p75, a Polypeptide Component of Karyoskeletal Protein-Enriched Fractions Associated with Transcriptionally Active Loci of *Drosophila melanogaster* Polytene Chromosomes

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A 75-kilodalton polypeptide has been identified which copurifies with karyoskeletal protein-enriched fractions prepared from *Drosophila melanogaster* embryos. Results of indirect immunofluorescence experiments suggest that this protein, here designated p75, is primarily associated with puffed regions of larval salivary gland polytene chromosomes. In nonpolytenized Schneider 2 tissue culture cells, p75 appeared to be localized throughout the nuclear interior during interphase. In mitotic cells, p75 was redistributed diffusely. A possible role for karyoskeletal elements in transcriptional regulation is discussed.

In recent years, it has become clear that the nucleus contains a proteinaceous substructure or karyoskeleton apart from the nucleic acids and membranous components. At the nuclear periphery, the karyoskeleton is represented by a network of intermediate filamentlike fibrils (1), termed the nuclear lamina. Major polypeptide components, known as lamins, have been identified in *Drosophila melanogaster* (36, 37) as well as a number of vertebrates (for reviews, see references 20 and 23). Nuclear pore complexes, passageways for the regulated exchange of macromolecules between nucleus and cytoplasm, are anchored to the nuclear lamina and are also apparently made up of a number of distinct proteins (12, 16, 17, 24, 38). The characteristic morphology of the nuclear lamina and pore complexes as well as the recently discovered homologies between the nuclear lamins and cytoplasmic intermediate filament proteins (18, 32) substantiate the designation of lamina and pore complexes as bona fide karyoskeletal elements, apparently homologous to cytoskeletal elements located outside the nucleus.

The existence of karyoskeletal elements in the nuclear interior has been a subject of intense controversy during the past decade. On the one hand, it has been possible to isolate a relatively amorphous structural entity distributed throughout the interior of nuclei that have been subjected to the same conditions as those required to identify nuclear pore complexes attached to an isolated lamina (19). On the other hand, it remains uncertain to what degree this internal structure, variously termed the internal nuclear matrix, nuclear cage, or nuclear scaffold, is representative of bona fide karyoskeletal elements of the intact cell or results from one or several more or less random denaturation artifacts (15, 30).

Recently, we reported the identification of DNA topoisomerase II as a major polypeptide component of karyoskeletal protein-enriched fractions prepared from *Drosophila* embryos (7). In situ analyses indicate that in contrast to the lamins, topoisomerase II is distributed throughout the interior of interphase nuclei. By examination of squash preparations derived from larval salivary gland giant cells, topoisomerase II appears to be distributed diffusely along the polytene chromosomes (7, 25). A karyoskeletal role for

DNA topoisomerase II has been proposed (30). Consistent with this suggestion, others have found that DNA topoisomerase II appears to be localized along the axes of metaphase chromosomes and isolated metaphase chromosome scaffolds (13, 14, 22).

In the present article, we report the identification of a novel component of karyoskeletal protein-enriched fractions prepared from *D. melanogaster*. Like DNA topoisomerase II, this protein, designated p75, appears to be distributed more or less diffusely throughout the interior of diploid nuclei. However, in contrast with DNA topoisomerase II, analyses of larval polytene chromosomes suggest that p75 is concentrated at transcriptionally active loci. During mitosis, p75 is redistributed diffusely throughout the mitotic cell. A possible role for the karyoskeletal association of protein factors in transcriptional regulation is discussed.

MATERIALS AND METHODS

Specific immunoglobulin G (IgG) fractions were from Cappel Laboratories (Cochranville, Pa.). Antibodies directed against the two largest subunits of *Drosophila* RNA polymerase II (39) were the generous gift of Arno Greenleaf (Duke University). Monoclonal antiribonucleoprotein (anti-RNP) antibodies (8) were obtained either from Terence Martin (University of Chicago) or Gary Zieve (Stony Brook). Antilamin antibodies were affinity purified as previously described (37) by using a cloned lamin- β -galactosidase fusion protein coupled to CNBr-activated Sepharose. Nitrocellulose was from Schleicher and Schuell, Inc. (Keene, N.H.). Acrylamide, methylene bisacrylamide, and Tri-X pan ASA 400 film were from Eastman Kodak Co. (Rochester, N.Y.). DNase I, phenylmethylsulfonyl fluoride, *N*-ethylmaleimide, L-1-tosylamide-2-phenylethylchloromethyl ketone, cyanogen bromide, DL-dithiothreitol, polyoxyethylene sorbitan monolaureate (Tween 20), fraction V bovine serum albumin (BSA), and calf alkaline phosphatase were from Sigma Chemical Co. (St. Louis, Mo.). CNBr-activated Sepharose CL-4B was from Pharmacia Fine Chemicals (Piscataway, N.J.). Hydroxylapatite Bio-Gel HTP was from Bio-Rad Laboratories (Richmond, Calif.). Formaldehyde and formic acid were from Fisher Scientific Co. (Springfield, N.J.). 5-Bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt, and *p*-nitro blue tetrazolium chloride were from United

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States Biochemical Corp. (Cleveland, Ohio). RNase A (RAF grade) was from Worthington Biochemical Corp. (Freehold, N.J.). Sodium dodecyl sulfate (SDS) was from British Drug House (Poole, England). All other chemicals were obtained commercially and were of reagent grade.

Many of the methods, including the explicit protocol for purification of the nuclear matrix-pore complex-lamina (NMPCL) fraction from *D. melanogaster* embryos, have been described previously (19, 30). *D. melanogaster* wild-type Oregon R strain P2 were grown in mass culture, and embryos were collected by the method of Allis et al. (2). SDS-polyacrylamide gel electrophoresis (PAGE) was done essentially by the method of Laemmli (27) with modifications (19). Proteins were transferred passively from SDS-polyacrylamide gels onto nitrocellulose sheets (19), and the resultant blots were probed with specific antibodies essentially by the method of Blake et al. (9) as modified (36) and as detailed in the individual figure legends. Calf alkaline phosphatase was glutaraldehyde conjugated to affinity-purified goat anti-IgG antibodies by the method of Avrameas (4); colorimetric detection of alkaline phosphatase activity on blots was done by the method of McGadey (31). Cyanogen bromide digestion of SDS-denatured polypeptides was done essentially as described by Nikodem and Fresco (34) as described previously (6). SDS-hydroxylapatite chromatography of SDS-denatured polypeptides was done essentially by the method of Moss and Rosenblum (33) and as described previously (5), with the following modifications. Samples were not alkylated prior to loading on the column, and all buffers included 1 mM dithiothreitol.

Affinity purification of anti-p75 antibodies. p75 was partially purified by SDS-hydroxylapatite chromatography (see Fig. 2) and peak fractions (50 to 68) were pooled and concentrated by trichloroacetic acid precipitation. p75 was further purified by preparative SDS-PAGE (7% polyacrylamide). The gel was stained with Coomassie brilliant blue (0.25%) in water and destained briefly, and the 75-kilodalton (kDa) band was identified and excised. The gel slice was homogenized with a tight-fitting Teflon pestle in 4 ml of water, after which the homogenate was adjusted to a final concentration of 100 mM NaHPO₄, pH 6.8, and 0.1% SDS. p75 was shake-eluted from the gel with three overnight incubations in 30 ml each of 100 mM NaHPO₄, pH 6.8, and 0.1% SDS. The pooled eluates were concentrated by SDS-hydroxylapatite chromatography and trichloroacetic acid precipitation, and p75 was resolubilized in 0.8 ml of 0.2 M NaHCO₃ (pH 9.5)–0.1% SDS. Approximately 400 µg of p75 was recovered in this way, all of which was coupled to 0.8 ml of CNBr-activated Sepharose CL-4B (Pharmacia) as described (29). Affinity purification of anti-p75 antibodies from unfractionated antiserum was done as described by Smith et al. (37).

Salivary gland squashes. Third instar larvae from *D. melanogaster* grown at 18°C were the source of salivary glands for squash preparations. For all of the experiments shown, salivary glands were dissected and fixed in 45% acetic acid containing 3.7% formaldehyde; this and subsequent steps were performed at room temperature unless otherwise specified. Within 1 min the glands were transferred to a drop of a 15% acetic acid–25% lactic acid solution on a clean cover slip and incubated for up to 2 min. The cover slip was then picked up with a clean slide, and the chromosomes were spread by tapping on the cover slip with the eraser end of a pencil and finally by application of continuous thumb pressure. Afterwards, the slide was frozen in liquid nitrogen, the cover slip was pried off, and the slide was immersed in 95%

ethanol for 10 min. Similar results were obtained with chromosomes fixed only in 140 mM NaCl–10 mM KHPO₄, pH 7.5 (PBS), containing 3.7% formaldehyde, as well as with PBS-formaldehyde, followed by 45% acetic acid (not shown). After fixation, slides were washed twice for 10 min each in PBS and then probed with antibody as described below.

Indirect immunofluorescence. Indirect immunofluorescence analyses were performed with permeabilized whole *Drosophila* Schneider 2 tissue culture cells (35), salivary gland squashes, and cryosections of third instar larvae. Exponentially growing Schneider cells were grown on gelatin-coated cover slips in Schneider medium (35). Cells were fixed and permeabilized by incubation in PBS containing 1% Triton X-100 and 3.7% formaldehyde for 1 min, followed by incubation in 45% acetic acid–3.7% formaldehyde for 1 min, and a final incubation in 95% ethanol–1% acetic acid for 10 min; this and subsequent procedures were performed at room temperature unless specified otherwise. Prior to incubation with the primary antibody, cover slips were washed three times for 3 min each in 140 mM NaCl–10 mM KHPO₄ (pH 7.5)–0.1% Triton X-100–0.02% SDS–10 mg of BSA per ml (BSA-Ab buffer). Cover slips were placed in the wells of microtiter plates and incubated with the primary antibody, diluted in BSA-Ab buffer as described in the figure legends, for 30 min at 37°C. Cover slips were washed for a total of 10 min in three changes of BSA-Ab buffer and then incubated with affinity-purified rhodamine-conjugated goat anti-rabbit IgG diluted in BSA-Ab buffer as described for the primary antibody. Cover slips were finally washed for a total of 10 min in three changes of 560 mM NaCl–10 mM KPO₄ (pH 7.5)–0.1% Triton X-100–0.02% SDS as described previously (19). Salivary gland squashes were probed with antibodies for immunofluorescence analyses essentially as described for tissue culture cells. Cryosections of *Drosophila* third instar larvae were prepared and processed for immunofluorescence analyses exactly as described previously (30, 36). Specimens were examined and photographed with a Leitz Ortholux II epifluorescence microscope and an Orthomat W camera.

RESULTS

Antisera raised against SDS-PAGE-purified 74-kDa *Drosophila* NMPCL protein(s) decorate both the nuclear envelope and multiple specific loci on larval polytene chromosomes. Antisera prepared against the major protein band at the 74-kDa position of one-dimensional SDS–7 to 15% polyacrylamide gradient gels of the *Drosophila* NMPCL fraction stain the nuclear periphery of a number of *Drosophila* cell and tissue types (19). The polypeptide components of this 74-kDa band can be resolved into two major species on low-percentage continuous-concentration polyacrylamide gels (36). Recently, these two polypeptides were identified as nuclear lamin isoforms Dm₁ and Dm₂. Both are derived posttranslationally from a common polypeptide precursor, lamin Dm₀ (37).

When some of these original anti-74-kDa polypeptide(s) antisera (19) were used to probe squashes of larval salivary gland giant cells in addition to the nuclear envelope, they apparently recognized multiple specific loci on the polytene chromosomes (Fig. 1a and b). However, the results of initial experiments with affinity-purified antilamin antibodies obtained from such antisera to probe larval cryosections (7) suggested that the polytene chromosome-staining activity had been lost in the affinity purification process. The lack of

FIG. 1. Antiserum directed against the 74-kDa NMPCL polypeptide(s) stains both the nuclear envelope and larval polytene chromosomes; affinity-purified antilamin IgG does not decorate polytene chromosomes. Salivary glands were dissected from larvae grown at 18°C, gently squashed, and prepared for indirect immunofluorescence as described in Materials and Methods. (a and b) Samples were probed with antiserum diluted 1:100. (a) Phase contrast; (b) fluorescence micrographs. The bar in panel b indicates 50 μ m and applies to both panels a and b. (c and d) Samples were probed with affinity-purified antilamin antibodies at a dilution of 1:100 relative to the unfractionated serum from which they were derived. (c) Phase contrast; (d) fluorescence. Film exposure for panel d was identical to that for panel b. The bar in panel d indicates 50 μ m and applies to both panels c and d.

reactivity of affinity-purified antilamin IgG with larval polytene chromosomes was confirmed by using squash preparations (Fig. 1c and d). There were at least two plausible explanations for this observation. First, it seemed possible that lamins or laminlike antigens might indeed be associated with multiple specific chromosomal loci and that the loss of the chromosome-staining activity was an artifact of affinity purification. Alternatively, we considered it likely that the original immunogen, a band of protein excised from the 74-kDa position on a one-dimensional SDS gel, contained other polypeptide species in addition to the lamins and that the antisera with which we were working were, hence, polyspecific.

SDS-hydroxylapatite chromatography of the *Drosophila* NMPCL fraction resolves the 74-kDa band into two antigenically distinct polypeptide species. To address the question of antigenic purity, we first used SDS-hydroxylapatite chromatography to resolve the *Drosophila* NMPCL fraction according to a second dimension of analysis. Based on the results of preliminary experiments, we eluted the SDS-hydroxylapatite column with a 0.1 to 0.3 M NaHPO_4 gradient; this was followed by 0.5 M NaHPO_4 step elution. Immunoblot analysis is shown in Fig. 2. The blot in Fig. 2A was probed with unfractionated anti-74-kDa NMPCL polypeptide antiserum; an identical blot, prepared in parallel, was probed with affinity-purified antilamin IgG. The unfractionated antiserum recognized a major species eluting between fractions 50 and 70 of this chromatogram as well as a polypeptide in the 0.5 M NaHPO_4 step. In contrast, the affinity-purified antilamin antibodies recognized only the 74-kDa polypeptide that eluted in the final step fraction.

Coomassie blue staining (not shown) demonstrated the presence of a major band of protein, henceforth designated p75, which coeluted with the immunoreactive material in fractions 50 to 70. Overall, this represented approximately 400 μ g, about 20% as much as the lamins obtained from the same preparation. Moreover, it appeared from the immunoblot in Fig. 2B that the column-purified p75 was completely free of contaminating lamin antigen. We therefore used the protein in fractions 50 through 68 of the profile shown to prepare an affinity column with CNBr-activated Sepharose as described in Materials and Methods.

Affinity purification and characterization of anti-p75 anti-

bodies. Specific anti-p75 antibodies were affinity purified from unfractionated anti-74-kDa NMPCL polypeptide antiserum by standard procedures (37; Materials and Methods) and compared with affinity-purified antilamin antibodies (Fig. 3). Immunoblots were prepared with either SDS-hydroxylapatite-purified lamins (lanes a) or SDS-hydroxylapatite-purified p75 (lanes b). Parallel strips were then probed with either unfractionated anti-74-kDa NMPCL polypeptide antiserum (lanes S), affinity-purified antilamin antibodies (lanes L), affinity-purified anti-p75 antibodies (lanes p75), or preimmune serum (lanes PI). As expected, the unfractionated serum reacted with both the lamins and p75. In contrast, the affinity-purified antilamin antibodies recognized only the lamins, while the affinity-purified anti-p75 antibodies recognized only p75. Preimmune serum controls were negative for both antigens. When affinity-purified anti-p75 antibodies were used to stain salivary gland squashes such as those shown in Fig. 1, only staining of specific chromosomal loci was observed. Detailed mapping of some of those loci is presented in Fig. 7 (see below). As already noted, and in contrast, the affinity-purified antilamin antibodies stained only the nuclear periphery (not shown; for examples, see references 5, 30, and 36) and were unreactive with polytene chromosomes (Fig. 1c and d).

CNBr peptide map comparison of p75 and the lamins. To complete the biochemical comparison between the nuclear lamins and p75, we used CNBr peptide mapping experiments. SDS-hydroxylapatite-purified p75 and lamins were partially digested with CNBr as described in Materials and Methods. The peptides were separated by SDS-7 to 15% polyacrylamide gradient PAGE, blot-transferred to nitrocellulose, and probed with appropriate affinity-purified antibodies (Fig. 4). No obvious or certain homologies were apparent after comparing the maps for these two proteins.

Identification of p75 as a polypeptide component of the NMPCL fraction. *Drosophila* NMPCL components are operationally defined as those proteins which resist extraction from isolated nuclei following nuclease digestion and extraction with Triton X-100 and 1 M NaCl. Most recently, we suggested that this definition be extended to stipulate that resistance to such extraction procedures depend on prior exposure of nuclei to moderately elevated temperatures (37°C) either in vitro or in vivo (i.e., heat shock [30]). We

FIG. 2. Quantitative separation of p75 from the lamins by SDS-hydroxylapatite chromatography. The NMPCL fraction was subjected to SDS-hydroxylapatite chromatography as described in Materials and Methods; 80 mg of protein was loaded onto a 25-ml column, which was subsequently eluted with a 0.1 to 0.3 M NaH_2PO_4 gradient. Following gradient elution, protein remaining bound to the column was eluted with one column-volume of 0.5 M NaH_2PO_4 . Shown are immunoblots of two gels run in parallel; 20- μl samples of the fractions indicated above each lane, including the 0.5 M step fraction, were subjected to SDS-PAGE (7% polyacrylamide) and immunoblot analysis. The blot in panel A was probed with the unfractionated anti-74-kDa NMPCL polypeptide antiserum diluted 1:1,000. The blot in panel B was probed with approximately 1 μg of affinity-purified antilamin IgG.

therefore set out to evaluate the behavior of p75 according to this paradigm.

Immunoblot analysis of the subnuclear distribution of p75 after nuclease digestion and subfractionation of nuclei as well as the effects of temperature on this distribution are shown in Fig. 5. As a control, parallel blots made from identical fractions were probed with antilamin antibodies (Fig. 5B). For both proteins, nuclease digestions were done at either 23 or 37°C as indicated. We have previously shown that the thermal stabilization of isolated nuclei is independent of whether the thermal incubation conditions are established prior to or during nuclease digestion (30).

Fractionation of p75 was indeed dependent on the temperature of incubation, and when such incubations were performed at 37°C, almost 100% of the detectable antigen was recovered in the NMPCL fraction (Fig. 5A). This was in contrast to the lamins (Fig. 5B). As reported previously (30), only approximately 40% of nuclear lamins Dm₁ and Dm₂ were associated with the NMPCL fraction under these conditions. When incubations were performed at 23°C, most of the nuclear p75 was recovered in the nuclease supernatant following digestion (Fig. 5A). This is again in contrast to the lamins, which were found exclusively in the 1 M salt supernatant following 23°C incubation (Fig. 5B). A likely interpretation of these data is that in the nucleus *in vivo*, p75 is primarily associated with chromatin, whereas lamins are largely involved in extrachromosomal protein-protein interactions. Hence, prior to thermal stabilization, p75 can be

extracted by fragmentation of the nucleic acid with nucleases, while lamins remain in a nucleic acid-independent structure. However, incubation of nuclei for 15 min at 37°C drives p75 into stable association with the NMPCL fraction. Presumably, this association reflects protein-protein interactions.

In situ localization of p75 during interphase and mitosis. To determine the subcellular localization of p75 in diploid (i.e., nonpolytenized) cells, we performed indirect immunofluorescence analyses on cryosections of *Drosophila* third instar larvae and on permeabilized *Drosophila* Schneider 2 tissue culture cells (Fig. 6). Diamidinophenylindole (DAPI) staining of the DNA is shown in panels a, c, e, and g. Immunofluorescence staining is shown in panels b, d, f, and h. Results obtained with larval cryosections (Fig. 6a and b) suggested that p75 was distributed more or less diffusely throughout the nuclear interior. This is in contrast with the exclusively peripheral localization of the lamins, as reported previously (5, 30, 36). Results obtained with permeabilized whole Schneider cells (Fig. 6e and f) also demonstrated a staining pattern consistent with internal nuclear localization. Preimmune controls shown for the cryosections and Schneider cells in panels c and d and panels g and h, respectively, were negative.

With the permeabilized Schneider cells, it was also possible to determine the localization of p75 during mitosis. Mitotic cells can be easily identified by DAPI staining, as indicated by the arrows in Fig. 6e and g. When cells were

stained with anti-p75 antibodies, immunofluorescence was observed throughout the mitotic cell (arrow, Fig. 6f). In this respect, p75 apparently resembles the *Drosophila* lamins (7).

p75 is associated with transcriptionally active loci of larval polytene chromosomes. After close examination of salivary gland squashes probed with affinity-purified anti-p75 antibodies, we found that the most intense immunofluorescence staining coincided with puffed regions of the chromosomes, suggesting that p75 might be involved in transcription. Detailed mapping of some of these loci is presented in Fig. 7. Salivary glands were obtained from larvae grown at 18°C. At low magnification, it was possible to recognize a complete grouping of chromosomes (Fig. 7A and B). By careful inspection, we could identify more than 100 bands of varying intensity. We focused our analysis on the left arm of chromosome 3 (Fig. 7C to F). Landmark puffs at positions 74 and 75 (Chinese lantern puffs [28]) stained intensely with antibody (Fig. 7C and D). This staining pattern was reproducible, as demonstrated in a separate but identical experiment (Fig. 7E and F). In addition to regions 74 and 75, regions of less intense antibody reactivity were also stained reproducibly (compare bands of antibody staining labeled a to h in panels D and F).

To further correlate the localization of p75 with transcription, we performed indirect immunofluorescence analyses similar to those described above on larvae which had been subjected to heat shock (not shown). Heat shock is known to result in transcriptional reprogramming, so that essentially all normal transcription ceases and new transcription is initiated at a defined set of heat shock loci (for a review, see reference 3). We therefore reasoned that if p75 were impor-

FIG. 3. Lamins and p75 are immunochemically distinct polypeptides. The lamins and p75 were purified from each other by SDS-hydroxylapatite chromatography as described in the legend to Fig. 2. Samples of each were electrophoresed separately on preparative SDS-7% polyacrylamide gels, and proteins were blot-transferred to nitrocellulose. After transfer, strips were cut from the nitrocellulose perpendicular to the protein bands. Strips labeled a were cut from the blot to which lamins had been transferred; strips labeled b contained p75. Strips were probed with various antibody fractions as follows: S, unfractionated anti-74-kDa NMPCL polypeptide antiserum; L, affinity-purified antilamin IgG; p75, affinity-purified anti-p75 IgG; PI, preimmune IgG.

FIG. 4. CNBr peptide map comparison of p75 and lamins; SDS-7 to 15% polyacrylamide gradient PAGE and immunoblot analysis. Lanes a and b were loaded with SDS-hydroxylapatite-purified p75 prior to (lane a) and after 2 h (lane b) of treatment with CNBr. Lanes c and d were loaded with SDS-hydroxylapatite-purified lamins prior to (lane c) and after 2 h (lane d) of treatment with CNBr. The blot containing lanes a and b was probed with affinity-purified anti-p75 IgG; the blot containing lanes c and d was probed with affinity-purified antilamin IgG.

tant in transcription, we might observe significant redistribution of p75 in response to heat shock. (It has been demonstrated that RNA polymerase II is apparently redistributed to heat shock loci under similar circumstances [10].) At low magnification, it could be seen that the number of intensely stained loci was dramatically reduced compared with results obtained prior to heat shock. Moreover, the two most intensely labeled bands could be tentatively identified as heat shock loci 87A and 87C. No assignment of the other stained loci could be made.

DISCUSSION

In this article, we have reported the identification of p75, a novel component of the *Drosophila* NMPCL fraction. Cell fractionation experiments indicate that under conditions known to promote structural stability of proteinaceous karyoskeletal elements in vitro (30), almost 100% of the p75 found in isolated nuclei is NMPCL associated. Results of indirect immunofluorescence analyses suggest that, like DNA topoisomerase II (7, 30), p75 is distributed throughout the nuclear interior. However, in contrast to DNA topoisomerase II, p75 appears to be concentrated at transcriptionally active loci of larval polytene chromosomes. Together, these observations define what appears to be a member of a new class of NMPCL polypeptide, i.e., one which is distributed throughout the nuclear interior like topoisomerase II but is nevertheless concentrated at specific chromosomal loci.

FIG. 5. Effect of 23 versus 37°C thermal incubation of nuclei on subnuclear distribution of p75 and the lamins. Nuclei were isolated from *Drosophila* embryos as described in Materials and Methods. NMPCL fractions were prepared following nuclease digestion of purified nuclei at either 23 or 37°C as indicated. Nuclear subfractions are designated as follows: N, nuclei; NS, nuclease supernatant; TXS, Triton X-100 supernatant; SS-1, first salt supernatant; SS-2, second salt supernatant. Four units (19) of each fraction were prepared for SDS-7% polyacrylamide PAGE and analyzed by immunoblotting. Fractions are designated above each lane. The blots in panel A were probed with affinity-purified anti-p75 IgG. The blots in panel B were probed with affinity-purified antilamin IgG.

During mitosis, the nuclear lamina disassembles and polypeptide components redistribute throughout the cell (for a review, see reference 23). DNA topoisomerase II seems to have a dual localization, with a portion being redistributed in a manner similar to the lamins (7), while a second fraction condenses with the chromatin to form the mitotic chromosome scaffold (13, 22). Based on the results shown in Fig. 6, it seems that p75 is redistributed along with the lamins. However, under the conditions of immunofluorescence analysis used, the identification of DNA topoisomerase II as a component of the mitotic chromosomes was obscured by the surrounding cytoplasmic staining (7). Therefore, we cannot exclude the presence of a portion of p75 in mitotic chromosomes.

Is p75 a karyoskeletal protein? The nuclear lamina has long been held to be a bona fide karyoskeletal element, based on its precise morphology and more recently on homologies

discovered between lamins and intermediate filament proteins (18, 32), as well as on the ability of lamina fibrils to assemble from solubilized, purified lamins in vitro (1). The designation of DNA topoisomerase II as a karyoskeletal protein is less certain. Nevertheless, we believe that the operational definition of DNA topoisomerase II as an NMPCL component has been rigorous and determined in parallel with comparable investigations of lamins.

The recently discovered effects of in vitro thermal incubation on the structural stability of isolated karyoskeletal elements (30) provides an additional operational criterion by which to evaluate NMPCL components. In this respect, lamins and DNA topoisomerase II behave identically (30). p75, while similar in its behavior, is not identical. In the absence of thermal stabilization, lamins and topoisomerase II are resistant to extraction with either nucleases or non-ionic detergents but can be quantitatively solubilized with 1

FIG. 6. Indirect immunofluorescence localization of p75 in third instar larval cryosections and in permeabilized Schneider 2 tissue culture cells. (a and c) DAPI-stained specimens; (b and d) immunofluorescence micrographs of third instar larval cryosections; (e and g) DAPI-stained specimens; (f and h) immunofluorescence micrographs of permeabilized whole Schneider 2 tissue culture cells. Samples in panels a, b, e, and f were probed with affinity-purified anti-p75 IgG at a titer of specific IgG equivalent to the unfractionated serum at 1:100. Samples in panels c, d, g, and h were probed with preimmune serum at 1:100. Arrows in panels e, f, and g indicate mitotic cells. Scale bar in panel d indicates 40 μ m and applies to panels a to d; bar in panel h indicates 12 μ m and applies to panels e to h.

M NaCl. In contrast, p75 is largely solubilized from isolated nuclei by nuclease digestion alone. This behavior of p75 seems inconsistent with a primary role as a karyoskeletal protein. Upon thermal incubation, however, p75, like the lamins and DNA topoisomerase II, is driven into tight association with the NMPCL components, so that it is among the most salt-resistant polypeptides in the fraction. It

remains to be determined whether this association is of any biological significance.

Is p75 a known protein or transcription factor? After its initial identification, we considered the possibility that p75 had been described previously in some other context. In this regard we performed a number of preliminary experiments. We first assessed whether p75 might be either a subunit or

FIG. 7. Indirect immunofluorescence localization of p75 in salivary gland polytene chromosomes taken from larvae grown at 18°C. (A, C, and E) Phase-contrast; (B, D, and F) fluorescence. Salivary glands were dissected from larvae grown at 18°C and subsequently fixed, squashed, and processed for immunofluorescence as described in Materials and Methods. Samples in all panels were probed with affinity-purified anti-p75 IgG as described in the legend to Fig. 6. Regions 74 and 75 of chromosomal arm 3L are indicated in panels D and F. The arrows labeled a through h in panels D and F show regions of antibody reactivity that were reproducibly obtained in such experiments. The scale bar in panel B indicates 50 μ m and applies to panels A and B; the bar in panel D indicates 7 μ m and applies to panels C and D; the bar in panel F indicates 10 μ m and applies to panels E and F.

subunit-derived breakdown product of RNA polymerase II. These possibilities were excluded as follows. Immunoblots comparable to those shown in Fig. 5 probed with antibodies directed against either the 140- or the 215-kDa subunit of *Drosophila* RNA polymerase II (39) failed to demonstrate any cross-reactivity between the polymerase II subunits and p75. Moreover, identification of the bona fide polymerase II subunits in these experiments revealed a different pattern of cell fractionation from that observed for p75 (P. Fisher, unpublished).

To assess the possibility that p75 might be a known RNP protein, we prepared extracts from *Drosophila* nuclei by two published procedures (40, 41). The results of this experiment proved relatively uninformative. Although p75 was effectively extracted by both procedures, so were the lamins (B. Benton, unpublished). Results obtained with a monoclonal anti-RNP antibody with demonstrable specificity for a 70-kDa mammalian RNP protein (8) were somewhat more useful. This antibody stained *Drosophila* larval polytene chromosomes, but with a pattern different from that observed with anti-p75 antibodies. This antibody failed to recognize purified p75 on nitrocellulose blots (B. Benton, unpublished).

In contrast to the negative impressions developed from the few results outlined above, comparison of p75 with a *Drosophila* 74-kDa chromosomal antigen described by Hill et al. (26) revealed a number of similarities. In addition to virtual identity of apparent mass and localization at transcriptionally active loci on polytene chromosomes, two-dimensional gels run in our laboratory and probed with affinity-purified anti-p75 antibodies (B. Benton, unpublished) revealed a pattern which was qualitatively and quantitatively indistinguishable from that reported by Hill et al. (26). Direct immunochemical comparisons between the 74-kDa chromosomal antigen and p75 will be necessary to elucidate their interrelationship.

What role might karyoskeletal association of proteins such as p75 play at transcriptionally active loci in vivo? In conclusion, we offer the following hypothesis to account for our current observations about p75. It has been proposed (11, 21) that one role of the karyoskeleton is to increase the local concentration of *cis*-acting regulatory elements in its immediate vicinity. Weak karyoskeletal binding sites for proteins may also exist, so that a second role might be to recruit *trans*-acting factors to the same regions through specific protein-protein interactions. It may be these interactions that are stabilized by incubation of nuclei at 37°C. Detailed chemical and biochemical analyses of these protein-protein interactions should be a focus for further investigation to address this question directly. A more complete molecular definition of putative internal karyoskeletal elements will be a prerequisite to these analyses.

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