

A Chromatin Binding Site in the Tail Domain of Nuclear Lamins That Interacts with Core Histones

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Abstract. Interaction of chromatin with the nuclear envelope and lamina is thought to help determine higher order chromosome organization in the interphase nucleus. Previous studies have shown that nuclear lamins bind chromatin directly. Here we have localized a chromatin binding site to the carboxyl-terminal tail domains of both A- and B-type mammalian lamins, and have characterized the biochemical properties of this binding in detail. Recombinant glutathione-S-transferase fusion proteins containing the tail domains of mammalian lamins C, B₁, and B₂ were analyzed for their ability to associate with rat liver chromatin fragments immobi-

lized on microtiter plate wells. We found that all three lamin tails specifically bind to chromatin with apparent K_d s of 120–300 nM. By examining a series of deletion mutants, we have mapped the chromatin binding region of the lamin C tail to amino acids 396–430, a segment immediately adjacent to the rod domain. Furthermore, by analysis of chromatin subfractions, we found that core histones constitute the principal chromatin binding component for the lamin C tail. Through cooperativity, this lamin–histone interaction could be involved in specifying the high avidity attachment of chromatin to the nuclear envelope *in vivo*.

THE nuclear lamina is a filamentous protein meshwork that lines the nucleoplasmic surface of the nuclear envelope (NE)¹ (reviewed by Gerace and Burke, 1988; McKeon, 1991; Nigg, 1992; Georgatos et al., 1994). The lamina is thought to provide a structural framework for the NE and an anchoring site at the nuclear periphery for interphase chromosomes, and therefore could play a major role in interphase nuclear organization. The lamina consists of a polymeric assembly of nuclear lamins, members of the intermediate filament (IF) protein superfamily (see McKeon, 1991; Nigg, 1992), as well as a number of less abundant lamina-associated polypeptides (discussed by Gerace and Foisner, 1994). Vertebrate lamins are classified as A- or B-subtypes based on their sequence and biochemical properties. B-type lamins (lamins B₁ and B₂) are present in somatic cells throughout development, while A-type lamins (lamins A and C) are expressed only during or after terminal differentiation in most cells. Mam-

malian lamins A and C appear to be alternative splice products of the lamin A gene. They are identical for the first 566 amino acids, and contain unique carboxyl termini of 98 residues and 6 residues, respectively. By contrast, the two mammalian B-type lamins of somatic cells are the products of separate genes (Hoger et al., 1990). Additional alternative splice variants of the lamin A and lamin B₂ genes have been characterized in mammalian germ cells (Furukawa and Hotta, 1993; Furukawa et al., 1994).

Like other IF proteins, lamins consist of a central α -helical rod domain flanked by an NH₂-terminal “head” domain and a COOH-terminal “tail” domain (see McKeon, 1991; Nigg, 1992; Heins and Aebi, 1994). The head domain of vertebrate lamins is usually \sim 30 amino acids, while the tail domain is typically \sim 180–275 residues. The basic lamin protomer is a homotypic dimer formed by parallel, unstaggered association of the rod domain to form a two-stranded coiled-coil α -helix. This subunit, which forms a rod-like structure with a length of \sim 50 nm, engages in both head-to-tail and lateral interactions to form lamin filaments and related structures with an axial periodicity of \sim 25 nm (Aebi et al., 1986; Gieffers and Krohne, 1991; Heitlinger et al., 1991; Moir et al., 1991). Thus, discrete regions of the lamin molecules (e.g., tail domains) are displayed at repeating intervals along lamin filaments. *In vitro* assembly studies with mutant lamins have shown that both the rod domain and head and tail domains are involved in filament formation (Gieffers and Krohne, 1991; Moir et al., 1991; Heitlinger et al., 1992).

The major functions of the lamina are likely to involve

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1. *Abbreviations used in this paper.* GST, glutathione-S-transferase; IF, intermediate filament; NE, nuclear envelope; NLS, nuclear localization sequence.

interactions with the inner nuclear membrane and chromatin, and these associations are beginning to be characterized in detail. Attachment of the lamina to the inner membrane is thought to involve lamin-binding integral membrane proteins (reviewed by Gerace and Foisner, 1994), and COOH-terminal isoprenylation of certain lamins (reviewed by McKeon, 1991; Nigg, 1992). A close association of the lamina with chromatin is suggested by structural studies (see Belmont et al., 1993 and references therein), and may preferentially involve discrete chromosomal regions (Hochstrasser and Sedat, 1987). Since recent *in vitro* binding studies have demonstrated specific interactions of lamins with various chromatin substrates (Burke, 1990; Glass and Gerace, 1990; Hoger et al., 1991; Yuan et al., 1991; Glass et al., 1993), the lamina-chromatin interaction is likely to involve lamins themselves. In addition, chromatin-binding integral membrane proteins of the lamina such as LAP2 (Foisner and Gerace, 1993; Furukawa et al., 1995) may participate in this association.

We previously found that the α -helical rod domain of lamins A/C contains a specific chromatin binding site, as measured by *in vitro* association of the recombinant rod domain with mitotic chromosome surfaces (Glass et al., 1993). In this study, we have demonstrated that a second chromatin binding region occurs in the tail domains of both A- and B-type lamins. Using solid phase binding to chromatin isolated from rat liver nuclei, we found that the tail domains of lamins C, B₁, and B₂ all bind specifically to isolated chromatin fragments, with apparent *K*_s of 120–300 nM. Furthermore, we mapped the chromatin binding region of the lamin C tail to an \sim 30-amino acid region flanking the COOH-terminal end of the rod domain, and showed that core histones are the major chromatin component responsible for the binding of this region. These results are discussed in relationship to the role of the lamina in higher order chromatin structure and the attachment of specific chromosomal regions to the NE *in vivo*.

Materials and Methods

Expression of Fusion Proteins and Recombinant Lamin Fragments in *Escherichia coli*

cDNA clones for human lamins A and C were provided by the laboratory of Dr. G. Blobel (Rockefeller University, NY), and cDNA clones for mouse lamins B₁ and B₂ were obtained from Dr. G. Krohne (University of Würzburg, Germany). Lamin fragment sequences were amplified by PCR, using synthetic oligonucleotide primers flanked by sequences for restriction enzyme sites for subcloning into the expression plasmid pGEX2T (Pharmacia LKB Biotechnology, Piscataway, NJ).

Polypeptides were expressed in the *E. coli* strain BL21 (Novagen, Inc., Madison, WI). Single colonies from transformed bacteria were grown to an A₆₀₀ of 0.5–0.7 at which point expression was induced by addition of 2 mM IPTG. Bacterial growth was continued another 2 h. Purification of GST fusion proteins was performed as described by Smith and Corcoran (1990). Bacterial cell pellets from 500-ml cultures were resuspended in 25 ml PBS (10 mM sodium phosphate, pH 7.4, 140 mM NaCl) containing 0.2 mM PMSF. Resuspended cells were sonicated with a Branson Sonifier for five 30-s periods on ice. Triton X-100 was added to 1%, and the solution was spun at 12,000 g for 10 min. The supernatant was incubated with 1.25 ml glutathione-agarose beads for 30 min at room temperature. Beads were then washed three times with PBS and fusion proteins were eluted by three sequential incubations with 5 ml of 50 mM Tris, pH 8.0, 200 mM NaCl, 5 mM reduced glutathione. Purified fusion proteins were stored at -20°C .

Isolation and Fractionation of Chromatin

Chromatin fragments were isolated from rat liver nuclei as described by Noll et al. (1975) and Zentgraf and Franke (1984). Rat liver nuclei, isolated as described (Gerace et al., 1982), were resuspended at 0.4 A₂₆₀ U/ml in 0.34 M sucrose, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM Tris, pH 7.5, 15 mM 2-mercaptoethanol. The suspension was brought to 1 mM CaCl₂ and digested with micrococcal nuclease (15 unit/ml) for 30 s at 37°C. Digestion was terminated by chilling on ice and addition of 0.1 M EDTA, pH 7.0, to a final concentration of 0.01 M. The nuclei were then centrifuged for 5 min at 4,000 g, and lysed by suspension in 0.2 mM EDTA, pH 7.0. Next, 0.5-ml samples were loaded on top of a 10–50% linear sucrose gradient containing 10 mM Tris 7.4, 100 mM NaCl, 1 mM EDTA, 0.2 mM PMSF. Centrifugation was performed at 4°C in a SW41 rotor (Beckman Instrs., Inc., Fullerton, CA) for 90 min at 40,000 rpm. Peak fractions with an absorbance at 260 nm were pooled and stored at -80°C .

For fractionation of chromatin, chromatin fragments were made 0.6 M NaCl and loaded on 10–50% linear sucrose gradients in 10 mM Tris, pH 7.4, 0.6 M NaCl, 1 mM EDTA, and 0.2 mM PMSF. Fractions at the top of the gradient were pooled to yield a histone H1-enriched fraction, and fractions sedimenting with the major nucleic acid peak were pooled to yield a core histone/DNA fraction. For isolation of core histones, chromatin fragments were applied to a hydroxylapatite column in 0.1 M potassium phosphate, pH 7.0, 0.1 M NaCl. After washing with the same buffer containing 0.6 M NaCl to remove H1 and other loosely bound proteins, core histones were eluted by the same buffer containing 2 M NaCl, pooled and stored at -80°C .

Solid-phase Binding Assay

Recombinant polypeptides were labeled with ¹²⁵I using the Iodogen reagent (Pierce Chem. Co., Rockford, IL) according to the manufacturer's instructions. After a 10-min labeling period, polypeptides were separated from free iodide by dialysis into the binding buffer containing 0.2 mM PMSF without BSA. Typical labeling yielded specific activities of 0.5–1.0 $\times 10^6$ cpm/ μg .

Lamin-chromatin binding was determined by a solid-phase binding assay originally described by Nachman and Leung (1982). Chromatin or chromatin subfractions (200 μl) at 10 mg/ml were immobilized in microtiter wells (Immulon 4; Dynatech Labs., Inc., Chantilly, VA) by incubating overnight at 4°C in coating buffer (10 mM Tris, pH 7.5, 100 mM NaCl, and 1 mM EDTA). H1-depleted chromatin and purified core histones (see above) were dialyzed from solutions containing high salt into coating buffer before adsorption to microtiter wells. Before ligand addition, the wells were blocked for 2 h with a binding buffer (20 mM Hepes, pH 7.2, 150 mM NaCl, 5 mM MgCl₂, and 30 mg/ml BSA), and washed once with the same buffer. Radiolabeled lamin fusion proteins were added in the absence or presence of unlabeled competitors and incubated for 2 h at room temperature. After three washes with binding buffer, the radioactivity in each well was measured by gamma counting. Nonspecific background was taken as the amount of ¹²⁵I-labeled ligand that remained bound per well when 2 $\mu\text{g/ml}$ radiolabeled ligand was incubated with a 50-fold excess of nonradioactive competitor. This is a suitable measure of nonspecific background, since a very similar level of background binding as defined in this manner was obtained for the ¹²⁵I-labeled GST-lamin C tail and ¹²⁵I-labeled GST-lamin C tail deletion mutants that were found to be deficient in chromosome binding (Fig. 6). Typical reactions yielded 4,000–6,000 cpm/well specifically bound after incubation with 2 $\mu\text{g/ml}$ radiolabeled GST-lamin tail constructs.

To examine possible binding of lamins to isolated DNA by a solid phase assay, a modified procedure was used to attach the DNA to microtiter wells, which involved adsorption of lightly biotinylated DNA to streptavidin-coated plates. This is because purified DNA did not significantly bind to microtiter wells under the conditions where purified proteins and protein-DNA complexes were efficiently adsorbed (see above). Purified DNA in water was lightly coupled with biotin by mixing with Photoprobe biotin (Vector Labs., Inc., Burlingame, CA) and illuminating for 10 min on ice as recommended by the manufacturer. Free biotin was then separated from the DNA by two extractions with isobutanol, and the DNA was precipitated with ethanol. The biotinylated DNA was then dissolved in water and adsorbed to the surface of Reacti-Bind streptavidin coated polystyrene strip plates (Pierce). Approximately 200 ng DNA per well was bound. Binding of radiolabeled lamin fusion proteins was then analyzed as above.

Individual binding experiments shown in this paper were carried out at

least three times, and the different repetitions yielded very similar results. Data from a single experiment is shown. Data points were taken in duplicates, and presented as the mean with standard deviation. To calculate apparent K_{ds} for the interaction of fusion protein with chromatin, data was expressed in linearizing plots for single site competitive interactions (Hulme and Birdsall, 1992).

Other Procedures

Protein concentrations were determined with a Bio-Rad protein assay kit. SDS-PAGE was performed by the method of Laemmli (1970).

Results

Analysis of Binding of the Lamin Tail Domain to Chromatin with a Solid Phase Assay

We developed a quantitative binding assay to investigate a possible interaction between the tail domains of nuclear lamins and chromatin. Previous studies of the lamin-chromatin interaction have involved intact lamins and lamin fragments containing the α -helical rod domain (Burke, 1990; Glass and Gerace, 1990; Hoger et al., 1991; Yuan et al., 1991; Glass et al., 1993). Since these proteins are insoluble under conditions of physiological salt and pH due to the strong tendency of the rod to self-associate, they are difficult to use for quantitative binding studies. Moreover, they generally require the use of buffers containing denaturants (e.g. urea) for isolation, and their capacity for complete *in vitro* renaturation after these treatments is un-

known. By contrast, we found that the lamin tail domains can be expressed as soluble fusion proteins in *E. coli* and purified under nondenaturing conditions. Since the tail domains do not strongly self associate, it was feasible to use recombinant fusion proteins containing the lamin tail domains for saturation binding analysis with isolated chromatin fragments. We immobilized the chromatin fragments on the surfaces of microtiter wells to analyze binding using a solid phase assay, because the strong tendency of chromatin to aggregate in physiological buffers (van Holde, 1989) made it difficult to carry out reproducible binding assays in solution. By contrast, the solid phase assay was highly reproducible (see Materials and Methods) and permitted the rapid analysis of a large number of samples.

Interphase chromatin used for these binding studies was prepared by micrococcal nuclease digestion of isolated rat liver nuclei followed by sucrose gradient centrifugation of the nuclease-released material (Fig. 1 A). The peak gradient fractions that were pooled and used for the binding studies (fractions 8–10) contained ~6–10-kb fragments of DNA, corresponding to 30–50 nucleosomes (Fig. 1 B). The overwhelmingly major proteins in these fractions were histone H1 and core histones (Fig. 1 C). Tail domains of lamins were expressed in *E. coli* as glutathione-S-transferase (GST) fusion proteins, which were purified from soluble bacterial extracts using glutathione affinity columns. The recombinant proteins that we analyzed were

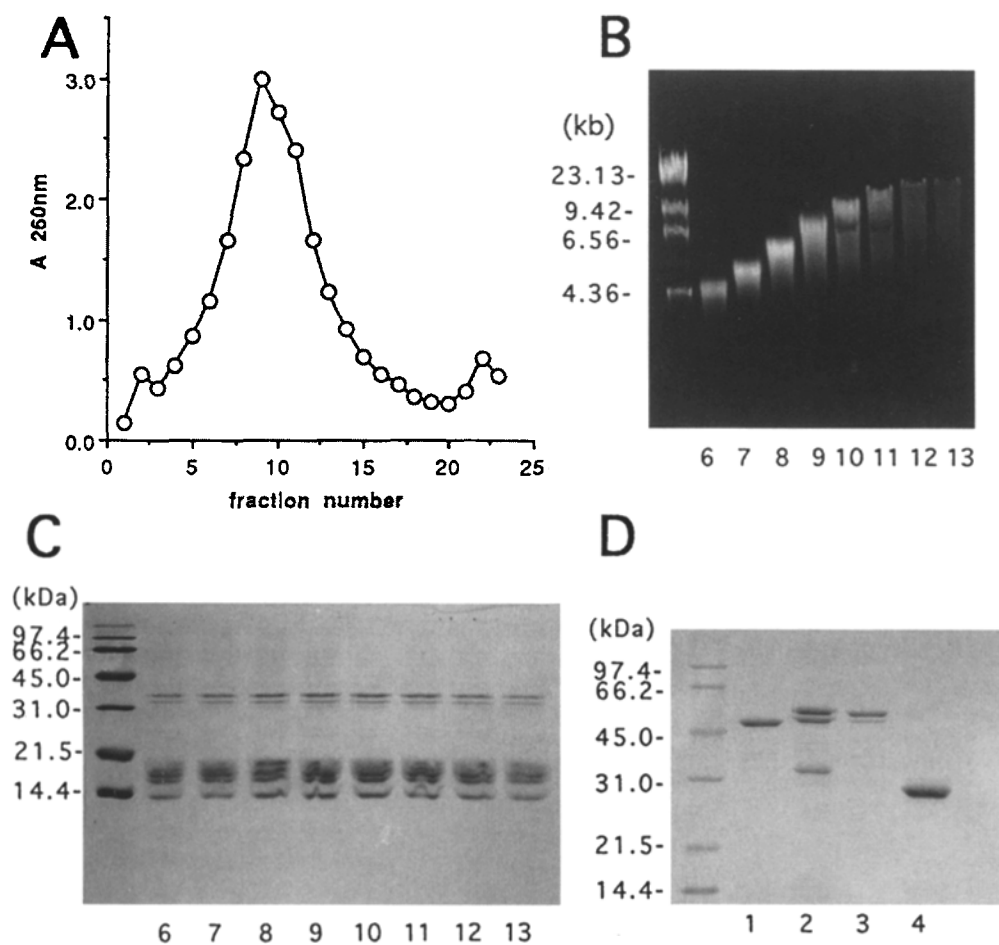


Figure 1. Preparation of interphase chromatin and recombinant fusion proteins containing the lamin tail domains. (A) Sucrose gradient sedimentation of chromatin fragments released from rat liver nuclei by micrococcal nuclease digestion. Chromatin was sedimented on a 10–50% sucrose gradient, and DNA was measured by absorbance at 260 nm. (B) Agarose gel electrophoresis of DNA present in different chromatin fractions. DNA from the peak region of the sucrose gradient (fractions 6–13) was analyzed by electrophoresis on a 0.3% agarose gel. (C) SDS-gel electrophoresis of proteins present in different chromatin fractions. Aliquots of sucrose gradient fractions 6–13 were analyzed on a 15% SDS-polyacrylamide gel, and proteins were visualized by Coomassie blue staining. (D) SDS-gel electrophoresis of purified recombinant GST-lamin tail fusions and GST. About 3 μ g of each protein was analyzed on a 12% SDS-polyacrylamide gel. Lane 1, GST-lamin C tail (389–572); lane 2, GST-lamin B₁ tail (391–588); lane 3, GST-lamin B₂ tail (382–596); lane 4, GST.

GST-lamin C tail (residues 389–572), GST-lamin B₁ tail (residues 391–588), GST-lamin B₂ tail (residues 382–596) and GST alone. The GST and GST-lamin C tail obtained in this fashion were mainly intact and migrated as single major bands at the expected mobilities, while the GST-lamin B₁ tail and GST-lamin B₂ tail preparations contained intact fusion protein and some faster migrating degradation products (Fig. 1 D).

In initial studies where immobilized chromatin was incubated with 2 $\mu\text{g/ml}$ ¹²⁵I-labeled GST-lamin C tail, GST-lamin B₁ tail, or GST-lamin B₂ tail, each of the three proteins bound to the chromatin at significant levels (Fig. 2 A). By contrast, very little radiolabeled GST by itself (<10% of the amount of the lamin tail constructs) associated with chromatin (data not shown). A substantial fraction of the GST-lamin tail binding appeared to be specific, since association of the radiolabeled ligand was competed by ~50% with a 50-fold excess of added nonradiolabeled GST-lamin tail constructs, but was not significantly competed with a 50-fold excess of nonradiolabeled GST (Fig. 2 A). The residual binding obtained with 2 $\mu\text{g/ml}$ radiolabeled ligand and a ~50-fold excess of unlabeled ligand was used as nonspecific background for the calculations in this study (see Materials and Methods).

The binding of the radiolabeled GST-lamin C tail construct to chromatin in the presence or absence of a 50-fold excess of nonradiolabeled competitor was examined under a variety of solution and washing conditions to further characterize this interaction (Fig. 2 B). Binding was similar

in solutions containing either 5 mM MgCl₂, 5 mM EDTA, or 5 mM EGTA, indicating that binding of the lamin tail to chromatin does not require divalent cations. Inclusion of 1% Triton X-100 in the standard assay decreased the specific binding about threefold. By contrast, most binding persisted when wells were washed with 0.25–1.0 M NaCl, and about 70% of the specific binding remained after washing in buffer containing 1.0 M NaCl. Analysis of a time course of the GST-lamin tail binding to chromatin showed that the maximal levels of binding were obtained at ~90–120 min (Fig. 2 C).

We then analyzed in detail the binding of GST-lamin tail constructs to immobilized chromatin by examining the displacement of radiolabeled constructs with increasing concentrations of the corresponding unlabeled polypeptides (Fig. 3). For all three lamin constructs, half maximal displacement of the labeled polypeptide in the concentration range tested (up to ~117 $\mu\text{g/ml}$) was achieved at ~7–12 $\mu\text{g/ml}$ unlabeled competitor. Data from these displacement experiments was expressed as linearizing plots for single site competitive interactions (Hulme and Birdsall, 1992). This analysis indicated that the GST-lamin C tail, GST-lamin B₁ tail and GST-lamin B₂ tail interacted with single classes of sites in chromatin with apparent *K_d*s of 300, 120, and 230 nM, respectively. Since the percentage of native protein in these preparations is unknown, and since the preparations of GST-lamin B₁ tail and GST-lamin B₂ tail contained significant amounts of degradation products as well as intact proteins, these values should be regarded

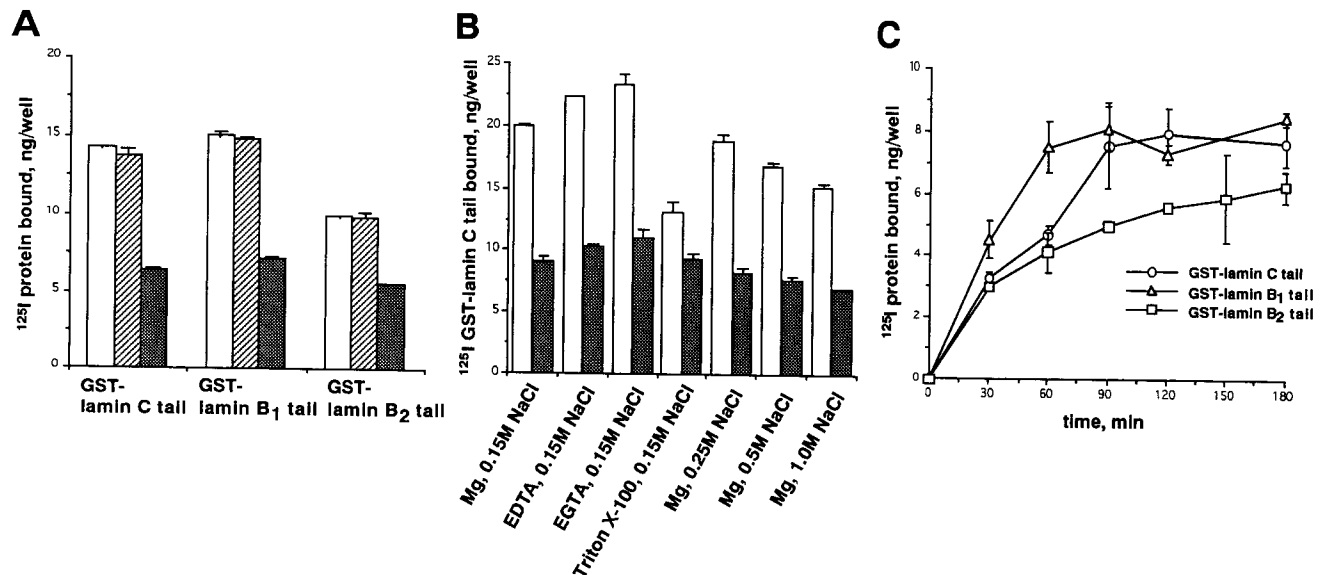


Figure 2. Characterization of lamin tail–chromatin binding. (A) Chromatin binding of ¹²⁵I-labeled GST-lamin C tail, GST-lamin B₁ tail and GST-lamin B₂ tail. ¹²⁵I-labeled fusion proteins (2 $\mu\text{g/ml}$) in binding buffer were incubated in microtiter wells containing immobilized chromatin fragments for 120 min (standard binding assay; see Materials and Methods) in the absence (open bars) or presence (solid bars) of a 50-fold excess of corresponding unlabeled polypeptides, or in the presence of a 50-fold excess of GST (hatched bars). (B) Binding to chromatin of ¹²⁵I-labeled lamin C tail under different solution and wash conditions. 2 $\mu\text{g/ml}$ of ¹²⁵I-labeled GST-lamin C tail was incubated in microtiter wells containing immobilized chromatin fragments for 120 min in the absence (open bars) or presence (solid bars) of a 50-fold excess of the unlabeled GST-lamin C tail under the following conditions: standard binding buffer (Mg, 0.15 M NaCl); solutions containing 5 mM EDTA (EDTA, 0.15 M NaCl) or 5 mM EGTA (EGTA, 0.15 M NaCl) instead of 5 mM Mg²⁺; standard buffer containing 1% Triton X-100 (Triton X-100, 0.15 M NaCl); or standard buffer followed by postincubation washes in 0.25–1.0 M NaCl (Mg, 0.25, 0.5, or Mg, 1.0 M NaCl). (C) Time course of GST-lamin tail binding to chromatin. 2 $\mu\text{g/ml}$ of ¹²⁵I-labeled GST-lamin C, B₁, and B₂ tail was incubated in the standard binding assay for various times. All points were corrected for nonspecific binding by subtraction of values obtained with a 50-fold excess of corresponding unlabeled polypeptides.

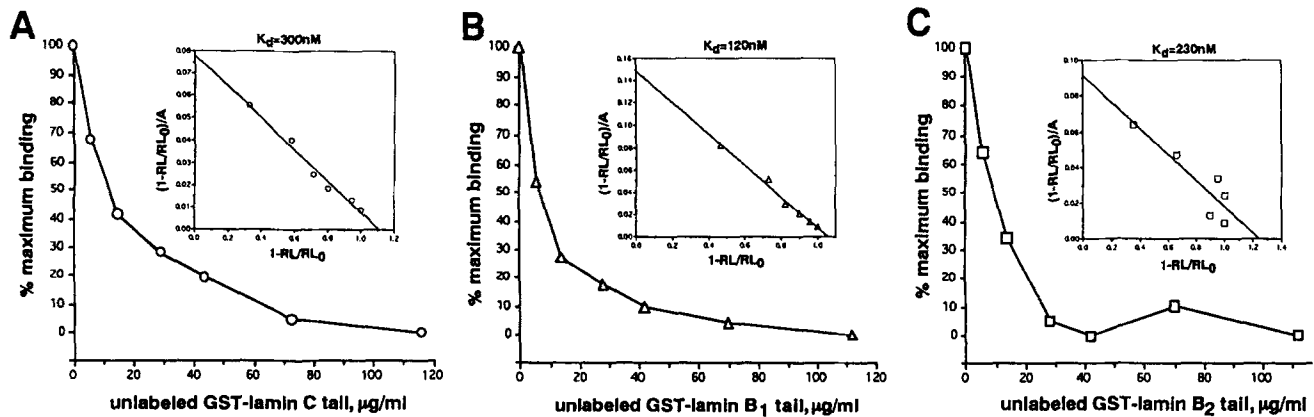


Figure 3. Displacement of ^{125}I -labeled GST-lamin C, B₁, and B₂ tails from chromatin by the corresponding unlabeled polypeptides, and calculation of dissociation constants. 2 $\mu\text{g/ml}$ of ^{125}I -labeled GST-lamin C tail, GST-lamin B₁ tail or GST-lamin B₂ tail (A–C, respectively) were incubated in the standard binding assay in the presence of various concentrations of the corresponding unlabeled GST-polypeptides. Binding at each point was corrected for nonspecific background by subtraction of values obtained with 117 $\mu\text{g/ml}$ unlabeled competitor (see Fig. 2). Insets: Data for specific binding was analyzed by plotting $(1-\text{RL}/\text{RL}_0)/A$ vs. $1-\text{RL}/\text{RL}_0$, where RL is the amount of radioactive fusion protein bound to chromatin at cold competitor concentration A, and RL_0 is the amount of radioactive fusion protein bound in the absence of unlabeled competitor. The slope given by this plot equals $-1/K_d$ (Hulme and Birdsall, 1992).

as approximate. It is not possible to calculate the amount of lamin bound to chromatin at saturation with this solid phase assay, because the fraction of sites within the immobilized chromatin fragments that was active for binding is unknown.

We tried to express the lamin A (389–664) tail as a GST fusion protein in *E. coli*, but found that it was extensively proteolyzed under all conditions tested, especially within the A-specific region. However, we successfully expressed largely undegraded lamin A tail without a fusion partner in insect cells using the baculovirus expression system, and also expressed undegraded lamin C tail without a fusion partner in *E. coli* using a pET vector. These lamin fragments were purified by ion exchange chromatography in the presence of 8 M urea, and were used for binding studies after removal of the urea. In binding displacement experiments, the curves for the lamin A tail and the lamin C tail were very similar to each other. When the data was expressed in linearizing plots for single site competitive interactions (see Fig. 3 legend), K_d s of ~ 400 nM were calculated for both the lamin A tail and lamin C tail (data not shown). The higher apparent K_d for the chromatin binding of the lamin C tail isolated in urea, compared with that for the GST-lamin C construct isolated under non-denaturing conditions, could be explained by incomplete renaturation of the lamin C tail after urea treatment. If the lamin A tail and lamin C tail refold to an active chromatin binding conformation to the same extent, this would indicate that the unique 98 and 6 residue extensions present on lamin A and lamin C, respectively, do not differentially influence chromatin binding.

Mapping the Chromatin-binding Region of the Lamin C Tail

To define the specific region of the lamin C tail involved in chromatin binding, we expressed as GST fusion proteins a series of deletion mutants of the lamin C tail lacking por-

tions of either the amino- or carboxyl-terminal ends (Fig. 4 A). These were purified as largely intact fusion proteins (Fig. 4 B) and were evaluated for their ability to displace a radiolabeled GST fusion protein containing the full-length lamin C tail (389–572) from chromatin, in comparison to the displacement by the unlabeled GST-lamin C tail itself (Fig. 4, C and D). The carboxyl-terminal deletions lamin C (389–480) and lamin C (389–430) had full displacement activity, whereas lamin C (389–416) was almost completely inactive (Fig. 4 B). The amino-terminal deletion lamin C (396–572) resulted in complete displacement, while the amino-terminal deletions lamin C (401–572) and lamin C (415–572) exhibited only partial displacement of binding. A final deletion, lamin C (431–572), had no displacement activity (Fig. 4 C). Together, the results with these amino- and carboxyl-terminal deletion mutants suggest that at least two regions between residues 396–430 of the lamin C tail are important for chromatin binding.

The nuclear localization sequence (NLS) of lamins A/C is present in the tail domain at amino acids 417–422 (KKRKLE). To determine whether the NLS is important for the chromatin binding activity, we prepared several lamin C tail constructs, including amino-terminal tail deletions, from which residues 417–422 were deleted (ΔNLS). These were GST-lamin C (389–572 ΔNLS), GST-lamin C (396–572 ΔNLS), and GST-lamin C (401–572 ΔNLS) (Fig. 5, A and B). Lamin C (389–572 ΔNLS) and lamin C (396–572 ΔNLS) both exhibited only partial displacement of the binding (Fig. 5 C). Combining the amino-terminal deletion of lamin C (401–572), which by itself had lost some displacement activity, with the ΔNLS mutant, which also was partially inactive, yielded the construct lamin C (401–572 ΔNLS) that lost almost all ability to displace the intact lamin C tail from chromatin (Fig. 5 C). These results together indicate that residues 396–401 and 417–422 (the NLS) both have important roles in the chromatin binding activity of the lamin C tail, and that the combined effects of these regions are additive.

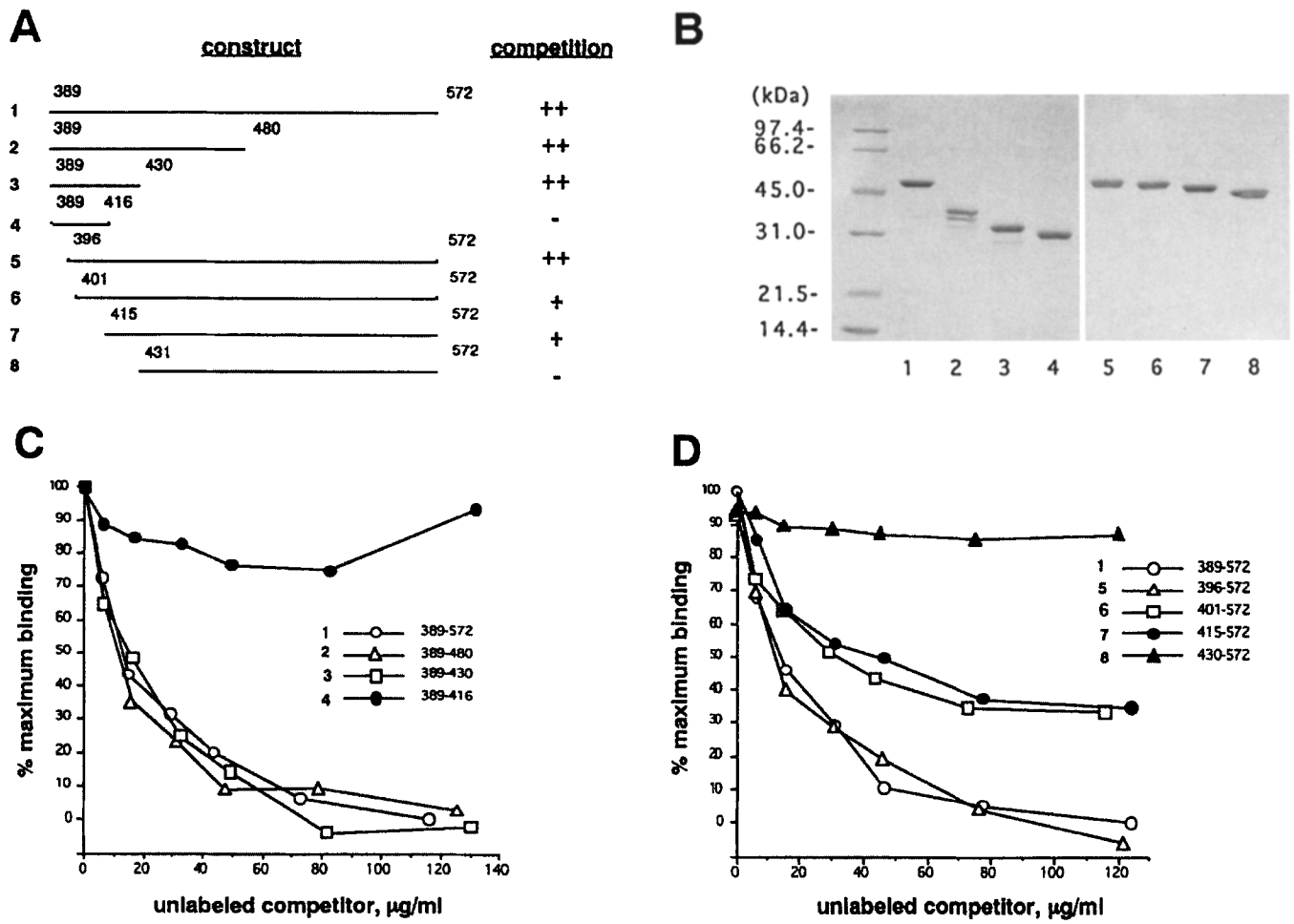


Figure 4. Displacement of ^{125}I -labeled GST-lamin C tail from chromatin by GST fusion proteins containing amino- or carboxyl-terminal deletions of lamin C tail. (A) Schematic diagram of the deletions analyzed. Terminal amino acids are numbered, and their displacement activity is summarized (*competition*). (B) Electrophoresis of purified recombinant GST fusion proteins on a 12% SDS gel. Proteins were visualized by staining with Coomassie blue. Lane numbers correspond to the number of each construct designated in A. (C–D) Binding of ^{125}I -labeled GST-lamin C tail ($2 \mu\text{g/ml}$) to chromatin was measured in the presence of various concentrations of each unlabeled recombinant polypeptide. Binding at each point was corrected for nonspecific background by subtraction of the value obtained with $122 \mu\text{g/ml}$ unlabeled GST-lamin C tail (see Fig. 2). (C) Displacement of the binding with constructs containing carboxyl-terminal deletions. (D) Displacement of the binding with constructs containing amino-terminal deletions.

We further prepared several bidirectional (NH_2 - and COOH -terminal) deletions to test in the binding displacement assay (Figs. 5, A and B). Lamin C (425–480), lamin C (415–480), and lamin C (411–480) were inactive in displacing the lamin C tail from chromatin (Fig. 5 D). By contrast, lamin C (396–480) and even lamin C (396–430) were sufficient for complete displacement of binding (Fig. 5 D). These data are consistent with the results of the amino- and carboxyl-terminal deletions (above), and further demonstrate that the minimal region required for complete displacement of chromatin binding by the lamin C tail in the context of these fusion proteins is residues 396–430.

To complement the results of the binding displacement experiments discussed above, we directly investigated the chromatin binding of several of the deletion mutants (Fig. 6). We radiolabeled GST fusion proteins containing lamin C (389–572), lamin C (389–430), lamin C (415–572), and lamin C (389–572 ΔNLS), and examined association of

these proteins with chromatin in the absence and presence of an excess of each unlabeled polypeptide to correct for nonspecific background (see Fig. 6 legend). The construct containing lamin C (389–430) showed a very similar level of specific chromatin binding compared to the full-length lamin C tail (389–572). By contrast, lamin C (415–572) and lamin C (389–572 ΔNLS), which were only partially active in competing for chromatin binding by the lamin C tail, were significantly reduced in their specific chromatin-binding activity. These results extend the binding competition studies presented above (Figs. 4 and 5) and verify that a short segment near the amino terminus of the lamin C tail contains the chromatin binding site of this lamin domain.

Identification of Core Histones as the Chromatin Binding Site for Lamin C

To identify the specific chromatin component that pro-

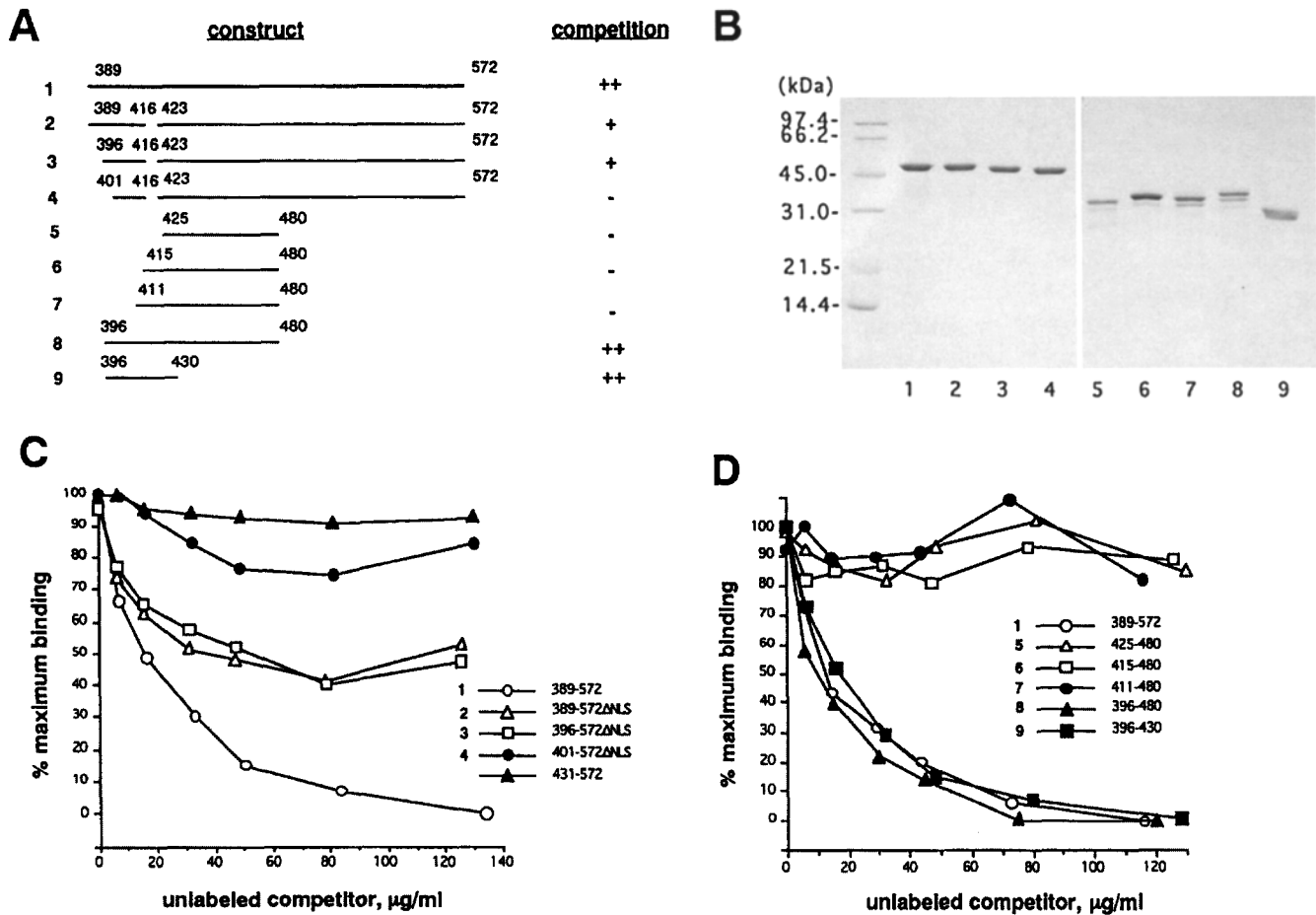


Figure 5. Displacement of ^{125}I -labeled GST-lamin C tail from chromatin by GST fusion proteins containing bidirectional and internal deletions of lamin C tail. (A) Schematic diagram of the deletions analyzed. Terminal amino acids are numbered, and their displacement activity is summarized (*competition*). (B) Electrophoresis of purified recombinant GST fusion proteins on a 12% SDS gel. Proteins were visualized by staining with Coomassie blue. Lane numbers correspond to the number of each construct designated in A. (C–D) Binding of ^{125}I -labeled GST-lamin C tail ($2 \mu\text{g/ml}$) to chromatin was measured in the presence of various concentrations of each unlabeled recombinant polypeptide. Binding at each point was corrected for nonspecific background by subtraction of the value obtained with $122 \mu\text{g/ml}$ unlabeled GST-lamin C tail (see Fig. 2). (C) Displacement of the binding with NLS and amino-terminal combined deletions. (D) Displacement of the binding by various bidirectional deletions.

vides the binding site for the lamin C tail (Fig. 7), we prepared several chromatin subfractions. A histone H1-enriched fraction (which also contained nonhistone chromosomal proteins) and a second fraction consisting of core histones bound to DNA were obtained by treatment of chromatin fragments with 0.6 M salt and sucrose gradient sedimentation (Fig. 7 A, lanes 1 and 2, respectively). A third fraction consisting of purified core histones was obtained by binding chromatin fragments to a hydroxylapatite column followed by salt elution (Fig. 7 A, lane 3; see Materials and Methods). These fractions were separately adsorbed to microtiter wells and analyzed in a binding displacement assay with the radiolabeled GST-lamin C tail (389–572), where the unlabeled competitors were GST fusions containing lamin C (389–572), lamin C (389–430), and lamin C (431–572). It should be noted that no attempts were made to normalize the amount of protein bound to the microtiter wells with the three histone fractions, so the amount of ligand bound to the three fractions cannot be directly compared. Also, raw binding data un-

corrected for nonspecific background is presented for all of the fractions analyzed (Figs. 7, B–D), since a priori we did not know which fraction displayed specific binding.

The radiolabeled lamin C tail construct bound to all three of the histone subfractions (Figs. 7, B and D). Most of the binding to the histone H1-enriched fraction (Fig. 7 B) appeared to be nonspecific, as it was only weakly displaced by lamin C (389–572) and lamin C (389–430). However, since even weak binding displacement was not obtained with lamin C (431–572), which lacks the chromatin binding site, it is possible that the histone H1-enriched fraction contains a specific chromatin binding component (possibly low amounts of contaminating core histones; see below). The high nonspecific background in this case precludes clear-cut conclusions. By contrast, binding of the radiolabeled lamin C tail to the core histone/DNA fraction (Fig. 7 C) and the purified core histone fraction (Fig. 7 D) was strongly displaced by lamin C (389–572) and lamin C (389–430). The degree of binding displacement in these cases was similar to that obtained when binding to intact

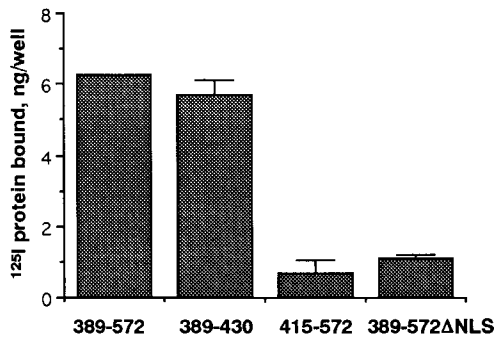


Figure 6. Chromatin binding of GST fusion proteins containing lamin C residues 389–572, 389–430, 415–572, or 389–572 deleted at residues 417–422. Binding of various ¹²⁵I-labeled fusion proteins (2 μg/ml) to chromatin fragments was measured in the standard binding assay. The constructs analyzed were GST–lamin C residues 389–572, 389–430, 415–572, and 389–572 lacking the nuclear localization sequence at residues 417–422 (389–572 ΔNLS). Shown is the specific binding, calculated by subtracting binding in the presence of a 50-fold excess of the corresponding unlabeled polypeptide from the total binding. The nonspecific binding, represented by the amount of ¹²⁵I-labeled ligand that remained bound per well when 2 μg/ml radiolabeled ligand was incubated with a 50-fold excess of the nonradioactive competitor, was nearly identical for all four ligands.

chromatin fragments was analyzed (compare to Fig. 2). Moreover, no displacement was obtained with lamin C (431–572), which also does not displace binding of the lamin C tail to intact chromatin (Fig. 4). The binding of GST–lamin C (389–430) to purified core histones was corrected for nonspecific background, and these data were expressed in a linearizing plot for single site competitive interaction (Hulme and Birdsall, 1992) to calculate a dissociation constant (Fig. 7 E). This analysis revealed a single class of binding sites with an apparent K_d of 320 nM. Thus, the binding specificity and affinity of the lamin C tail for purified core histones is very similar to that for chromatin fragments.

In other experiments, we extracted the DNA from the chromatin fragments used for these binding studies, biotinylated it, adsorbed it to the surface of streptavidin-coated microtiter wells, and then evaluated its ability to bind the radiolabeled lamin C tail (see Materials and Methods). No specific binding of the lamin tail was observed (data not shown), indicating that bulk sequence DNA does not contain detectable binding sites for the lamin C tail. These results, together with the histone fraction binding results discussed above, indicate that core histones are the predominant binding site for the lamin C tail in isolated chromatin. However, it should be noted that our analysis might not detect binding sites in chromatin for the lamin tail that are either low abundance or low affinity.

Discussion

Specific Chromatin Binding of Lamin Tail Domains

Previous work has established that vertebrate nuclear lamins specifically interact with several different chroma-

tin substrates (Burke, 1990; Glass and Gerace, 1990; Hoger et al., 1991; Yuan et al., 1991). In this study we have defined and characterized an interaction between the tail domains of mammalian lamins and rat liver chromatin fragments. Our analysis involved the use of recombinant tail domains expressed as soluble GST fusion proteins. Unlike intact lamins and lamin fragments containing the rod domain, which are insoluble under conditions of physiological salt and pH, the tail domains do not strongly self-associate and therefore could be used for quantitative binding analysis. Our use of soluble fusion proteins also avoided possible complications of protein refolding after solubilization in chemical denaturants such as urea (see below).

Using a solid phase binding assay, we found that the tail domains of lamins C, B₁, and B₂ specifically interact with chromatin, with apparent K_d s of 120–300 nM. Furthermore, by using binding displacement and direct binding analyses with a series of deletion constructs, we mapped the chromatin binding region of the lamin C tail to a 35–amino acid region distal to the rod domain, corresponding to residues 396–430 (Fig. 8). Our studies showed that two amino acid stretches in this segment (residues 396–401 and residues 417–422, the latter comprising the NLS), which both are enriched in basic amino acid residues, are particularly important for the ability of recombinant fusion proteins to compete for the chromatin binding of the intact lamin C tail domain (Fig. 8, *bold sequences*). It is unclear whether these amino acid stretches directly interact with chromatin, or whether they are important for the proper folding of the region containing residues 396–430 into a chromatin-binding structure. The finding that the NLS is important for the chromatin binding of the lamin tail supports the possibility that many NLSs may be multifunctional (discussed in Gerace, 1992). Interestingly, the chromatin binding site in the lamin C tail does not involve the highly charged region at its COOH terminus (residues 551–572), which contains a cluster of eight acidic residues followed by a second cluster of six basic residues.

We have not analyzed the regions of the lamin B tails that are responsible for their chromatin binding, and do not know whether they coincide with the chromatin binding domain of the lamin A/C tail. A comparison of the sequences of human, mouse and chicken lamins A, B₁, and B₂ in the region corresponding to residues 396–430 of human lamins A/C is shown in Fig. 8. The only highly conserved sequence in this region is the NLS (residues 417–422 of human lamins A/C). The region corresponding to residues 396–401 of human lamins A/C contains at least two basic residues in all lamins but varies in their spacing and surrounding residues, while the areas between these sequences and distal to the NLS are enriched in serine/threonine residues but differ considerably in exact sequence.

Using specific histone and DNA fractions isolated from rat liver chromatin, we determined that the specificity and K_d for the binding of the lamin C tail to purified core histones was very similar to that for chromatin fragments. Since the lamin tail did not bind to isolated DNA, we infer that core histones represent the predominant chromatin binding site for the lamin C tail in isolated chromatin. While our analysis showed no specific binding of the lamin

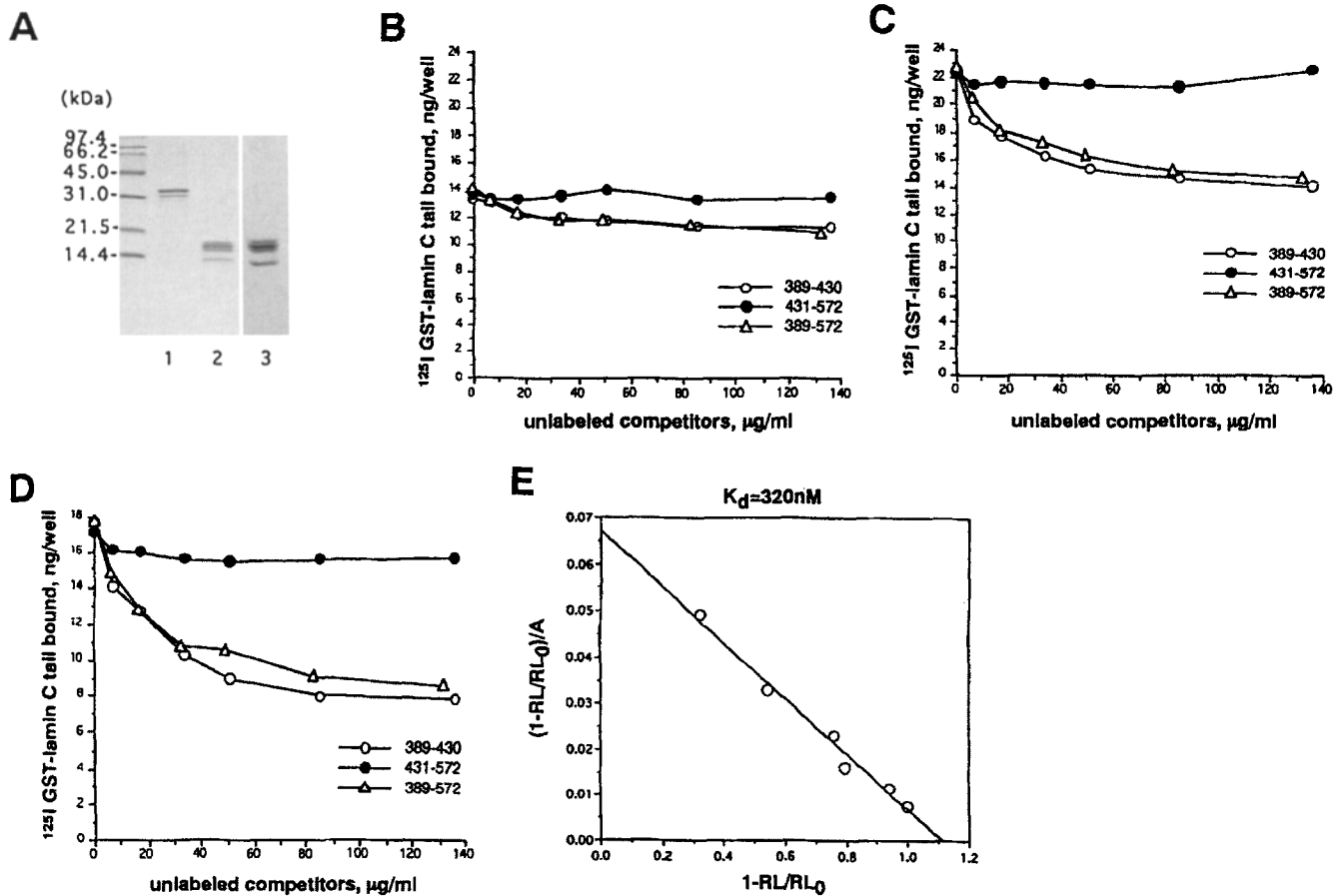


Figure 7. Displacement of ^{125}I -labeled GST-lamin C tail from various chromatin subfractions by GST fusion proteins containing various lamin C tail regions. (A) Protein composition of chromatin subfractions. The histone H1-enriched fraction, core histone-DNA fraction and purified core histones were isolated as described in Materials and Methods. Proteins were analyzed by electrophoresis on a 15% SDS-gel and staining with Coomassie blue. Lane 1, histone H1-enriched fraction; lane 2, core histone-DNA fraction; lane 3, purified core histones. (B–D) Chromatin subfractions were immobilized on microtiter wells in coating buffer, and incubated with ^{125}I -labeled GST-lamin C tail (389–572) at a concentration of 2 $\mu\text{g/ml}$ in the presence of various concentrations of unlabeled GST-lamin C residues 389–572, 389–430, or 431–572. Shown are uncorrected binding data. (B) Displacement of ^{125}I -labeled GST-lamin C tail from the histone H1-enriched fraction by unlabeled fusion proteins. (C) Displacement of ^{125}I -labeled GST-lamin C tail from the core histone-DNA fraction by unlabeled fusion proteins. (D) Displacement of ^{125}I -labeled GST-lamin C tail from purified core histones by unlabeled fusion proteins. (E) Determination of a dissociation constant for the binding of ^{125}I -labeled GST-lamin C (389–430) to purified core histones. The data for each point obtained with ^{125}I -labeled GST-lamin C tail (389–430) in D was corrected for nonspecific background by subtracting the binding obtained in the presence of 125 $\mu\text{g/ml}$ unlabeled GST-lamin C tail. These data were used to plot $(1-\text{RL}/\text{RL}_0)/A$ vs. $1-\text{RL}/\text{RL}_0$ to determine the K_d (see Fig. 3).

C tail to bulk double-stranded DNA, we cannot exclude an interaction with a specific DNA sequence class that is not sufficiently enriched in bulk sequence DNA to be detectable with our assay. We note that lamins and other proteins containing coiled-coiled α -helices, including cytoplasmic intermediate filament proteins, have been found to interact *in vitro* with single stranded DNA (Shoeman and Traub, 1990; Luderus et al., 1994), as well as with A/T-rich SAR/MAR DNA sequences (Luderus et al., 1992), which have a propensity to form single-stranded regions (Kohwi-Shigematsu and Kohwi, 1990). Whether single-stranded SAR/MAR sequences occur *in vivo* is unknown. However, an interaction of SAR/MAR sequences with the nuclear lamina was not observed in studies involving an approximately physiological buffer with divalent cations (Izaurralde et al., 1988).

Relationship to Other Investigations of the Lamin–Chromatin Interaction

In a previous study where chromatin association was assayed by dialysis of solubilized recombinant lamin constructs into a solution of physiological pH and salt in the presence of mitotic chromosomes, we showed that the rod domain of nuclear lamins A/C by itself specifically interacts with chromatin (Glass et al., 1993). However, since association of the rod domain with chromatin occurred with significantly lower efficiency than seen for constructs containing both rod and tail domains (Glass et al., 1993), it was possible that the lamin tail also contributed to chromatin binding. This notion was consistent with studies showing that certain deletions in the tail domains of *Xenopus* lamin A and L₁₁ compromised their ability to associate

Human lamin A/C	389	LSPSPTS	QRSRGR	ASSHSSQTQGGGSVT----	KKRKLE	STESRSSF	430
Mouse lamin A/C	389	LSPSPTS	QRSRGP	ASSHSSQ--GGGSVT----	KKRKLE	SSESRSFF	428
Chicken lamin A/C	388	LSPSPSS	QRGAR-	-SSGLQHS-GAGSA-----	KKRRLE	DGEGRERG	425
Human lamin B ₁	391	LSPSPSS	RVTVSR	ASSRSVRTRG-----	KRKRVD	VEESEASS	429
Mouse lamin B ₁	389	LSPGPSS	RVTVSR	ASSRSVRTRG-----	KRKRID	VEESEASS	427
Chicken lamin B ₁	382	LSPGPSS	RVTVSR	ASSRSVRTRG-----	KRKRID	VEESEASS	428
Mouse lamin B ₂	383	LSPSPSS	RITISR	ATSSSSSSSGVGMVGG	KRRRLE	TEDTSGSP	427
Chicken lamin B ₂		LSPSPSS	RVTVSR	ATSSSSSSSTSLVRSSRG-	KRRRLE	AEELSGSG	

Figure 8. Comparison of the amino acid sequences of different vertebrate lamins near the chromatin binding region of human lamin C (residues 396–430). Shown are sequences from the tail domains human lamins A and C (Fisher et al., 1986), human lamin B₁ (Pollard et al., 1990), mouse lamins B₁ (Hoger et al., 1988) and B₂ (Hoger et al., 1990), chicken lamins B₁ (Peter et al., 1989) and B₂ (Vorburger et al., 1989), and mouse lamin C (Riedel and Werner, 1989). Bold regions in the human lamin C sequence are those important for chromatin binding activity based on the results of deletion experiments. The first seven amino acids of these sequences correspond to the highly conserved regions that mark the NH₂-terminal ends of the lamin tail domains.

with minichromosomes assembled in vitro (Hoger et al., 1991).

The results of the present study directly establish the existence of a chromatin binding site in the lamin tail. Interestingly, at least in the case of lamin C, the chromatin binding site of the lamin tail is immediately adjacent to the rod domain. This raises the possibility that the rod and tail domains interact with the same chromatin structure (i.e., core histones), possibly as two components of a larger composite binding site. Unfortunately, it is not possible to compare the affinity of the rod vs. tail domain binding to chromatin, because the rod domain binding can be measured only in an association assay involving both lamin–chromatin and lamin–lamin associations.

Studies with turkey erythrocyte lamins isolated in the presence of urea showed a specific association of avian lamin A with chromatin fragments, in a fashion that was consistent with a K_d of ~ 1 nM (Yuan et al., 1991). This contrasts with the results of the present study, which show a significantly lower apparent affinity (300 nM) of lamin tails with chromatin fragments. While the basis for this discrepancy is unknown, it is possible that the former assay measured a cooperative association of lamin A with chromatin rather than simple binding of a lamin protomer. This would suggest an affinity that is substantially higher than the actual chromatin binding affinity of a lamin protomer that does not self assemble during chromatin binding. Consistent with this interpretation, other studies have shown that intact lamins self-associate when they interact with chromatin in vitro (Glass and Gerace, 1990; Hoger et al., 1991) and that chromatin lowers the critical concentration for self-assembly (Glass and Gerace, 1990).

The inability of turkey erythrocyte lamin B isolated in the presence of urea to interact with chromatin (Yuan et al., 1991) contrasts with our results, which showed chromatin binding for both A- and B-type lamin tails when the latter were expressed as soluble GST fusions. This difference may be explained by differences in the self-association capacity of the different avian lamin isotypes (see above), or the possibility that A- and B-type lamins have

different abilities to refold into a native structure during dialysis from urea solutions. We have found that pretreatment of the GST–lamin C tail fusion protein with 8 M urea does not influence its capacity for chromatin binding, while pretreatment of the GST–lamin B₁ tail fusion protein with urea results in loss of all detectable chromatin binding activity (our unpublished observations).

Role of Lamin–Chromatin Binding in Interphase Chromosome Structure

We believe that the in vitro interaction of lamin tail domains with chromatin measured in this study is likely to be physiologically significant, as our studies involved a native chromatin substrate analyzed under approximately physiological solution conditions. Moreover, the interaction was found to be salt-stable, and specific by multiple criteria (see above). Considering the abundance of nuclear lamins at the inner nuclear membrane, the interaction we have characterized is expected to be of major importance for anchorage of chromatin at the NE during interphase.

A priori, it is likely that chromatin has a high avidity, stable interaction with the nuclear lamina in vivo (see Gerace and Burke, 1988). Moreover, light microscope studies have indicated that the NE–chromatin interaction can involve specific chromosomal regions (Hochstrasser et al., 1986). By contrast, our in vitro studies have shown that monomeric lamin tails bind to chromatin with intermediate rather than high avidity, and that the interaction (at least for lamin C) involves core histones, which are ubiquitous chromosomal components. A simple model can accommodate both these in vivo and in vitro features. This speculative model (Fig. 9) proposes that higher order chromatin architecture is involved in determining the chromosomal regions that interact with the lamina in vivo, and can convert the intermediate affinity interactions of monomeric binding units into a high-avidity association. Specific chromosomal regions that are packaged in a higher order structure capable of cooperatively associating with the repeating array of binding sites on lamin fila-

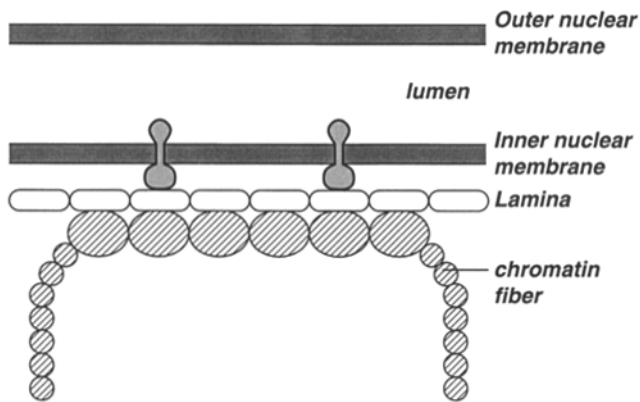


Figure 9. Speculative model for specification of the lamin-chromosome interaction by higher order chromatin structure. The lamina, depicted as a polymeric array of nuclear lamins with a regular longitudinal repeat, is shown to be attached to the inner nuclear membrane by integral membrane proteins. According to this model, chromatin packaged into higher order structures that can cooperatively bind to the lamina (*large hatched spheres*) can become stably associated with the latter. Chromatin packaged into structures that are unable to cooperatively bind to the lamina (*small hatched spheres*) do not become stably associated with the latter.

ments could engage in a stable, high-avidity interaction with the nuclear lamina, while chromosomal regions that lack the higher order structure needed for cooperative binding could not interact with the lamina strongly and stably.

In support of this model is the observation that a discrete higher-level chromatin structure, heterochromatin, is preferentially associated with the NE in a wide range of cells (Hochstrasser and Sedat, 1987; Johns, 1988). By contrast, unfolded (active) chromatin is localized throughout the nuclear interior and is not preferentially associated with the NE (e.g., Roshbash and Singer, 1993). While cooperative binding involving a specific higher order chromatin structure may be fundamental to the lamina-chromatin interaction, additional features and components could also help to specify this interaction. For example, lamins could have a preferred interaction with certain posttranslationally modified forms of histones, and lamina-associated integral membrane proteins that bind chromatin (Foisner and Gerace, 1993) could also contribute to the chromatin attachment.

It should be noted that an intermediate (rather than high) affinity interaction of the lamin protomer for histones is appealing from a physiological perspective. In this case, lamins that are imported into the nucleus after biosynthesis could migrate throughout the nuclear interior with relatively little restriction because of weak nucleosome binding until they ultimately reach the site (the lamina) where they could engage in cooperative assembly with lamins and integral membrane proteins, and thus be stably incorporated into a supramolecular structure.

We consider it likely that the interaction of chromatin with the lamina mediates one of the highest levels of chromosome organization in the interphase nucleus, since the number of chromosomal regions associated with the NE in

interphase cells can be relatively low. For example, the *Drosophila* polytene chromosome set contains 15–20 sites closely associated with the NE with high frequency (Hochstrasser et al., 1986; Hochstrasser and Sedat, 1987). Thus it is unlikely that the lamina-chromosome association is involved defining the 10–100-kb “loops” that are thought to comprise units of chromosome folding and function (van Holde, 1989). Understanding the functions of the lamin-chromatin interaction more precisely will require *in vivo* studies in which it is specifically disrupted.

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