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In vivo association of lamins with nucleic acids in *Drosophila melanogaster*

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SUMMARY

A ^{32}P-labeling strategy was developed to study the interaction(s) in tissue culture cells between proteins and nucleic acids. Interphase and mitotic nuclear lamins were studied in *Drosophila* K_c cells. After bromodeoxyuridine incorporation and in vivo photo-crosslinking with 366 nm light, it was found that interphase lamins were associated with nucleic acid. Interactions with DNA as well as RNA were detected. In contrast, interaction of nucleic acids with mitotic lamin was not observed. Photo-crosslinking in the presence of antibiotics distamycin and/or chromomycin suggested that interphase lamins interacted with both A-T-rich DNA and G-C-rich DNA; interactions with G-C-rich DNA predominated. These results have implications for understanding the interphase organization of the higher eukaryotic cell nucleus as well as the transition of cells from interphase to mitosis. A model of nuclear organization, consistent with our results, is proposed.

Key words: Lamin, *Drosophila*, Photo-crosslinking, Nucleic acid

INTRODUCTION

Two lamin genes have been found in *Drosophila melanogaster.* (Unless indicated otherwise, for *Drosophila*, the term lamin refers to protein products encoded by the *Drosophila* lamin Dm_0 gene and/or cloned cDNA). One gene encodes lamin Dm_0 (Gruenbaum et al., 1988; Osman et al., 1990). Lamin Dm_0 is modified post-translationally such that two isoforms, lamins Dm_1 and Dm_2, are associated with interphase nuclei (Smith et al., 1987). Lamin Dm_0 and derivatives contain a COOH-terminal CaaX motif (CAIM) and are stably isoprenylated, at least during interphase (Havel et al., 1992); except for mature sperm, they are expressed in all *Drosophila* cell types and tissues examined to date (Whalen et al., 1991; Riemer et al., 1995). Ubiquitous expression of protein is reflected by ubiquitous mRNA expression (see Whalen et al., 1991). Lamin Dm_0 and derivatives are apparently homologous to the B-type lamins of vertebrates.

During both meiosis and mitosis (M phase), interphase lamins Dm_1 and Dm_2 are converted to what is apparently a single M-phase isoform, lamin Dm_{mit} (Smith and Fisher, 1989). In contrast with interphase lamin isoforms, purified lamin Dm_{mit} is soluble in vitro (Lin and Fisher, 1990). Conversion in vivo from interphase to M-phase lamins is accompanied by both phosphorylation and dephosphorylation (Smith and Fisher, 1989); conversion was reconstituted in vitro (Maus et al., 1995). Most recently, it was demonstrated that in vitro phosphorylation of a specific lamin fragment by *cdc2*-kinase blocked head-to-tail polymerization of that fragment with another complementary but unmodified fragment (Stuurman et al., 1996). Similar results were found with protein kinase A (Stuurman, 1997). Head-to-tail polymerization is thought to be a component of lamina formation in vivo.

A separate gene encodes *Drosophila* lamin C (Bossie and Sanders, 1993; Riemer and Weber, 1994; Riemer et al., 1995). In contrast with lamin Dm_0 and derivatives, lamin C lacks a COOH-terminal CaaX motif and analogous with vertebrate lamins of the A/C-type, expression of lamin C is highly regulated (Riemer et al., 1995). Lamin C is only seen at certain developmental stages and in specific cells. Selective expression of protein is reflected by selective expression of mRNA.

In vitro studies demonstrated that polymeric interphase lamins could bind DNA non-covalently in a sequence-specific manner (Ludérus et al., 1992, 1994; Baricheva et al., 1996; Zhao et al., 1996). Results of previous studies suggested that the vertebrate nuclear envelope and/or lamins also interacted with DNA in vivo (Galcheva-Gargova and Dessev, 1987; Wedrychowski et al., 1989; Christova et al., 1992). Lamins can bind chromatin in vitro (Glass and Gerace, 1990; Höger et al., 1991; Yuan et al., 1991; Glass et al., 1993; Taniura et al., 1995).

To better understand the role of extrachromosomal karyoskeletal proteins in the organization of the interphase nucleus, we investigated the interaction of lamins with nucleic acids in vivo; previous studies have focussed on in vitro interactions. Typically, *Drosophila* tissue culture cells were grown in the presence of bromodeoxyuridine (BrdU) and

exposed to 366 nm light while still viable. Cells were then lysed, treated with micrococcal nuclease, and lamins immunoprecipitated with specific antibodies. Nucleic acid fragments covalently crosslinked (linked) to proteins and co-immunoprecipitated with lamins were ^{32}P-labeled by incubation with T4 polynucleotide kinase and [γ-^{32}P]ATP. Though suitable targets for ^{32}P-labeling, nucleic acid fragments left by nuclease treatment were empirically determined to be too small to alter SDS-PAGE migration of attached proteins significantly. Hence, ^{32}P-labeled material in immunoprecipitates was analyzed by SDS-PAGE and autoradiography/fluorography. Interphase lamins Dm_1 and Dm_2 were strongly labeled in a BrdU/366 nm light-dependent way. Antibiotics distamycin and chromomycin were used to gain insight into the sequence preferences of lamin-DNA interactions. In contrast, mitotic lamin Dm_{mit} resisted labeling. Our observations have implications for understanding the role of extrachromosomal karyoskeletal proteins in organizing the interphase nucleus as well as the transition of higher eukaryotic cells from interphase to mitosis. They suggest that in living cells, lamins are closely associated with both DNA and RNA.

MATERIALS AND METHODS

Antibodies

Affinity-purified rabbit anti-lamin Dm_0 and derivatives IgG (pH 2.3 eluate) was prepared essentially as previously described (Fisher and Smith, 1988), using bacterially expressed protein immobilized on glutaraldehyde-activated glass beads (Whalen et al., 1991). Monoclonal antibody BMG6H8 directed against BrdU was obtained from Boehringer-Mannheim (Germany) and was used at a 1:75 (v:v) dilution. Alkaline phosphatase-conjugated goat anti-mouse IgG and alkaline phosphatase-conjugated goat anti-rabbit IgG were from Kirkegaard and Perry (Gaithersburg, MD). The anti-lamin IgG used for these studies recognized both lamin Dm_0 and derivatives as well as lamin C (see e.g. Bossie and Sanders, 1993; Riemer and Weber, 1994; Riemer et al., 1995). Moreover, both lamins are expressed in K_c cells (Riemer et al., 1995). Hence, since lamins Dm_1 and C comigrate after SDS-PAGE, the results reported in this article potentially pertain to both *Drosophila* lamins. However, studies with a monoclonal antibody specific for lamin C (Riemer et al., 1995) were used to evaluate its contribution to the labeling patterns seen. No labeling of lamin C was detected (R. Rzepecki and P. A. Fisher, unpublished), thus suggesting that our conclusions pertain only to lamin Dm_0 and derivatives.

Nucleases and nuclease digestion

Nucleases used were micrococcal nuclease, RNase A and DNase I. Micrococcal nuclease was from Boehringer-Mannheim and was of the highest grade commercially available (catalog number 107 921). RNase A was from Worthington Biochemical Corp. and was RASE grade (catalog number LS005679). Before use, RNase A was determined empirically to be completely free of DNase activity (M. McConnell and P. A. Fisher, unpublished; see also Meller et al., 1994; Meller and Fisher, 1995). DNase I was from Sigma Chemical Co. and was RNase-free grade (catalog number D7291). To study the sensitivity of ^{32}P-labeled nucleic acid photo-crosslinked to lamin (see Fig. 3), micrococcal nuclease digestion was performed for 30 minutes at 37°C with a final concentration of 45 μg/ml enzyme, RNase A digestion was performed for 30 minutes at 37°C with a final concentration of 60 μg/ml enzyme and DNase I digestion was performed for 30 minutes at 37°C with a final concentration of 66 μg/ml enzyme. All nuclease digestions were performed in Buffer IPA (see below) supplemented with 5 mM $MgCl_2$ and 2.5 mM $CaCl_2$.

Methods

Much of the methodology was as detailed previously. *Drosophila melanogaster* (Oregon R, P2 strain) were maintained in mass culture and embryos collected according to Allis et al. (1977). SDS-PAGE was according to Laemmli (1970) as modified by Fisher et al. (1982) on polyacrylamide minigels. Proteins were transferred electrophoretically from gels to sheets of nitrocellulose (Harlow and Lane, 1988) and resulting immunoblots were processed and probed with primary antibodies. Bands of reactivity were visualized colorimetrically (McGadey, 1970) with calf alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (Blake et al., 1984; Smith and Fisher, 1984) and a commercial one-solution alkaline phosphatase substrate (Kirkegaard and Perry, Gaithersburg, MD).

Tissue culture cell maintenance

Drosophila K_c cells were the generous gift of Dr John Watson (University of California at San Francisco) and were maintained in suspension culture exactly as previously described (Berrios et al., 1991). To enrich for mitotic cells, vinblastine was added to a final concentration of 2 μg/ml and culturing continued for 18-20 hours (Hanson and Hearst, 1973; see also Smith and Fisher, 1989). For routine BrdU incorporation (except where indicated otherwise) 200 ml of exponentially growing cells ($1.2\text{-}1.8\times10^6$ cells/ml) were supplemented with 20 μM 5-bromo-2-deoxyuridine (BrdU) and maintained in culture for an additional 26-27 hours. For experiments with distamycin and chromomycin, cell cultures supplemented with 20 μM BrdU and 20 μM 5-bromo-2-deoxycytidine (BrdC) were maintained as above.

Nitrocellulose 'dot-blot' analysis to measure BrdU incorporation

To measure incorporation of BrdU into cellular DNA, 1×10^7 cells that had been cultured in BrdU for the times indicated in Fig. 1 were washed three times each in 3 ml of PBS, finally resuspended in 100 μl of PBS and lysed by addition of an equal volume of 10 % (w/v) SDS and 40 mM DTT. Samples were then boiled for 10 minutes. After cooling, trichloroacetic acid was added to a final concentration of 10% (w/v). Samples were then incubated for 10 minutes on ice and precipitated material collected by centrifugation for 10 minutes at 10,000 ***g***. The supernatant was discarded and pellets were dissolved in 100 μl of solution containing 100 mM Tris-HCl, pH 7.5, 5 mM EDTA and 0.5% (w/v) SDS. After complete resolubilization, proteinase K (Worthington, Freehold, NJ) was added to a final concentration of 50 μg/ml and samples were incubated for 60 minutes at 50°C. The nucleic acids were then recovered by standard phenol/chloroform extraction and ethanol precipitation (Sambrook et al., 1989) and dissolved in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Dissolved nucleic acids were adsorbed to nitrocellulose in a commercial 'dot-blot' manifold (Schleicher and Schuell, Keene, NH) and BrdU incorporation quantified by scanning densitometry after standard antibody detection with a commercially available monoclonal anti-BrdU antibody and goat anti-mouse IgG conjugated to alkaline phosphatase. Colorimetric detection of alkaline phosphatase was exactly as described for immunoblot analysis.

In vivo photo-crosslinking without and with antibiotics

In vivo photo-crosslinking was performed with a hand-held lamp (UVGL-58, UVP Inc., San Gabriel, CA) emitting 366 nm light. All quantities refer to 200 ml of cell culture starting material. Before photo-crosslinking, cells were harvested by centrifugation at 4°C for 5 minutes at 900 ***g***, resuspended in chilled 10 mM KPO_4, 140 mM NaCl, pH 7.5 (PBS) and washed three times by recentrifugation and resuspension. Finally, the cell pellet was resuspended in 8 ml of PBS and the cells transferred to a plastic Petri dish (10 cm in diameter). The Petri dish was placed on ice and remained uncovered; photo-crosslinking was by illumination for 30 minutes with the hand-held

lamp suspended such that the bottom surface of the lamp was 3 cm above the top of the cell suspension.

In experiments with antibiotics (distamycin and chromomycin), cells were grown in 20 μM BrdU and 20 μM BrdC, harvested, washed and resuspended in 8 ml of PBS, exactly as described above. However, before illumination, cell suspensions were supplemented with either 50 μM distamycin A3 (Sigma Chemical Co.) or 50 μM chromomycin (Sigma Chemical Co.), or 50 μM each of both. After antibiotic addition, cell suspensions were incubated for 30 minutes at room temperature and then subjected to illumination with 366 nm light as described above.

Immunoprecipitation and ^{32}P labeling

Cells were harvested by centrifugation, resuspended in PBS, lysed and denatured by addition of SDS and DTT; samples were then boiled for 10 minutes. Typically, about 6×10^8 cells were harvested, resuspended in 1.5 ml PBS and denatured by addition of an equal volume of solution containing 10% (w/v) SDS and 40 mM DTT. Whole cell lysates prepared in this way were stored at −20°C until use. Lysates, each derived from about 1.5×10^7 cells, were thawed by boiling for 10 minutes. Samples were cooled to room temperature and trichloroacetic acid was added to a final concentration of 10% (w/v). Samples were then incubated for 10 minutes at room temperature and precipitated proteins collected by centrifugation, also at room temperature, for 10 minutes at 10,000 ***g***. The supernatant was discarded, 5 μl of 1.5 M Tris-HCl, pH 8.8, was added to the protein pellet and the pellet was dissolved in 200 μl of solution containing 20 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% (v/v) Triton X-100 and 0.02% (w/v) SDS (Buffer IPA). Immediately before use, Buffer IPA was supplemented to a final concentration of 2.5 mM $CaCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM L-1-tosylamide-2-phenylethylchloromethyl ketone, 1 mM L-1-tosyl-L-lysyl-chloromethyl ketone, 1 μg/ml leupeptin and 1 μg/ml pepstatin. Unless indicated otherwise, 5 μl of micrococcal nuclease (680 μg/ml; Boerhinger, Mannheim, Germany) was then added and samples were incubated for 60 minutes at 37°C.

Immunoprecipitation was performed essentially as previously described (Smith et al., 1987). Specifically, protein A-Sepharose (Pharmacia, Piscataway, NJ) was hydrated in Buffer IPA supplemented with 5 mM EDTA (final concentration) (Buffer IPB), washed by suspension and brief centrifugation and finally resuspended in Buffer IPB. 2-4 μg of affinity-purified IgG were added to 50 μl packed protein A-Sepharose beads. The total volume was brought to 350 μl with Buffer IPB and the protein A-Sepharose-IgG mixture was incubated with gentle agitation for 90 minutes at 37°C. Unbound IgG was removed by washing first with Buffer IPB (once) and then with Buffer IPA (twice). Micrococcal nuclease-treated K_c-cell lysate was then added to the washed protein A-Sepharose-IgG beads and the mixture was incubated for 90 minutes at 37°C. The beads with IgG and antigen bound (immunoprecipitate) were recovered by centrifugation, washed three times with 350 μl of Buffer IPB followed by three identical washes with Buffer IPA.

After the final wash in Buffer IPA, the washed immunoprecipitate was resuspended in 80 μl of 1.5× concentrated T4 polynucleotide kinase buffer (New England Biolabs, Beverly, MA). To this resuspended immunoprecipitate was added 1-2 μl of [γ-^{32}P]ATP (4500 Ci/mmol; 10 μCi/μl; ICN Pharmaceuticals, Costa Mesa, CA) and 5 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA). No unlabeled ATP was added. ^{32}P-labeling with T4 polynucleotide kinase was for 30 minutes at 37°C. After this incubation, the ^{32}P-labeled immunoprecipitates were washed three times with 350 μl of Buffer IPB and finally resuspended in 45 μl of 2.5× concentrated standard SDS-PAGE loading buffer, followed by electrophoresis on an SDS-7% polyacrylamide gel and electrophoretic transfer to nitrocellulose for autoradiography or phosphorimager (Molecular Dynamics 445 SI) and immunoblot analyses.

Quantification by scanning densitometry

Immunoblots (dot-blots) were quantified using an LKB Ultrascan XL laser densitometer (LKB Instruments Inc., Gaithersburg, MD). Quantitative data are expressed in arbitrary units, except where indicated in the text and figures.

Partial purification of K_c cell lamins

To partially purify interphase lamins Dm_1 and Dm_2, nuclei were purified from K_c cells essentially as previously described for fresh embryos (McConnell et al., 1987), except that a final concentration of 1% (v/v) Triton X-100 was used in the extraction solution to ensure cell breakage. A typical purification was initiated with 3×10^8 K_c cells grown in BrdU. After purification, nuclei were resuspended in 1 ml of 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM L-1-tosylamide-2-phenylethylchloromethyl ketone, 1 mM L-1-tosyl-L-lysyl-chloromethyl ketone, 1 μg/ml leupeptin and 1 μg/ml pepstatin. The resuspended nuclei were supplemented with 34 μg/ml micrococcal nuclease and 80 μg/ml RNase A, after which they were incubated for 60 minutes at 23°C. Insoluble material (digested nuclei) was recovered from the pellet fraction after centrifugation at 4°C (10,000 ***g*** for 10 minutes), resuspended in chilled 20 mM Tris-HCl, pH 7.5, 1% (v/v) Triton X-100, incubated on ice for 10 minutes, and again subjected to centrifugation (4°C, 10,000 ***g***, 10 minutes). After centrifugation, the supernatant was discarded and the pellet resuspended in 3 ml of 20 mM Tris-HCl, 0.5 M NaCl; the resuspended pellet was allowed to incubate for 10 minutes on ice. This resulted in significant solubilization of interphase lamins (see also Lin and Fisher, 1990). The sample was subjected to centrifugation (4°C, 10,000 ***g***, 10 minutes) and the lamin-containing supernatant was withdrawn, adjusted to 5% (w/v) SDS, 20 mM DTT and boiled for 10 minutes. After cooling, proteins were precipitated with 10% (w/v) trichloroacetic acid, resolubilized in 100 mM $NaPO_4$, 1 mM EDTA, 0.1% (w/v) SDS, pH 6.8, and subjected to hydroxyapatite chromatography in the presence of 0.1% (w/v) SDS, exactly as previously described (Berrios et al., 1983; Filson et al., 1985) and essentially according to Moss and Rosenblum (1972). Lamin-containing fractions were pooled and subjected to immunoprecipitation and ^{32}P-labeling as described above.

Bacterial expression and purification of lamin Dm_0

Full-length lamin Dm_0 was expressed in *E. coli* from the plasmid pETDmLFL (Stuurman et al., 1996). Purification of cDNA clone-encoded lamin Dm_0 from bacterial extracts was also as described (Stuurman et al., 1996). Bacterially expressed lamin Dm_0 was shown to be similar to embryo lamins Dm_1 and Dm_2 with respect to in vitro DNA binding (Baricheva et al., 1996), and could participate in head-to-tail polymerization and sedimentation, which is presumably dependent on lateral interactions (aggregation) (Stuurman et al., 1996). Binding of DNA to lamin Dm_0 was confirmed by sedimentation (not shown). After purification, 15 μg of bacterially expressed lamin Dm_0 was treated with 34 μg micrococcal nuclease in 250 μl IPA supplemented with 2.5 mM $CaCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM L-1-tosylamide-2-phenylethylchloromethyl ketone, 1 mM L-1-tosyl-L-lysyl-chloromethyl ketone, 1 μg/ml leupeptin and 1 μg/ml pepstatin; incubation was for 120 minutes at 37°C. After micrococcal nuclease treatment, samples were denatured by addition of SDS to 5%, DTT to 20 mM, and boiling for 10 minutes. Lamin Dm_0 was then recovered by trichloroacetic acid precipitation and resolubilized in 150 μl of 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA. Without this micrococcal nuclease pretreatment, bacterially expressed lamin Dm_0 contained readily detectable bound nucleic acid, presumably of endogenous origin (not shown). This interaction obscured further analysis but proved that lamin Dm_0 synthesized in *E. coli* could bind nucleic acid in vivo.

After purification, exhaustive micrococcal nuclease pretreatment,

denaturation and return to nondenaturing conditions, clone-encoded lamin Dm_0 was incubated for 120 minutes at 37°C with various amounts of *Bam*HI/*Hin*dIII (New England Biolabs, Beverly, MA)-fragmented pCL (SAR-containing) DNA (Bode et al., 1992; see also Rzepecki et al., 1995). Restriction enzymes were used according to the vendor's instructions. Activity of the restriction enzymes on pCL DNA was confirmed by agarose gel electrophoresis (not shown). After incubation of lamin Dm_0 with pCL fragments, performed under nondenaturing conditions, clone-encoded lamin Dm_0 was denatured by addition of SDS to 5%, DTT to 20 mM, and boiling for 10 minutes, recovered by trichloroacetic acid precipitation and subjected to standard immunoprecipitation and ^{32}P-labeling (see above).

RESULTS

Quantification of BrdU incorporation in *Drosophila* K_c tissue culture cells

Dot-blot analysis with a monoclonal anti-BrdU antibody was used to quantify BrdU incorporation into K_c cell DNA. Cells were grown in the continuous presence of BrdU and assayed at various times, for BrdU incorporation, as described in Materials and methods (Fig. 1). Though conceptually difficult to interpret, results showing an optimum time of K_c cell growth for BrdU incorporation into nuclear DNA were highly reproducible. A representative example is shown, where optimal incorporation was found after 27 hours of incubation. Immunofluorescence analyses with the monoclonal anti-BrdU antibody suggested that at the end of this time, the majority of individual cells incorporated detectable amounts of BrdU (not shown).

Detection of nucleic acids bound to lamins

Immunoprecipitation and ^{32}P-labeling combined with SDS-PAGE were used to evaluate the interaction with nucleic acids of *Drosophila* lamin Dm_0-derivatives in vivo. This strategy is described in the Introduction. Scanning densitometry was used for quantification. Based on initial results, cell growth in BrdU followed by exposure to 366 nm light led to a 3.1-fold increase in labeling relative to lamin from cells that were not exposed to either BrdU or 366 nm light (not shown but see e.g. Fig. 2). Both interphase isoforms, lamins Dm_1 and Dm_2, were labeled (not shown but see Fig. 4B, lane 2). ^{32}P-labeling could only be detected after initial treatment of lysates with micrococcal nuclease and/or RNase A, both of which leave a 5′-OH group on the remaining nucleic acid fragments. No labeling was detected when DNase I was used for initial treatment of lysates (not shown). DNase I, though effective for DNA fragmentation, leaves a 5′-PO_4 group. T4 polynucleotide kinase is known to phosphorylate a 5′-PO_4 much less effectively than a 5′-OH.

We judged that denaturation in SDS and DTT followed by precipitation with trichloroacetic acid, further denaturation and SDS-PAGE was a sufficiently harsh treatment to remove any nucleic acid fragments associated noncovalently with lamin. To exclude further noncovalent interactions, lamin was first partially purified by extraction from micrococcal nuclease/RNase A-treated K_c cell nuclei and subjected to chromatography on hydroxyapatite in the presence of 0.1% (w/v) SDS; before analysis, cells were grown in medium containing BrdU (see Materials and methods). As shown in Fig. 2, partially purified lamin could be readily labeled with an initial exposure to 366 nm light (Fig. 2B, lane 2). As expected, labeling after exposure to 366 nm light was far more intense than labeling without exposure (Fig. 2 B, compare lane 2 with lane 1), even though approximately equal amounts of lamin, as revealed by immunoblot analysis, were recovered from immunoprecipitation and subjected to SDS-PAGE (Fig. 2A, compare lane 2 with lane 1).

Both DNA and RNA apparently reside next to lamin in vivo

To elucidate further the nucleic acid composition of K_c cell nuclear proteins that were immunoprecipitated and ^{32}P-labeled after cell growth in BrdU as well as exposure to 366 nm light, labeled proteins were treated again (an additional time after labeling) with excess amounts of various nucleases. The results demonstrated that the greatest diminution of labeling occurred after another treatment with micrococcal nuclease (Fig. 3B, compare lane 2 with lane 1). Micrococcal nuclease degrades both DNA and RNA; treatment with RNase A resulted in discernible but lesser decreases (Fig. 3B, compare lane 3 with lane 1), as did treatment with DNase I (Fig. 3B, compare lane 4 with lane 1). Nuclease sensitivity results indistinguishable from those shown in Fig. 3 were obtained with K_c cells that

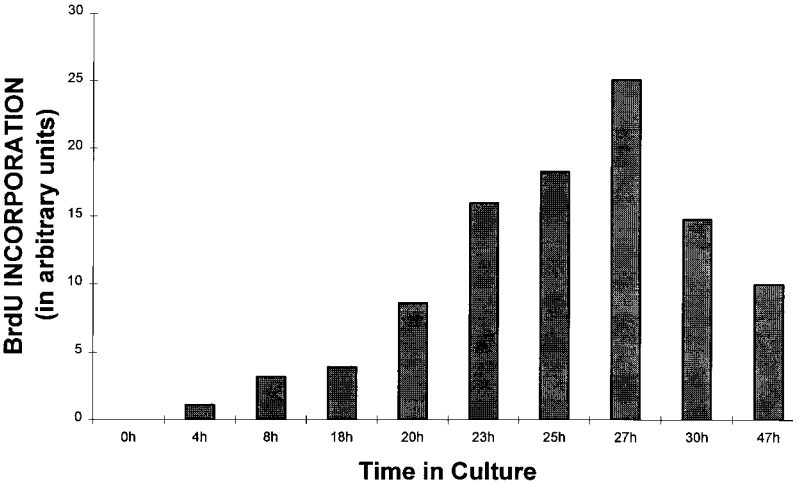

Fig. 1. Accumulation of BrdU in nuclear DNA of *Drosophila* K_c tissue culture cells. K_c cells in suspension culture (1.5×10^6 cells/ml) were supplemented with 20 μM BrdU at 0 hours, as indicated. After continued growth for the indicated times, cells were harvested by centrifugation, washed three times with PBS solution and lysed as described in Materials and methods. BrdU incorporation was analyzed with a monoclonal anti-BrdU antibody (BMG6H8), also as described (Materials and methods). At maximal incorporation (27 hours), the majority of cells exhibited nuclear BrdU incorporation by immunofluorescence (not shown).

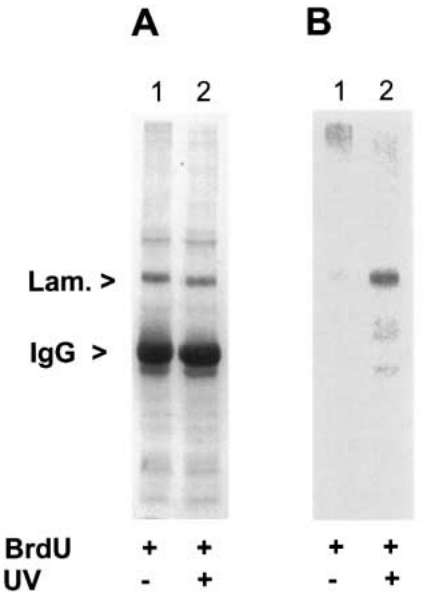

Fig. 2. Lamin labeling after nuclear purification, protein extraction and SDS- hydroxyapatite chromatography. K_c cells were grown in suspension culture in 20 μM BrdU and lysed under nondenaturing conditions (see Materials and methods). Nuclei were purified, processed and extracted as described (Materials and methods). Lamins were further purified by SDS-hydroxyapatite chromatography. Immunoprecipitation was with affinity-purified anti-lamin IgG. Both immunoblot analysis (A) and autoradiography of the immunoblot (B) are shown. The immunoblot was probed with affinity-purified anti-lamin IgG. Migration positions of lamins (Lam.) and rabbit IgG heavy chain (IgG) are indicated. UV refers to exposure to 366 nm light; when performed, this was before cell lysis.

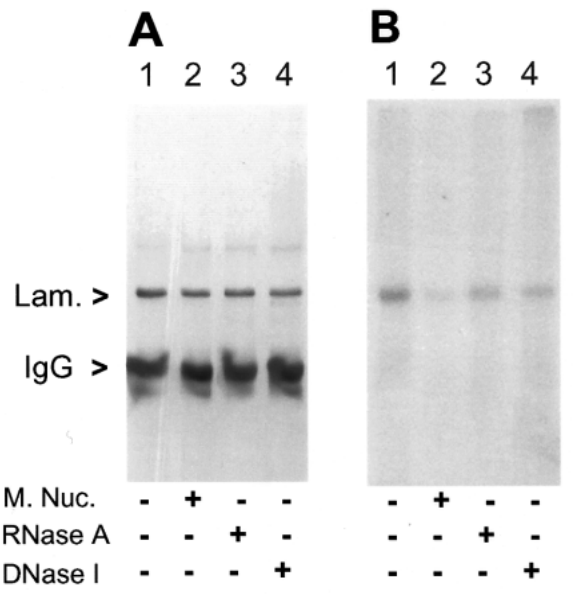

Fig. 3. ^{32}P-label associated with lamins is sensitive to treatment with micrococcal nuclease, DNase I and RNase A. Immunoprecipitation was with affinity-purified anti-lamin IgG. Both immunoblot analyses (A) and autoradiography of the respective immunoblots (B) are shown. The immunoblot in A was probed with affinity-purified anti-lamin IgG. Before lysis, all cells were maintained in 20 μM BrdU and exposed to 366 nm light as described (Materials and methods). Treatment with nucleases was as indicated below each panel. Quantification was by scanning densitometry. The migration positions of lamins (Lam.) and rabbit IgG heavy chain (IgG) are indicated.

had not been exposed to any nucleoside analogs (e.g. BrdU) as well as with *Drosophila* embryo lamin. Embryos were also not exposed to nucleoside analogs.

Densitometric quantification of the data shown indicated that micrococcal nuclease retreatment removed more than 95% of the ^{32}P label while RNase A removed 40% of the label and DNase I removed 70% of the label. Mung bean nuclease and RNase H had no effect on ^{32}P label bound to lamin (not shown). Treatment after labeling of samples with proteinase-K before SDS-PAGE completely eliminated ^{32}P-labeled bands from resulting autoradiograms (not shown). Loss of ^{32}P-labeled species correlated exactly with loss of lamin, as revealed by immunoblot analysis.

After denaturation bacterially expressed lamin Dm_0 does not bind DNA non-specifically during immunoprecipitation

To assess whether lamin binding to nucleic acid could occur as an in vitro artifact, we subjected bacterially expressed lamin Dm_0 to the same lysis/denaturation procedure used for K_c cells. Purified, bacterially expressed lamin Dm_0 was treated with large amounts of micrococcal nuclease to remove any endogenously bound nucleic acid, denatured by addition of SDS and DTT, followed by boiling and precipitation with trichloroacetic acid, and finally resolubilized in a nondenaturing solution (see Materials and methods). Purified bacterially expressed lamin was then incubated in this solution with varying amounts of fully restricted pCL DNA, followed by standard immunoprecipitation and ^{32}P-labeling. No binding of DNA to bacterially derived lamin Dm_0 was detected (not shown). Without removal of endogenously bound nucleic acid, binding of DNA to an identical amount of lamin Dm_0 was easily observed by immunoprecipitation and ^{32}P labeling (not shown).

Nucleic acid can be found associated with interphase but not mitotic lamin

To address the question of what happens to lamin-nucleic acid interactions when the nuclear envelope disassembles during mitosis, soluble mitotic lamin Dm_{mit} was compared with insoluble interphase lamins Dm_1 and Dm_2. All were purified by immunoprecipitation from appropriate K_c cell extracts and ^{32}P-labeled using standard protocols (see Materials and methods). Briefly, cells grown in BrdU were treated with vinblastine to enrich for mitotic cells (the mitotic index of the resulting population was about 40%), exposed to 366 nm light, and fractionated by centrifugation into a 10,000 ***g*** nuclei containing pellet and a soluble 100,000 ***g*** supernatant. Fractions were then treated with micrococcal nuclease, and lamins were subjected to standard immunoprecipitation and ^{32}P labeling. Although immunoblot analysis revealed that similar amounts of lamin were subjected to SDS-PAGE (Fig. 4A), only labeling of interphase lamins Dm_1 and Dm_2 was detected (Fig. 4B, lane 2). No labeling of mitotic lamin Dm_{mit} was observed (Fig. 4B, lane 1).

Identical results to those shown were obtained when cell

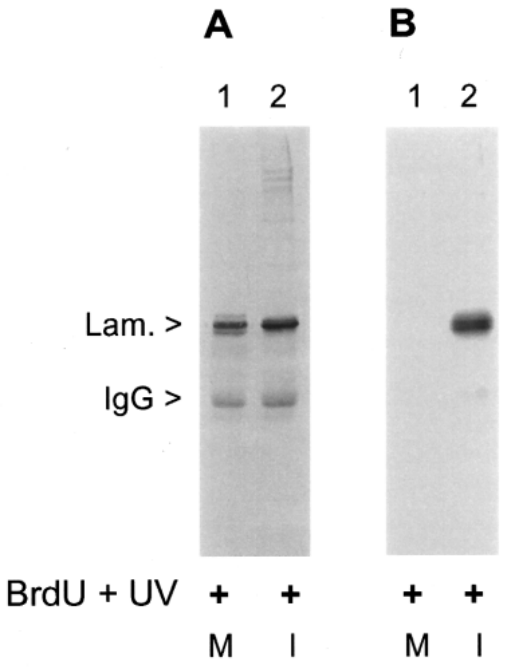

Fig. 4. ^{32}P labeling of interphase lamins Dm_1 and Dm_2 but not mitotic lamin Dm_{mit}. K_c cells were grown in vinblastine until about 40% were blocked in mitosis (the remainder were still in interphase), as determined microscopically. Cells were also maintained in 20 μM BrdU, exposed to 366 nm light and lysed. Samples were subjected to immunoprecipitation with affinity-purified anti-lamin antibodies and ^{32}P labeling as described (Materials and methods). Both immunoblot analysis (A) and autoradiography of the immunoblots (B) are shown. The immunoblot was probed with affinity-purified anti-lamin IgG. Migration positions of lamins (Lam.) and rabbit IgG heavy chain (IgG) are indicated. UV refers to exposure to 366 nm light. Lanes 1 were loaded with mitotic lamin Dm_{mit} as designated below the panels (M). Lanes 2 were loaded with interphase lamins Dm_1 and Dm_2, also as designated below the panels (I).

lysates were treated with micrococcal nuclease before instead of after fractionation (not shown). This experiment was performed to address the possibility that some lamin Dm_{mit} remained associated with chromosomes during mitosis but partitioned into the 10,000 ***g*** pellet after centrifugation. By treating samples with micrococcal nuclease before fractionation mitotic chromosomes would be digested, thus liberating any lamin Dm_{mit} associated, along with attached nucleic acid fragments, into the 10,000 ***g*** supernatant.

Lamin interacts with both A-T-rich and G-C-rich DNA in vivo

Previously, we showed both in vitro and in situ, that *Drosophila* lamin was associated with a particular A-T-rich DNA sequence (Baricheva et al., 1996). Both strands of duplex DNA were involved in binding (Bogachev et al., 1996). These observations were consistent with previous results of others (Ludérus et al., 1992, 1994). To obtain insight into the base composition of the nucleic acid with which lamin interacts in vivo, we studied the effects on lamin labeling of two antibiotics, distamycin and chromomycin. Distamycin is thought to disrupt protein interactions with A-T-rich DNA while chromomycin is thought to disrupt interactions primarily with G-C-rich DNA (Van Dyke et al., 1982; Van Dyke and Dervan, 1983; Käs and Laemmli, 1992). Cells were first grown in both BrdU and BrdC, and subjected to illumination in vivo with 366 nm light. In control experiments, separate incorporation of either BrdU (radiolabeling intensity set at 100%) or BrdC (85% labeling intensity relative to BrdU) led to similar intensities of lamin labeling as did incorporation of both analogs (140% relative to BrdU alone). Lamins were purified by immunoprecipitation with anti-lamin antibodies and subjected to standard ^{32}P labeling as described in the Introduction (also see Materials and methods). Lamin recovery was monitored by immunoblot analysis (Fig. 5A,C). ^{32}P incorporation was quantified with a PhosphorImager (Fig.

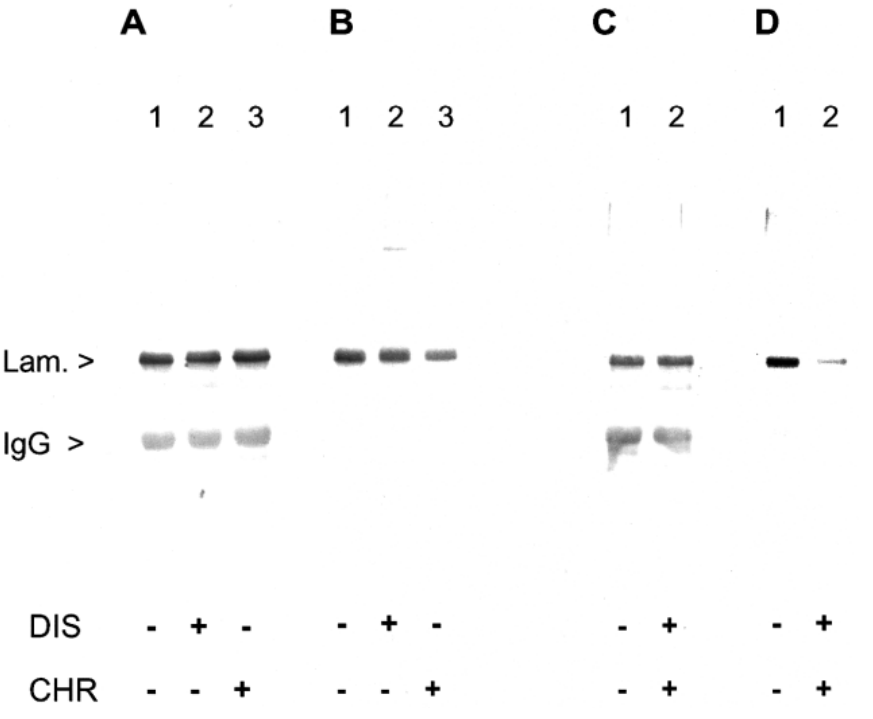

Fig. 5. Effects of distamycin and chromomycin on ^{32}P-labeling of interphase lamins Dm_1 and Dm_2. K_c cells were grown in suspension culture in 20 μM BrdU and 20 μM BrdC, incubated for 30 minutes at room temperature in either 50 μM distamycin or 50 μM chromomycin, or 50 μM each of both antibiotics, as indicated below each panel (distamycin, DIS; chromomycin, CHR). Immunoblot analyses with affinity-purified anti-lamin IgG (A and C) and autoradiographs of immunoblots (B and D) are shown. Migration positions of lamins (Lam.) and rabbit IgG heavy chain (IgG) are indicated.

5B,D). Although neither antibiotic eliminated lamin labeling completely, both led to a reproducible decrease in the ratio, determined densitometrically, of ^{32}P-lamin to immunoreactive material recovered (Fig. 5A, compare with B). For cells incubated in distamycin, lamin labeling was reduced 30%. For cells incubated in chromomycin, lamin labeling was reduced 55%. For cells incubated in both antibiotics, lamin labeling was reduced 81%. These results show that both antibiotics are able to interfere with lamin-nucleic acid interactions and suggest that lamin resides close to both A-T-rich DNA and G-C-rich DNA in vivo.

As a control, we investigated the effects of distamycin and chromomycin on in vivo labeling of DNA topoisomerase II. The results were different from those obtained for lamin and were exactly consistent with reports of previous studies performed in an entirely different way (Käs and Laemmli, 1992). These results will be presented in detail subsequently (R. Rzepecki and P. A. Fisher, in preparation).

DISCUSSION

In vivo, intense labeling of lamin was seen after incorporation of BrdU and/or BrdC followed by exposure to 366 nm light. While we consider it unlikely, it is formally possible that lamins labeled with ^{32}P are derived from an atypical subpopulation of K_c cells (e.g. apoptotic cells) present in our cultures and that the conclusions derived from our studies are therefore not pertinent to the overall population. Where feasible, experiments were also performed with *Drosophila* embryos, for which identical conclusions were reached (R. Rzepecki and P. A. Fisher, unpublished). Similar observations to those reported in the preceding article were recorded for K_c cells not exposed to any nucleoside analogs, for *Drosophila* embryos (also not exposed to nucleoside analogs) and for *Drosophila* DNA topoisomerase (topo) II (R. Rzepecki and P. A. Fisher, in preparation). Previously, topo II was shown indirectly to interact in vitro with both RNA and DNA in the unfractionated cell nucleus (Meller and Fisher, 1995; see also Meller et al., 1994). Lamin was shown to bind DNA, both in vitro and in situ (Ludérus et al., 1992; 1994; Baricheva et al., 1996). Our results corroborate these previous observations. In addition, they are the strongest demonstration that we are aware of that associations between lamins and nucleic acids apparently occur in vivo. Moreover, they demonstrate that when the nuclear envelope and molecular components such as lamin disassemble during M-phase, protein-nucleic acid interactions readily detected during interphase are abrogated (or at least substantially weakened). An extraordinary amount of attention has previously been focussed on the mechanisms by which post-translational modification, e.g. of lamins, alters protein-protein interactions required for cell cycle-dependent nuclear envelope disassembly and reassembly (see Nigg, 1992, for a review). Our observations suggest that similar attention to the cell cycle-dependent modification of protein-nucleic acid interactions is now warranted. In a related study that supports these conclusions, it was suggested that lamins had to be in a polymeric state to bind DNA specifically (Zhao et al., 1996).

Results of nuclease sensitivity experiments (Fig. 3) indicate that both RNA and DNA are involved in interactions in vivo with lamins; 70% of the detectable labeling was DNase I-sensitive while only 40% of the label was RNase A-sensitive. Moreover, resistance to mung bean nuclease suggests the involvement of double-stranded but not single-stranded DNA, while resistance to RNase H argues against involvement of RNA-DNA hybrids.

Although quantitative data were presented regarding sensitivities to DNase I and RNase A, we think for several reasons that they may be relatively unreliable. Firstly, as noted above, under the conditions used, a substantial majority of ^{32}P labeling was BrdU- (and/or BrdC-) and 366 nm light-dependent. BrdU, however, is a DNA synthesis precursor, and for incorporation into RNA considerable metabolic processing must first occur. Secondly, the efficiency with which T4 polynucleotide kinase phosphorylates DNA may be very different from that with which it modifies RNA. Thirdly, total labeling detected (DNA plus RNA) was greater than 100%, a theoretical impossibility and, most probably, a technical artifact limiting quantification by desitometric scanning. Based on these uncertainties, we urge that our observations only be interpreted qualitatively. It is also impossible from our studies to speculate about the stoichiometry with which lamins bind nucleic acids during interphase. This is due most fundamentally to the difficulty in determining crosslinking efficiency after BrdU incorporation.

Two important control experiments were performed. Both had negative results. First, we found that to be effectively labeled using our immunoprecipitation/^{32}P-labeling protocol, lamin must be covalently crosslinked to nucleic acid, presumably while non-denatured. Bacterially synthesized lamin Dm_0 that was able to bind DNA, as demonstrated either by sedimentation or endogenously in *E. coli*, could not be ^{32}P-labeled without crosslinking, even after prolonged cell-free incubation under non-denaturing conditions with relatively high concentrations of DNA. Second, labeling of lamin was highly cell cycle-dependent. Mitotic lamin Dm_{mit} resisted labeling whereas interphase lamins Dm_1 and Dm_2 did not (Fig. 4). The first observation demonstrates that the ability of lamin to bind DNA through immunoprecipitation/^{32}P-labeling/SDS-PAGE analysis requires stabilization by covalent crosslinking. This is consistent with the dependence of lamin labeling on exposure to UV light (Fig. 2). Both of these observations, coupled with the lack of lamin labeling after initial lysate treatment with DNase I, suggest that the labeling seen was not a consequence of direct protein modification by T4 polynucleotide kinase or some unknown contaminant of the commercial kinase preparation.

Photo-crosslinking between BrdU-containing nucleic acid and protein only occurs when the two principal molecules are within several Å of each other (see e.g. Simpson, 1979). Thus, we conclude that lamins are intimately associated with nucleic acids in vivo. Attempts were made to investigate $gp210_D$ (Berrios et al., 1995) by a similar strategy. Based on several criteria, including immunoelectron microscopic localization and protease sensitivity (see also Greber et al., 1990; Greber and Gerace, 1992), $gp210_D$, although a nuclear envelope protein like lamin, would be expected not to be closely associated with nucleic acids. Unfortunately, neither of the anti-$gp210_D$ monoclonal antibodies currently available, AGP-26 and AGP-78 (Filson et al., 1985), was effective for immunoprecipitating $gp210_D$ using our current immunoprecipitation/^{32}P-labeling protocol (R. Rzepecki and P.

A. Fisher, unpublished). Thus, although we feel this to be an important experiment, preparation of new anti-gp210$_D$ antibodies and/or development of new immunoprecipitation and labeling strategies will be required before this experiment can profitably be repeated.

However, the negative results (i.e. non-labeling) with the extremely large amounts of IgG present in the immunoprecipitates during our labeling reaction suggest that lamin labeling is specific. Similarly compelling was our observation that *Drosophila* lamin C, although it could be immunoprecipitated, was fully refractory to labeling with [γ-^{32}P]ATP (see above; R. Rzepecki and P. A. Fisher, unpublished). This negative result with lamin C is, in our estimation, an ideal control for the specificity of lamin Dm$_0$-derivative labeling, since both *Drosophila* lamin types are intermediate filament proteins, closely related in primary amino acid sequence and highly similar in subcellular localization.

Based on the results of in vitro studies, it was concluded that lamin could bind primarily to A-T-rich DNA. Current results with distamycin and chromomycin (Fig. 5) suggest that although associations with A-T-rich DNA occur, lamin resides primarily next to G-C-rich DNA in vivo. Moreover, the abrogation of these interactions during mitosis suggests that they are certainly of significance in the organization of the interphase nucleus. We propose that it is now appropriate to consider both A-T-rich DNA and G-C-rich DNA as potential targets for lamin binding in vivo. Further experiments will be required to define specific nucleotide sequence motifs to which the lamina is attached in the cell.

In conclusion, we would like to offer a model for the organization of the *Drosophila* nucleus. This model, while not entirely novel, has obvious implications for elucidating such diverse aspects of nucleic acid metabolism as DNA replication, repair and transcription as well as RNA splicing and transport. Although based in large part on data presented in this article and previously, it is entirely hypothetical and is presented primarily to stimulate further research. Accordingly, nuclei are hypothesized to contain, at a minimum, three topologically distinct domains. These are (1) the chromatin; (2) the extrachromosomal karyoskeleton; and (3) the nucleoplasm or diffusible intranuclear domain. The chromatin contains all of the cell's nuclear DNA and the information encoded therein. The chromatin also contains those proteins intimately involved in maintaining DNA structure, e.g. histones and HMG proteins. We postulate that the primary function of the cell nucleus is to provide regulated access to the information stored in nuclear DNA. The extrachromosomal karyoskeleton is largely proteinaceous, gives form to nuclei and organizes elements of chromatin such that accessibility of DNA is optimized. The soluble intranuclear domain contains those molecules that act on chromatin. A partial list includes DNA polymerases and associated replication factors, repair proteins, RNA polymerases, and associated transcription factors and proteins that modulate chromatin structure (e.g. condensation and decondensation). We further hypothesize that these domains interact so that changes in one (e.g. the karyoskeleton) regulate function in another (e.g. the chromatin). Physical interactions between karyoskeletal proteins and nucleic acids, such as were documented in the preceding article, would obviously be predicted by this model.

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