a possible interaction of lamins with inert charged surfaces. Histone modified microspheres were prepared by cross-linking histone (type II-S; Sigma Chemical Co.) to amino microsphere particles using 5 mM disuccinimidyl suberate at a ratio of 160 μg histone per 0.3 ml of a 2.5% microsphere suspension. The reaction was incubated at 22°C for 30 min and terminated by the addition of 100 mM triethanolamine pH 7.4. DNA-histone beads were prepared by incubating histone-modified microspheres with 5 mg/ml pBR322 DNA for 30 min, followed by centrifugation at 10,000 g for 5 min in a minifuge (Beckman Instruments). Approximately 0.3 OD units of DNA were bound to 30 μl of histone microsphere suspension. Each lamin assembly assay contained 30 μl of microsphere suspension in a final volume of 100 μl KHM buffer.

Quantitation of Lamin Assembly and SDS Gel Analysis

To determine the amount of insoluble assembled lamins, after incubation for 2 h samples were spun at 10,000 g in a Beckman minifuge-12 for 5 min. Pellets were then washed once with 500 μl of KHM buffer, solubilized in SDS gel sample buffer (2.0% SDS, 150 mM Tris-HCl pH 8.8, 10% sucrose, 10 mM DTT) and separated by SDS-PAGE according to the method of Laemmli (1970). After electrophoretic transfer onto nitrocellulose (Burnette, 1981), lamin proteins were detected using anti-lamin rabbit sera, followed by ^{125}I-labeled protein A (10^6 cpm/ml). The bands revealed by autoradiography were excised and counted in a gamma counter (LKB Instruments, Gaithersburg, MD). Purified lamins of known concentration were run in parallel lanes to generate a standard curve for estimation of lamin mass in the test samples. Two dimensional gel electrophoresis was performed as previously described (Burke and Gerace, 1986), except that ampholytes (LKB Instruments) 5-7 and 3.5-9.5 were used in a 1:1 ratio.

Micrococcal Nuclease Treatment

To extract chromatin after lamin-chromosome association, assembly reactions were carried out using either rat liver lamins A and C or metaphase S140, 0.3 OD$_{260}$ units of metaphase chromosomes and 3 μg/ml lamins in a final volume of 100 μl. After 2 h at 33°C, 1 mM CaCl$_2$ (final concentration) and 50 units of micrococcal nuclease (Calbiochem-Behring Corp.) were added, and samples incubated for a further 15 min at 33°C. The KCl concentration was adjusted to 0.5 M, and the samples processed immediately for either immunofluorescence or immunoblot analysis.

For analysis of nuclease sensitivity of lamin binding sites, chromosomes were treated with micrococcal nuclease before the addition of S140 by resuspending 0.2 OD$_{260}$ units of chromosomes in 100 μl KHM buffer containing 1 mM CaCl$_2$ and 50 U of micrococcal nuclease. After digestion at 33°C for 1 min, nuclease activity was inhibited by the addition of EGTA to a final concentration of 10 mM. S140 was then added to the tubes (final lamin concentration of 4 μg/ml), samples incubated for 2 h at 33°C and then processed for immunofluorescence microscopy. Parallel samples were centrifuged at 10,000 g for 5 min, the pellets were subjected to SDS gel electrophoresis and stained with Coomassie blue.

Protease Treatment

Chromosomes were resuspended in KHM buffer at a concentration of 0.25 OD$_{260}$/50 μl to which TPCK-trypsin (Sigma Chemical Co.; stock solution, 1 mg/ml in KHM buffer) was added to a final concentration of 0.5 μg/ml. After incubation at 23°C for various times the trypsin was inactivated by the addition of a 10-fold excess by weight of soybean trypsin inhibitor (Sigma Chemical Co.).

Immunofluorescence Microscopy

Samples were centrifuged for 30 s at 1,000 rpm onto 18 × 18 mm glass coverslips in a centrifuge (model J6-B; Beckman Instruments) equipped with a J55.2 rotor (Beckman Instruments). The samples were then fixed for 4 min with 3% formaldehyde (Polysciences, Inc.) in PBS. The coverslips were washed three times with PBS containing 0.2% gelatin, and incubated

with rabbit antiserum against lamins A and C (1:100 dilution in PBS containing 0.2% gelatin) for 30 min. After three washes with PBS the samples were incubated for 30 min with rhodamine-conjugated goat anti-rabbit IgG (Cappel Laboratories, Malvern, PA). After three washes with PBS, samples were incubated with the DNA-specific Hoechst dye #33258 (Calbiochem-Behring Corp.) at a concentration of 1 μg/ml in PBS. Samples were examined using a Zeiss Axiophot microscope equipped with epifluorescence optics, and photographs taken using Kodak T-Max film at ASA 400.

Results

Association of Lamins A and C with Chromosome Surfaces in the Absence of Membranes

Previous studies using lysates of mitotic CHO cells to study nuclear envelope assembly suggested that when lamin B is immunodepleted from the assembly system, nuclear membrane assembly is greatly reduced, whereas lamins A and C continue to interact with the surfaces of chromosomes (Burke and Gerace, 1986). To further characterize lamins A and C association with chromosomes, we prepared a fractionated nuclear assembly system containing mitotic chromosomes mixed with a membrane-free postmicrosomal supernatant. A postmicrosomal supernatant (S140) of mitotic CHO cell homogenates contained greater than 95% of lamins A and C while the microsomal pellet contained almost all of lamin B (Fig. 1 A) as expected from previous fractionation studies (Gerace and Blobel, 1980; Burke and Gerace, 1986).

When a metaphase S140 (either undiluted or diluted to as low as 2.5 μg/ml lamins) was incubated with metaphase chromosomes at 33°C, lamins A and C completely coated the chromosome surfaces in a time-dependent fashion as visualized by immunofluorescence microscopy (Fig. 1 B). After 1 h of incubation some chromosomes had a patchy distribution of lamins at their surfaces, while others were completely surrounded by lamins. After 2 h, every chromosome in the reaction mixture was uniformly coated with lamins. Longer incubation did not lead to any detectable changes in this pattern even though the reaction contained excess lamins relative to chromosome binding sites (see Materials and Methods). No membranes were present at the chromosome periphery at the end of the incubation as seen by thin-section electron microscopy as expected from the absence of membranes in the S140 fractions (data not shown). These data show that lamins A and C associate with chromosome surfaces in vitro in the absence of membranes and lamin B. Chromosomes are uniformly coated with lamins and no distinct chromosomal regions (e.g., centromeres or telemeres) appear to be preferred initiation sites for lamin interaction.

Telophase-like dephosphorylation of lamins occurred in mitotic cytosol during a 33°C incubation, as shown by two-dimensional isoelectric focusing/SDS-PAGE (Fig. 2). Similar dephosphorylation occurred if chromosomes were present (data not shown). Initially, lamins A and C migrated as the hyperphosphorylated acidic isoforms of metaphase cells (containing 2-3 mol of phosphate per mol of lamin; Ottaviano and Gerace, 1985) (Fig. 2 A). However, after two hours of incubation at 33°C, the lamins migrated as more basic isoforms characteristic of the interphase dephosphorylated lamins. Thus, lamins become efficiently dephosphorylated by phosphatases present in mitotic cytosol, in a time frame that roughly corresponds to assembly around chromosomes. These
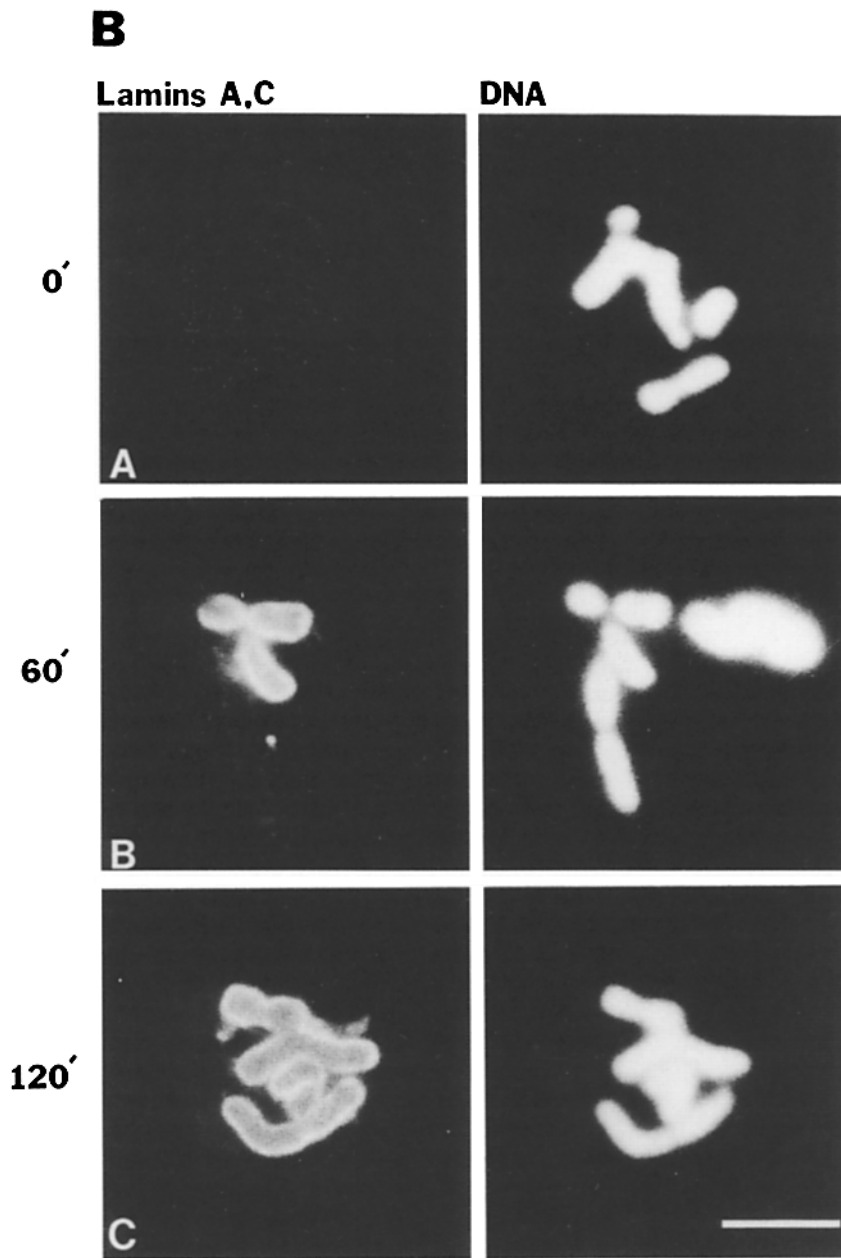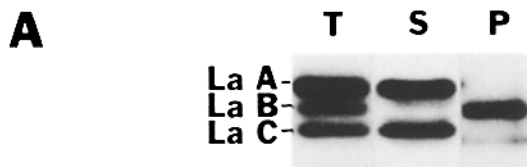
Figure 1. Association of lamins A and C with metaphase chromosomes in vitro. (*A*) Metaphase CHO cells were homogenized in KHM buffer and the homogenate was centrifuged at 140,000 *g* to yield a post-microsomal supernatant and microsomal pellet. Samples then were electrophoresed on an SDS gel and processed for immunoblotting with lamin A-, B-, and C-specific antibodies (*A*). Shown are samples of initial homogenate (*T*), postmicrosomal supernatant (*S*), and microsomal pellet (*P*). Lamins are indicated (*La A, B, C*). (*B*) A postmicrosomal supernatant of metaphase CHO cells was diluted in KHM buffer containing metaphase CHO chromosomes to yield a final lamin concentration of 3 μg/ml. Samples were incubated at 33°C for the times indicated and then processed for immunofluorescence microscopy using lamin-specific antibodies (lamins A, C) or the DNA-specific dye Hoechst #33258 (DNA). Bar, 5 μM.

data further support the notion that lamin dephosphorylation promotes lamina assembly (Gerace and Blobel, 1980; Burke and Gerace, 1986).

The association of lamins A and C with chromosomes described above may be due to a direct binding of lamins themselves to chromosomes, or may be mediated by other soluble proteins present in the homogenate that act as "linkers." To distinguish between these possibilities, we analyzed whether purified rat liver lamins A and C could associate with metaphase chromosomes without soluble components present in a metaphase post-microsomal supernatant. A solution of rat liver lamins A and C (Fig. 3 *A*) at 3 μg/ml in a buffer of alkaline pH and high salt that maintains lamin solubility was mixed with chromosomes, and immediately dialyzed against a solution of approximately physiological pH and ionic strength before analysis of lamin assembly by im-
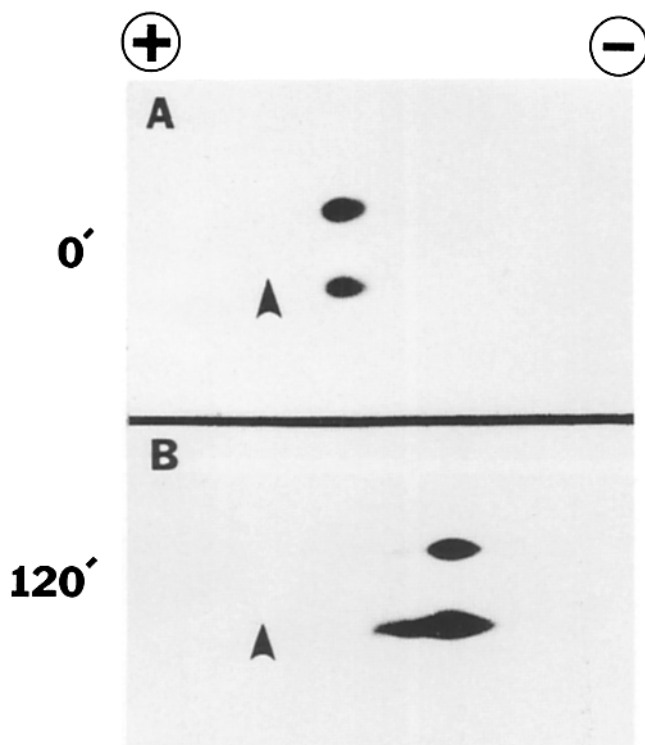
Figure 2. Dephosphorylation of lamins A and C in a metaphase post-microsomal supernatant. Metaphase CHO cell S140 was diluted into KHM buffer to yield a final lamin concentration of 3 μg/ml and incubated at 33°C. Samples were taken before incubation (0') (A) and after 120' of incubation (B), separated by two-dimensional electrophoresis and processed for immunoblot analysis using rabbit antibodies against lamins A and C. The position of BSA determined by Ponceau S-staining of the nitrocellulose filters is indicated by the arrow.

munofluorescence microscopy. After 15 min of dialysis (Fig. 3 B) lamins were distributed in a patchy fashion on the chromosome surfaces. After 30 min, when the pH had equilibrated to 7.4, all chromosome surfaces were uniformly coated with lamins, similar to results obtained when a metaphase S140 was used as a source of lamins (compare Fig. 1 B, C; with Fig. 3 B, C). Incubation for longer times did not result in any changes in the lamin distribution as detected by this immunofluorescence assay (data not shown) even though substantial quantities of unassembled lamins remained. These data show that lamins A and C themselves can associate with chromosomes without any soluble accessory proteins.

Assembly of Lamins into a Structure around Chromosomes

Association of lamins with chromosome surfaces may reflect binding of lamin protomers to chromosomes without assembly into a supramolecular structure or, alternatively, formation of a lamina-like structure that can remain intact after chromatin is removed. To distinguish between these possibilities, chromosomes were incubated for two hours with either purified rat lamins A and C or diluted metaphase S140 at a lamin concentration where all chromosome surfaces become coated with lamins. Samples then were digested with micrococcal nuclease and extracted with 0.5 M NaCl to solubilize

the chromatin. Whereas these conditions completely extracted histones (Fig. 4 B), lamins were not appreciably solubilized when either purified rat liver lamins A and C or metaphase S140s (Fig. 4 A) were used for assembly. Distinct chromosome silhouettes resembling the size and shape of intact chromosomes were visible by phase-contrast microscopy after chromatin extraction (Fig. 4 C). These "chromosome ghosts" contained lamins as seen by immunofluorescence microscopy, yet did not stain with a DNA-specific dye (Fig. 4 C), confirming that most chromatin had been extracted. Based on these operational criteria, a lamina-like supramolecular structure is assembled around chromosomes during the course of these reactions.

Effect of Lamin Concentration on Lamin–Chromosome Association

To investigate whether chromosome surfaces promote (i.e., facilitate) lamin assembly into a supramolecular structure, we analyzed the concentration dependence for appearance of lamins in a form pelletable at 10,000 g in the absence or presence of chromosomes (see Materials and Methods). Assembly of lamins into insoluble structures was determined after 120 min of incubation, when the amount of insoluble lamins had reached a plateau in samples either lacking or containing chromosomes (data not shown).

When purified rat lamins at a concentration of 3 μg/ml or less were incubated in the absence of chromosomes (Fig. 5 B) no lamins were detected in the pellet. At and above 4 μg/ml, there was a linear increase in the amount of insoluble lamins with increasing lamin concentration (Fig. 5 B, closed symbols). These insoluble structures are lamin paracrystals as seen by electron microscopy (data not shown), similar to previous observations (Aebi et al., 1986). Thus, purified lamins appear to have a critical concentration for forming insoluble structures, similar to other intermediate filament proteins. A similar critical concentration for lamin assembly of ∼4 μg/ml was found in an appropriately diluted S140 fraction (Fig. 5 A). Lamins remaining in the 10,000-g supernatant after assembly with either mitotic S140 or purified lamins sedimented at 4–5S on sucrose velocity gradients (data not shown), as expected for unassembled protomers (Gerace and Blobel, 1980; Aebi et al., 1986).

When purified lamins or diluted metaphase S140s were incubated in the presence of chromosomes, lamins entered an insoluble structure at significantly lower concentrations than in samples lacking chromosomes (Fig. 5, open symbols). Association of lamins with chromosomes was first measured at ∼0.5 μg/ml, and reached an initial plateau between 2–3 μg/ml. Above 4 μg/ml, a further increase in insoluble lamins was seen, paralleling the appearance of insoluble lamins in samples lacking chromosomes.

When these samples were visualized by immunofluorescence microscopy, chromosomes incubated with 0.5–2 μg/ml lamins had a patchy lamin coating (Fig. 5 C). Chromosomes acquired a complete lamin coating when incubated with ∼2.5 μg/ml lamins (Fig. 5 C; and Figs. 1 C and 3 C), corresponding to the plateau in amounts of pelletable lamins. When chromosomes were incubated with lamins at concentrations above the critical concentration for self-assembly (i.e., >4 μg/ml), they also became uniformly coated with lamins (Fig. 5 C). In addition small insoluble bodies that were
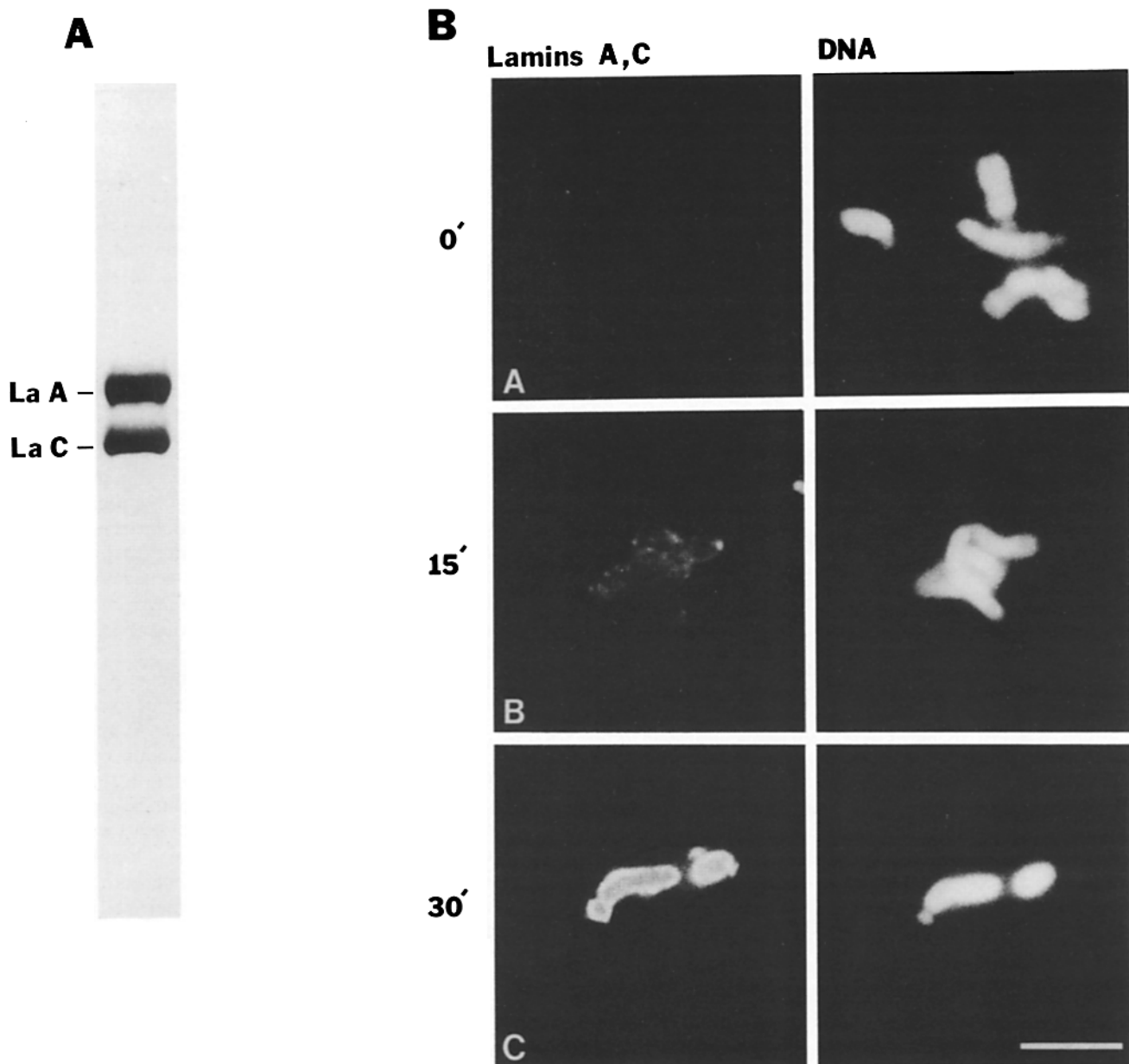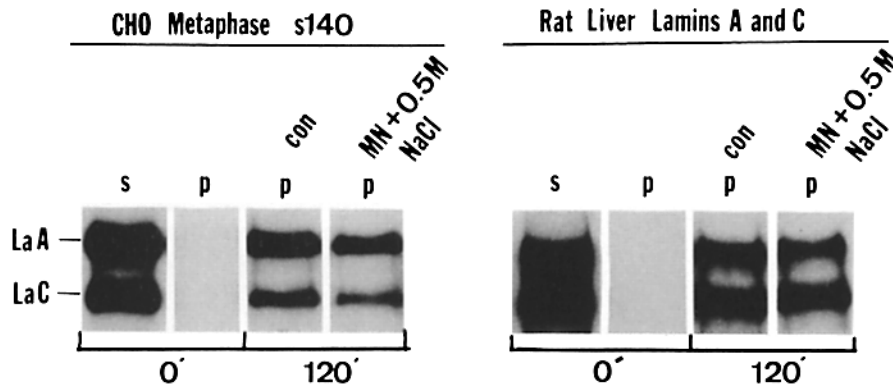
Figure 3. Association of purified rat liver lamins A and C with metaphase chromosomes in vitro. (*A*) Purified lamins A and C from rat liver nuclear envelopes were electrophoresed on a SDS gel and stained with Coomassie blue. A representative lamin preparation used in the assembly assay is shown. (*B*) rat liver lamins A and C were diluted to 3 μg/ml in pH 8.8 buffer containing metaphase chromosomes, and samples were dialyzed against pH 7.4 buffer for the indicated times. Samples were then processed for immunofluorescence microscopy using lamin-specific antibodies (lamins A, C) or the DNA-specific dye Hoechst #33258 (DNA). Bar, 5 μM.

not associated with chromosomes (representing lamin paracrystals) were visible by immunofluorescence microscopy at these high lamin concentrations (data not shown). Whatever the concentration of lamins used for assembly, lamin-containing structures never extended outward from surfaces of lamin-coated chromosomes as seen by immunofluorescence microscopy (e.g., Fig. 1 *B, C*; Fig. 5 *C*) and EM (not shown). This indicates that lamin assembly involves periodic and frequent binding sites on chromosome surfaces and that chromosome surfaces are not simply nucleating assembly of lamin filaments that grow outward from chromosomes.

Loss of Lamin Assembly Alteration of Native Chromosome Structure

We investigated the chromosomal binding site(s) that promote lamin assembly by modifying chromosome structure with a variety of different procedures. Limited tryptic digestion of the chromosomes resulted in inhibition of lamina association with the chromosome surfaces when diluted S140 (Fig. 6 *A*) or purified lamins (data not shown) were used for assembly. After two minutes of digestion, only ~60% of the chromosomes became coated with lamins (Fig. 6 *A, B*). Af-

A. Immunoblot

CHO Metaphase s140

Rat Liver Lamins A and C
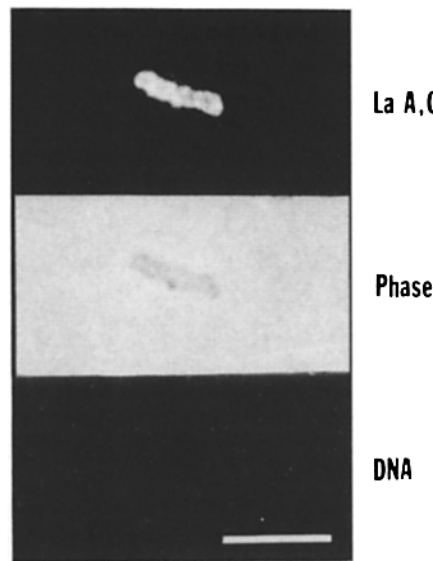

B. Coomassie Blue
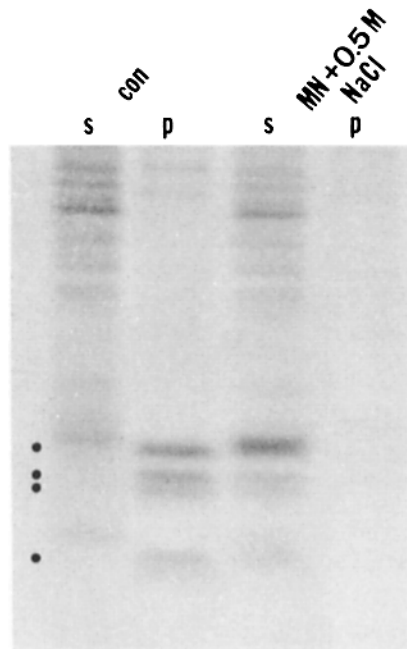
C. Microscopy

Figure 4. Formation of a supramolecular lamina structure at chromosome surfaces. (*A*) Purified rat liver lamins A and C, or metaphase CHO cell S140s were diluted to a final lamin concentration of 3 μg/ml and incubated for 120 min with 2.0 OD_{260} U/ml metaphase chromosomes. After 0 min or 120 min of incubation, samples were centrifuged at 10,000 g to yield supernatants (*S*) and pellets (*P*). Some samples that had been incubated for 120 min were digested with micrococcal nuclease and extracted with 0.5 M NaCl before centrifugation (*MN + 0.5 M NaCl*); other samples (*CON*) were centrifuged without nuclease digestion and salt extraction. Samples then were subjected to SDS gel electrophoresis and immunoblot analyses to detect lamins A and C. Shown are the supernatants and pellets after 0 min incubation, and pellets only after 120 min incubation. (*B*) Samples were subjected to SDS gel electrophoresis and stained with Coomassie blue. Core histones are indicated by dots to left of the first lane. (*C*) A sample of chromosomes incubated for 120 min with CHO S140 was digested with micrococcal nuclease, extracted with 0.5 M NaCl, and labeled for immunofluorescence microscopy with antibodies to lamins A and C (*lamins A, C*) or the DNA-specific dye Hoechst #33258 (*DNA*). Bar, 10 μM.

ter 5 min of trypsin treatment (Fig. 6 *A, C*) or longer (data not shown) no chromosomes became coated with lamins. Analysis of chromosomal proteins by SDS gel electrophoresis after 5 min of trypsin digestion showed that the bulk of histone was not degraded (Fig. 6 *B*), although chromosomes become visibly "swollen" (Fig. 6 *A*), indicating that their structure was perturbed. These data show that the capacity of chromosomes to direct lamin assembly disappears coincident with alteration of higher order chromosome structure by trypsinization.

As an alternative method to modify chromosome structure we briefly digested chromosomes with micrococcal nuclease, which converts chromosomes to large round masses of chromatin (Fig. 7). Despite these structural changes the majority of chromosomal proteins were still present in a pelletable form (Fig. 7 *B*). When incubated with diluted S140 (Fig. 7) these digested chromosomes showed very little association of lamins with their surfaces (Fig. 7 *A, + MN*).

These results suggest that native chromosome structure is required for lamin assembly in these systems. Lamins do not associate with chromosomes due to nonspecific aggregation with charged macromolecules at chromosome surfaces, since limited treatment of chromosomes with either trypsin or micrococcal nuclease abolishes lamin assembly even though most constituents of chromosomes are still retained. Further demonstrating this point, we found that in vitro lamin assembly with mitotic S140 or purified lamins does not occur around 3 μm diameter microsphere beads that are negatively or positively changed, or 3 μm beads coupled with histone or histone and DNA (data not shown, see Materials and Methods).
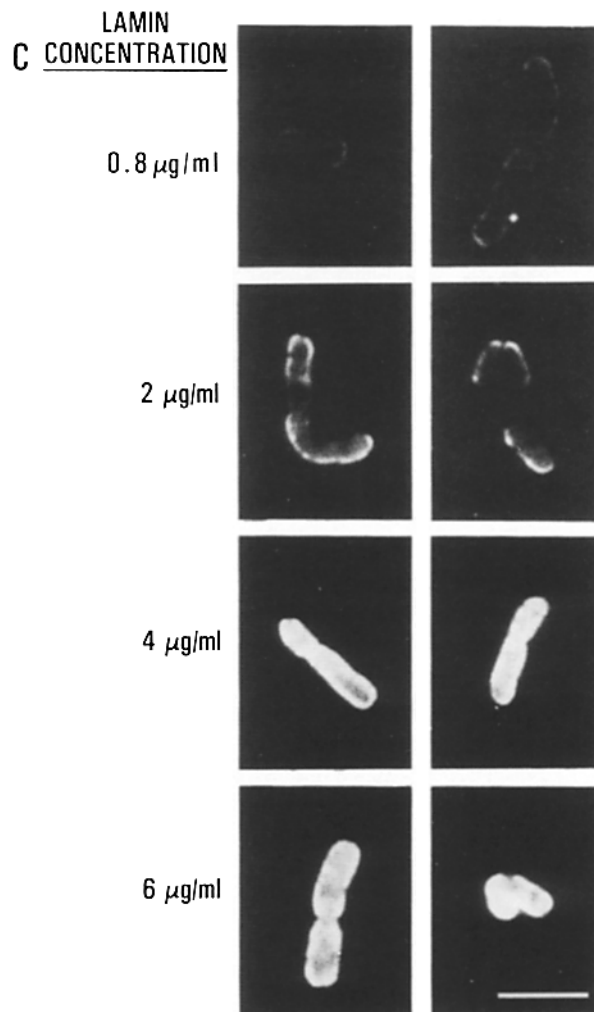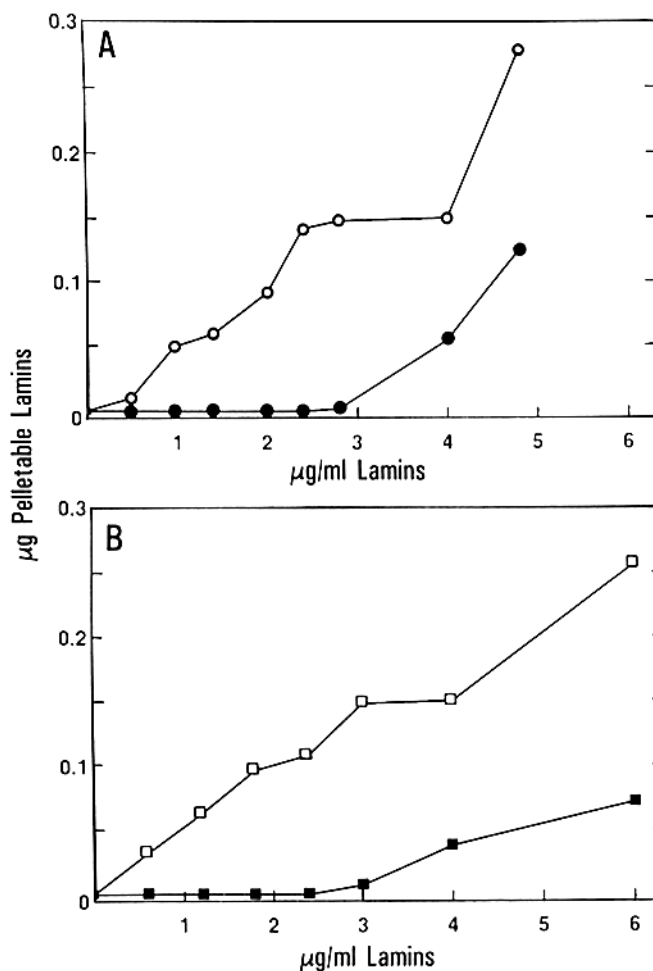
Figure 5. Dependence of self-assembly and association with chromosomes on the lamin concentration. Metaphase S140 (*A*) or rat liver lamins A and C (*B*) were adjusted to the concentrations indicated either in the presence (*open symbols*) or absence (*closed symbols*) of 2.5 OD_{260} U/ml of metaphase CHO chromosomes. After incubation for 120 min samples were centrifuged at 10,000 *g* for 5 min and amount of lamins in the pellets was determined by quantitative immunoblot analysis. *C* shows the immunofluorescence pattern of lamins at the surface of mitotic chromosomes when chromosomes were incubated with metaphase S140 for 120 min at 33°C at the lamin concentrations shown. An indistinguishable pattern was seen when rat liver lamins were used in the assay. Bar, 5 μM.

Discussion

Assembly of Lamins A and C at Chromosome Surfaces In Vitro

In this study we have used two systems to analyze an association of nuclear lamins with mitotic chromosomes. The first of these involves incubating an S140 fraction from mitotic cells with metaphase chromosomes. In essence, this is a fractionated in vitro nuclear assembly system that lacks membranes and lamin B. Burke (1990) has recently described a similar assay in which lamin–chromosome association can be measured in the absence of membrane assembly. The second system involves dialysis of purified rat lamins A and C from dissociating conditions into a buffer that permits assembly in the presence of mitotic chromosomes. In both systems structures containing lamins A and C are formed at the chromosome surfaces that appear very similar by microscopy, stability upon subsequent nuclease/high salt extraction of chromatin, and dependence of assembly on lamin concen-

tration. Together, these data show that lamins A and C themselves directly interact with mitotic chromosomes in vitro.

The major difference between lamin assembly occurring with mitotic S140 compared to purified lamins is the rate of assembly at the chromosome surfaces. When the metaphase S140 is used as a source of soluble lamins, complete lamin coating of chromosomes requires between 1 and 2 h. In contrast, chromosome coating is complete by 30 min when purified rat liver lamins are used. This difference in rate may reflect differences in the initial phosphorylation state of lamins in the two samples. Metaphase lamins A and C are hyperphosphorylated, and presumably must become dephosphorylated to become assembly competent (Burke and Gerace, 1986). The dephosphorylation of mitotic lamins that occurs in vitro may be rate limiting for assembly. In contrast, rat liver lamins initially have a lower level of phosphorylation since they are purified from interphase nuclei, and assemble into filaments and related structures as soon as they are dialyzed from chemical dissociating conditions into buffers of more physiological pH and salt (Aebi et al., 1986).
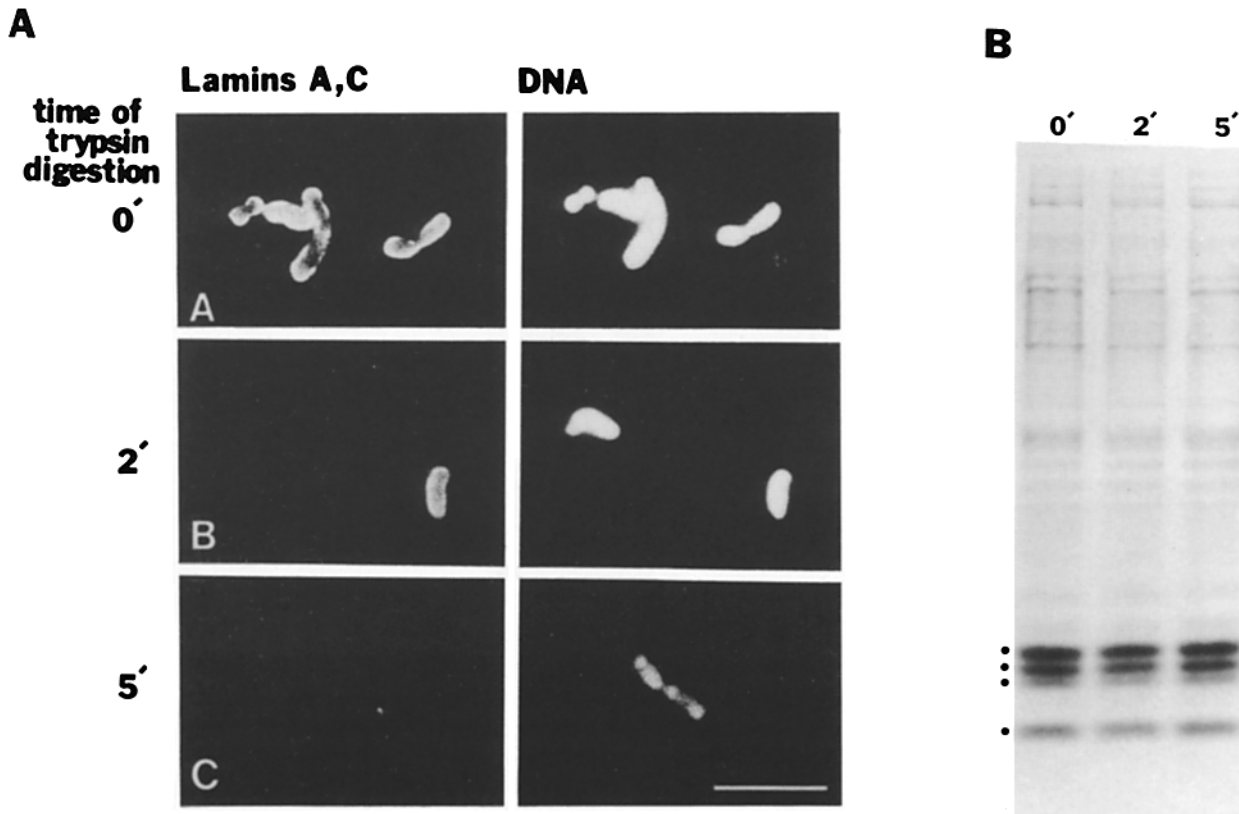
Figure 6. Treatment of chromosomes with trypsin inhibits assembly of lamins A and C. (*A*) CHO chromosomes were digested with trypsin for the times indicated, metaphase S140 was added to a final concentration of 3 μg/ml, and samples were incubated for 120 min at 33°C. The samples were then processed for immunofluorescence microscopy to visualize lamins A and C or DNA. Bar, 10 μM. (*B*) Parallel samples of chromosomes that had been digested with trypsin were mixed with S140 and incubated for 2 h at 33°C. After centrifugation at 10,000 *g*, pellets were subjected to SDS gel electrophoresis and stained with Coomassie blue. Dots to the left of the first lane indicate the core histones.

Association of lamins A and C with chromosomes in these assays results in the formation of an operationally defined supramolecular structure containing lamins that is resistant to solubilization when chromatin is extracted by nuclease digestion followed by high salt treatment. By these criteria the lamin-containing structure formed in vitro is similar to the native nuclear lamina. However, these two structures cannot be identical since the native nuclear lamina contains lamin B and other nuclear envelope components (Gerace and Burke, 1988; Kaufmann, 1989; Senior and Gerace, 1989). We have not successfully visualized the structures formed from lamins during these reactions by EM.

Assembly of lamins A and C at chromosome surfaces occurs at concentrations up to eightfold lower than the apparent critical concentration for lamin self-assembly in the absence of chromosomes, which is ∼4 μg/ml. This suggests that chromosome surfaces promote lamin assembly, as well as spatially direct this process. Chromosomal binding sites for lamins apparently become saturated at ∼2.5 μg/ml lamins based on immunofluorescence and biochemical assays. This value represents a lamin dimer concentration of ∼20 nM, indicative of a high-affinity lamin–chromosome interaction. At and above this concentration, chromosomes become completely coated with lamins. Taken together these data indicate that chromosome surfaces are preferred sites for lamin assembly in vitro. This supports the notion that a lamin–

chromosome interaction in part specifies targeting of lamins to chromosomes during nuclear reassembly in telophase (see below).

The lamin-containing structure that assembles at chromosome surfaces in vitro is always closely associated with chromosomes and does not extend outward as seen by light or EM whatever the lamin concentration in the assembly reactions. Therefore, chromosomes do not merely provide nucleating sites for assembly of lamin filaments. Rather, multiple interactions of the lamin-containing structure with the surface of chromosomes probably are essential for coating of chromosomes with lamins. Thus, chromosome-directed lamin assembly may be a cooperative process mediated by both lamin–lamin and lamin–chromosome interactions. However, this cooperativity would not necessarily be apparent in our measurements due to limitations in our assay and possible heterogeneity of binding affinities at different regions of the chromosome surface.

In preliminary studies, we found that preparations of lamin B purified from rat liver nuclear envelopes do not associate with the surfaces of mitotic chromosomes, unlike purified lamins A and C (Glass, J., and L. Gerace, unpublished). Furthermore, the lamin B preparation does not assemble into filaments and paracrystals as described for lamins A and C (Aebi et al., 1986). This does not necessarily mean that lamin B lacks the ability to bind to chromosomes or assem-
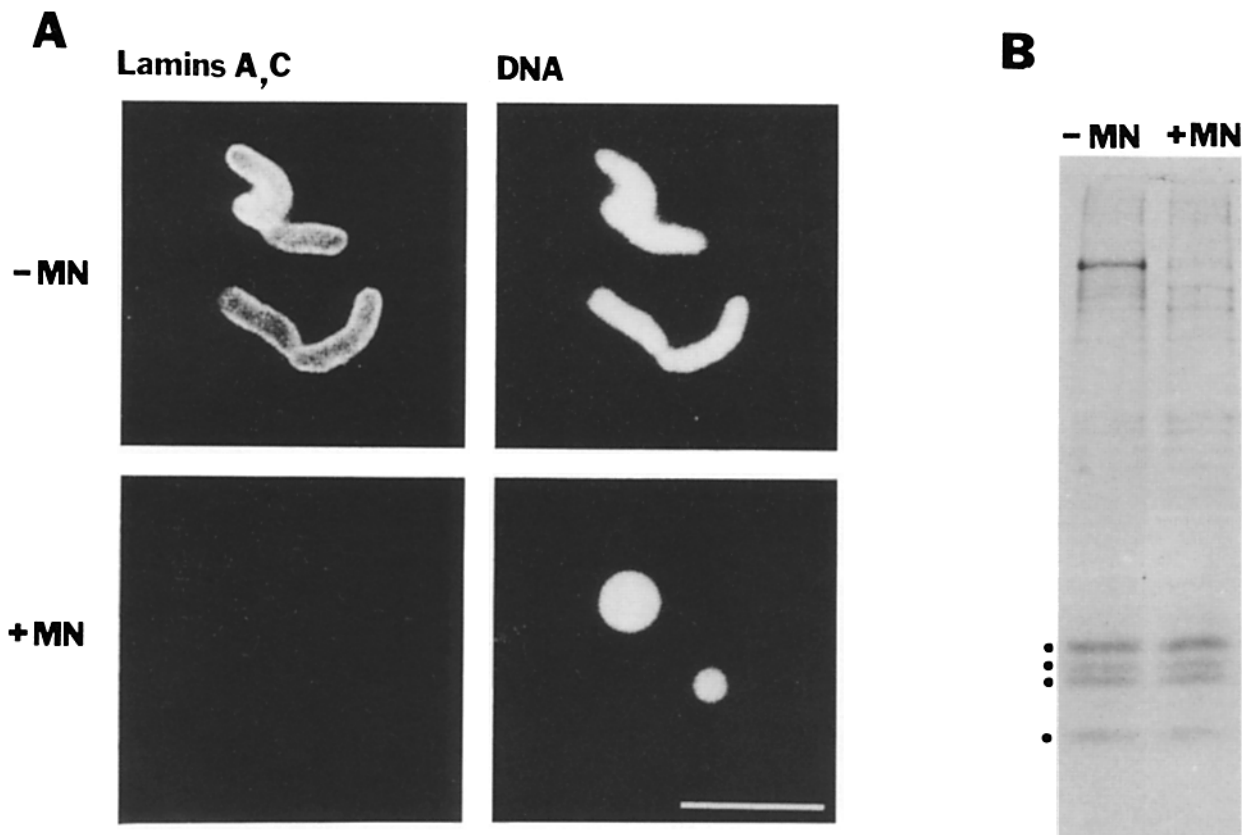
Figure 7. Treatment of chromosomes with micrococcal nuclease inhibits assembly of lamins A and C. (*A*) Chromosomes were treated with micrococcal nuclease, and metaphase cell S140 was added to yield a final lamin concentration of 4 μg/ml. After 2 h of incubation at 33°C, samples were processed for immunofluorescence microscopy to visualize lamins A and C or DNA. Bar, 10 μM. (*B*) Parallel samples of micrococcal nuclease-digested (*+MN*) or untreated (*−MN*) chromosomes were mixed with metaphase S140 and incubated for 2 h. After centrifugation at 10,000 *g* for 5 min, pellets were subjected to SDS gel electrophoresis and stained with Coomassie blue. The SDS-PAGE protein profile from many samples of chromosomes treated with micrococcal nuclease shows no significant selective loss of any protein band. Dots to the left of the first lane indicate the core histones.

ble into filaments, since the lamin B in these preparations is not native. Lamin B is carboxy-methylated in vivo (Chelsky et al., 1987), and becomes demethylated by alkaline treatment (see also Ottaviano and Gerace, 1985). Furthermore, native lamin B is membrane associated during mitosis, unlike the purified protein. Perhaps lamin B requires methylation, a structural change that occurs when it is bound to membranes, or unknown cofactors that promote chromatin binding and filament formation.

Nature of the Lamin Binding Site in Chromosomes

In principle, the lamin binding site on the chromosome may be either protein, nucleic acid or a nucleic acid–protein complex. Histones, DNA, or the nucleosome are attractive candidates for lamin binding sites because they are globally present on the surface of mitotic chromosomes. Alternatively, lamin binding may involve more minor proteins concentrated at the chromosome periphery, such as peripherin (Chaley et al., 1984) or perichromin (McKeon et al., 1984). If the lamin binding site were DNA (see Lebkowski and Laemmli, 1982), specific DNA sequences could be preferred for binding (e.g., satellite DNA of heterochromatin; however, see Newport, 1987).

Whatever the nature of the lamin binding site, it is plausi-

ble that it must be present in a topologically defined fashion on the chromosome surfaces to promote lamin assembly. Limited digestion of chromosomes with trypsin or micrococcal nuclease abolishes chromosome-directed lamin assembly. These treatments extract or degrade different components of chromosomes, yet both disrupt the higher order structure of mitotic chromatin. Indeed, the proper presentation of lamin binding sites for chromosome-directed assembly may be determined by higher order chromatin folding. Thus, the heterochromatin that comprises mitotic chromosomes and that is associated with the nuclear periphery during interphase may contain an appropriate array of lamin binding sites, while interphase chromatin that is not associated with the nuclear envelope may lack this array.

Functions of Lamin–Chromatin Interaction

Since nuclear envelope reformation during telophase occurs in juxtaposition to all exposed surfaces of telophase chromosome masses, it is likely that interactions between chromosome surfaces and specific nuclear envelope components are important for membrane assembly. The ability of chromosome surfaces to promote assembly of lamins A and C described in this paper supports the idea that formation of the lamina around chromosomes in vivo during anaphase and

telophase is important for directing assembly of nuclear membranes (Burke and Gerace, 1986). According to this model, coordinated assembly of lamins A and C together with the membrane-bound lamin B at the chromosome surfaces would target nuclear membrane vesicles to chromosomes. In cell types that lack lamins A and C (Guilly et al., 1987; Stewart and Burke, 1987), membrane assembly could be directed by possible binding of lamin B or other minor lamin isotypes (Kaufmann, 1989; Lehner et al., 1986) to chromatin. This model specifically predicts that if the chromatin binding of all lamins (major and minor) were disrupted, nuclear membranes would not associate with chromosomes at the end of mitosis.

Lamin–chromatin interactions could be important for interphase chromosome structure as well as nuclear envelope reformation, assuming that a subset of the interactions formed during telophase are subsequently maintained. Anchoring of chromosomes to the interphase lamina could help to maintain chromosomes in separate domains of the nucleus as recently described for *Drosophila* (Hochstrasser et al., 1986; Hochstrasser and Sedat, 1987) and human cells (Manuelidis and Borden, 1988) to prevent them from becoming entangled during the course of interphase metabolism (transcription and replication). In addition, the lamina may actually influence chromatin structure since it could provide an array of binding sites for chromatin at the nuclear periphery (see above). The number or affinity of these sites may be altered by the developmentally controlled expression of lamins A and C, which occurs only after differentiation in many cell types (Rober et al., 1989; Stewart and Burke, 1987) when an increased amount of heterochromatin is observed (Johns, 1988). The assays described in this paper should permit identification of chromosomal binding sites for lamins and the specific region of the lamin molecules involved in this binding. This could further illuminate how lamins influence higher order chromatin structure and regulate telophase nuclear envelope assembly.

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