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Localization and Posttranslational Modifications of Otefin, a Protein Required for Vesicle Attachment to Chromatin, during *Drosophila melanogaster* Development

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Otefin is a peripheral protein of the inner nuclear membrane in *Drosophila melanogaster*. Here we show that during nuclear assembly in vitro, it is required for the attachment of membrane vesicles to chromatin. With the exception of sperm cells, otefin colocalizes with lamin Dm_0 derivatives in situ and presumably in vivo and is present in all somatic cells examined during the different stages of *Drosophila* development. In the egg chamber, otefin accumulates in the cytoplasm, in the nuclear periphery, and within the nucleoplasm of the oocyte, in a pattern similar to that of lamin Dm_0 derivatives. There is a relatively large nonnuclear pool of otefin present from stages 6 to 7 of egg chamber maturation through 6 to 8 h of embryonic development at 25°C. In this pool, otefin is peripherally associated with a fraction containing the membrane vesicles. This association is biochemically different from the association of otefin with the nuclear envelope. Otefin is a phosphoprotein in vivo and is a substrate for in vitro phosphorylation by cdc2 kinase and cyclic AMP-dependent protein kinase. A major site for cdc2 kinase phosphorylation in vitro was mapped to serine 36 of otefin. Together, our data suggest an essential role for otefin in the assembly of the *Drosophila* nuclear envelope.

In eukaryotic cells, DNA replication and RNA processing occur in the nucleus, while protein synthesis occurs in the cytoplasm. These activities are physically separated by the nuclear envelope, which is composed of inner and outer membranes, nuclear pores, and a nuclear lamina (reviewed in reference 18). The nuclear lamina is located between the inner nuclear membrane and the peripheral chromatin (reviewed in references 13, 17, and 31). The best-studied proteins of the nuclear lamina are the lamins. The composition of lamina varies in different cell types and is under developmental regulation in both vertebrate (18) and in invertebrate (9, 38) cells. Two lamin genes are known in *Drosophila melanogaster*, and they code for lamins Dm_0 and C (5, 19). During development, lamin Dm_0 derivatives are expressed in most cells, while lamin C is first detected in late stage 12 embryos in oenocytes, hindgut and posterior spiracles, and, subsequently, in other differentiated tissues (39). *Drosophila* lamin Dm_0 undergoes posttranslational modifications that give rise to at least three distinct isoforms, termed Dm_1, Dm_2, and Dm_{mit}, which differ in their phosphorylation patterns. Dm_1 and Dm_2 are polymeric and are present in most types of interphase nuclei (43, 46). Dm_{mit} is soluble (depolymerized) and is present in the maternal pool and in mitotic cells (44).

The nuclear lamins show affinity to several proteins of the inner nuclear membrane, including LBR (also referred to as p58/p54) (2, 51), LAP 1 (14), LAP 2 (14), and otefin. Otefin is a 45-kDa protein with a mobility of 53 kDa upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). It is localized peripherally on the nucleoplasmic side of the *Drosophila* inner nuclear membrane and colocalizes with lamin Dm_0 in interphase nuclei and during mitosis (1, 20, 35). Otefin has no apparent homology to known proteins. It is highly hydrophilic with a relatively high content of serine and threonine residues, and it includes several putative sites for phosphorylation by cdc2 kinase and protein kinase A (PKA).

There is evidence that lamins and lamina-associated proteins are involved in nuclear envelope assembly. Antibodies directed against A- or B-type mammalian lamins blocked vesicle binding to chromatin in mammalian cell extracts (8). Similarly, anti-lamin Dm_0 antibodies blocked the interaction between vesicles and chromatin in a *Drosophila* cell-free system (48). The role of lamin proteins in the association between nuclear vesicles and chromatin in *Xenopus laevis* extracts is controversial. Depletion of lamin B3 from the assembly extract did not prevent formation of nuclear envelopes consisting of membranes and nuclear pores. These lamin B3-depleted nuclei were small, fragile, and failed to replicate their DNA (22, 29, 33). In contrast, Dabauvalle et al. (11) were able to block the formation of nuclear envelopes by using an antibody directed against both lamins B2 and B3. A major reason for the discrepancy between the above studies could be that *Xenopus* extracts contain lamin B2, in addition to lamin B3 (26 [reviewed in references 31 and 50]). LAP 2 protein associates with chromosomes prior to assembly of most lamins, which suggests a role for LAP 2 in initial events of nuclear envelope reassembly (14). A recent study suggests a role for LBR in providing the docking sites of vesicles to chromatin (37).

In the present study, possible otefin function(s), its distribution during *Drosophila* development, its association with the membrane vesicle pool, and its possible posttranslational modifications were investigated. To analyze otefin function(s), a *Drosophila* embryonic extract competent to assemble nuclei in vitro was incubated with antiotefin antibodies. Analysis of the

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assembly process in extracts depleted of otefin activity revealed that although chromatin decondensation proceeded normally, membrane vesicles did not bind to the surface of the chromatin. From stages 6 to 7 of *Drosophila* egg chamber maturation until 6 to 8 h of embryonic development, otefin is found in large amounts in the cytoplasm, as well as in the nuclear envelope. In the nuclear envelope, the relative amounts of otefin and lamin Dm_0 derivatives remain roughly constant. During egg chamber development, otefin is also localized to the nucleoplasm, similar to lamin Dm_0 derivatives (44). In the cytoplasm, otefin is bound to the membrane vesicles in a salt-sensitive manner. Otefin and lamin Dm_0 derivatives are present in all cells examined from embryos, larvae, and adult flies, with the exception of sperm cells. In highly polytenized cells, such as salivary glands, fat, and hindgut, otefin appears in reduced amounts compared with those in diploid cells. Otefin is phosphorylated in vivo in *Drosophila* Kc cells and can be phosphorylated in vitro by cdc2 kinase and by PKA. The major site for cdc2 kinase phosphorylation in vitro is serine 36. Our results suggest that in *Drosophila*, otefin activity is required, in addition to lamin activity, for the proper attachment and assembly of the nuclear membrane vesicles around decondensed chromatin.

MATERIALS AND METHODS

Antibodies. A monoclonal antibody (MAb) specific for *Drosophila* lamin Dm_0 derivatives (611A3A6), a MAb specific for *Drosophila* otefin (618A2O7), and affinity-purified polyclonal antibodies specific for *Drosophila* lamin Dm_0 have been previously described (20, 30, 44). Affinity-purified polyclonal anti-*Drosophila* otefin antibodies were prepared by injecting 500 μg of bacterially expressed otefin into rabbits. Purified immunoglobulin G (IgG) was obtained by chromatography on a protein A–Sepharose CL-4B column (Pharmacia). The IgG fraction was then dialyzed against 0.1 M NaH_2PO_4 (pH 6.8) and loaded on a column of otefin bound to CNBr-Sepharose 4B (Pharmacia). The immunoaffinity-purified antibodies were eluted from the column with 0.1 M triethanolamine (pH 11.6) into 0.1 M NH_2PO_4 (pH 6.8). Cy_3A-conjugated goat anti-rabbit IgG (heavy and light chains [H+L]), Cy_3A-conjugated rabbit anti-mouse IgG, and alkaline phosphatase-conjugated goat anti-rabbit IgG (H+L) were purchased from Jackson Labs, West Grove, Pa.

Nuclear assembly extracts and inactivation of otefin. The preparation of extracts from 0- to 6-h-old *Drosophila* embryos and sperm chromatin was performed exactly as described in reference 48. To inhibit otefin activity in the embryonic extract, anti-*Drosophila* otefin antibodies were preincubated with occasional mixing for 60 to 90 min on ice with extracts supplemented with an ATP-regenerating system. A typical assay included 20 μl of otefin-inactivated crude extract or cytoplasm centrifuged at 150,000 × *g*, 3 μl of the ATP-regenerating system, and 1 μl of sperm chromatin (~1,000 cells). The reaction mix was then incubated for an additional 2 to 3 h at 22°C. Preparation of samples for fluorescence microscopy or for electron microscopy was performed essentially as described previously (48).

Isolation and subfractionation of *Drosophila* egg chambers. *Drosophila* (Oregon R or Canton S) egg extracts were prepared as previously described (27). Briefly, virgin females (Oregon R) 3 to 7 days old were chilled on ice and homogenized in phosphate-buffered saline (PBS) containing 140 mM NaCl, 10 mM K_2HPO_4 (pH 7.5), 10 mg of polyvinylpyrrolidone per ml, 1 mM phenylmethylsulfonyl fluoride, and 100 μM TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone). The homogenate was filtered through 250-μm-pore-diameter nylon mesh, and stage 11 to 14 egg chambers were then collected by passing the filtrate through 125-μm-pore-diameter nylon mesh. The filtrate was then passed over 64-μm-pore-diameter mesh which retained egg chambers from germarium through growth stage 10 (g-10). The dissected egg chambers were washed with DWB (240 mM β-glycerophosphate [pH 7.2], 60 mM EGTA, 45 mM $MgCl_2$, 1 mM dithiothreitol) and Dounce homogenized. Nuclei were separated by centrifugation at 10,000 × *g*. Subfractionation of the nonnuclear fraction into membrane-vesicle and membrane-free fractions was performed by centrifugation at 100,000 × *g* in a TLA-100 Beckman rotor at 4°C. Preparation of protein lysate and immunoblot analysis were performed as previously described (20).

Immunofluorescence analysis. Egg chambers were staged according to the method of King (24) and fixed in 0.4% formaldehyde for 30 min at 22°C. Salivary glands, fat cells, imaginal discs, and gut tissues were dissected from 3rd instar larvae. Testis and gut tissues were dissected from adult males (Canton S). Larva and adult tissues were fixed for 15 min at 22°C in PBS containing 3.7% formaldehyde and 1% Triton X-100. Tissues were then incubated with MAb 618A2O7 (antiotefin) or with MAb 611A3A6 (antilamin) and stained with Cy_3-conjugated rabbit anti-mouse IgG. DNA was stained with a 1-μg/ml solution of 4′,6′-diamidino-2-phenylindole (DAPI). Some of the egg chambers were then embedded in Tissue Tek (Miles Laboratories, Naperville, Ill.), immediately frozen at −80°C, and cut into 8-μm-thick sections at −20°C. Whole tissues and sections were covered with glycerol containing 2% *n*-propyl gallate and viewed in a Leitz microscope equipped with epifluorescence illumination and a 63×/NA = 1.4 Planapo oil immersion objective. Photographs were taken with Kodak Technical Pan 2415 film and developed with an HC110 developer.

Confocal microscopy. *Drosophila* egg chambers were immunolabeled as described above and viewed with an ACAS 570 (Meridian Instruments, Okemos, Mich.) confocal scanning microscope system, with a 100×/NA = 1.3 oil immersion objective. Step sizes were 0.7 μm for the *x* and *y* axes and 2 μm for the *z* axis. The pinhole was either 40 or 100 μm in diameter, and 32 illuminations per point were averaged. The excitation wavelength was at 514 nm, and the emission was collected as the reflected light from a 575-nm dichroic mirror. Larva and adult tissues were viewed with a Bio-Rad MRC-1024 confocal scanhead coupled to a Zeiss Axiovert 135M inverted microscope. A 63×/NA = 1.4 oil immersion objective was used. Excitation light was provided by a 100-mW air-cooled argon ion laser run in the multiline mode. The 514-nm line of the laser was used for excitation and was selected with an interference filter. The emission filter was D580/32 (32-nm bandpass centered about 580 nm). The confocal iris diameter was between 1.3 and 3.5 mm, with the larger opening used for weaker signals. Vertical resolution was between 0.5 and 1 μm, depending on the iris setting. If needed, two to four images were averaged in order to reduce point noise. Images of 512 × 512 pixels were acquired with a hardware zoom of 3 to 10 (0.106 to 0.032 μm/pixel). When needed, a median filter (3 by 3) was used to remove point noise from the digital images. The relative signals of lamin and otefin were determined by calculating the average fluorescence intensity per pixel in the region of the nuclear envelope, as resolved by the Image Pro for Windows software. These analyses were always performed with neighboring tissues in the same preparation.

Developmental immunoblot analysis. (i) Postembryonic stages. *Drosophila* larvae, pupae, or adults (0.3 ml) were collected and Dounce homogenized in homogenizing buffer (50 mM NaCl, 50 mM Tris-HCl [pH 7.5], 250 mM sucrose, 5 mM $MgCl_2$, 2.5 mM *N*-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM TPCK, 0.1 mM leupeptin) on ice. The homogenates were centrifuged at 4,000 × *g* for 10 min at 4°C. To the cytoplasmic supernatant, 0.3 ml of 5× SB buffer (400 mM Tris-HCl [pH 6.8], 12% SDS, 700 mM β-mercaptoethanol, 25% glycerol) was added, and the sample was incubated for 3 min at 95°C. The pellet was resuspended in 1× SB buffer to a final volume of 0.3 ml. The nuclear fraction was then incubated for 3 min at 95°C, followed by breakage of DNA by passing the sample through a 21-gauge needle several times. Amounts of supernatant and nuclear fractions equivalent to 5 to 10 μl of packed larvae, pupae, or adult flies from the same preparation were subjected to immunoblot analyses, with an SDS–8 to 14% polyacrylamide gradient gel and MAbs (antiotefin or antilamin), followed by incubation with ^{125}I-labeled sheep anti-mouse antibodies and exposure of the blot to X-ray films (Agfa).

(ii) Embryonic stages. Packed and dechorionated *Drosophila* embryos from the different ages were washed three times with buffer NM (250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl [pH 8], 1 mM dithiothreitol, 10 μM leupeptin, 10 μg of aprotinin per ml) and Dounce homogenized on ice. The homogenates were centrifuged at 15,000 × *g* for 15 min at 4°C. The nuclear pellet was washed three times in 5 volumes of buffer NM, the nuclei were brought to the initial volume of embryos with buffers NM and 4× SB, and the samples were heated for 5 min at 95°C. Subfractionation of the supernatant into a membrane-vesicle pellet fraction and a soluble supernatant fraction was performed as described previously (48). Possible contamination of the cytoplasmic fraction with nuclei was ruled out by staining with the DNA-specific dye DAPI and viewing with a fluorescence microscope. Amounts equivalent to 5 μl of packed embryos of nuclei, membrane vesicle pellet, or membrane-free cytoplasmic supernatant were subjected to SDS–10% PAGE and immunoblot analysis with specific MAbs.

Preparation and extraction of membrane vesicles. Membrane vesicles and soluble membrane-free cytoplasm were prepared as previously described (48). The purity of the membrane fraction was analyzed on a sucrose gradient and by electron microscopy (47a). Biochemical extractions from membrane vesicles were performed by washing the 150,000 × *g* pellet twice with buffer NM and then incubating the pellet with buffer NM modified as indicated in the legend to Fig. 5 for 30 min at 4°C. The supernatant and pellet were separated by centrifugation for 20 min at 150,000 × *g*. The pellet was brought to the initial volume of the homogenate, and equivalent volumes of pellet and supernatant were subjected to SDS–10% PAGE and immunoblot analysis with specific MAbs.

Expression of otefin in *E. coli* cells and its purification. The AAAATG translation start sequence in otefin cDNA (30) was PCR mutated to create an *Nde*I site. The *Nde*I-*Xmn*I fragment of this otefin cDNA was cloned into pET20b+ vector (Novagen). This created an in-frame fusion between amino acids 1 to 387 of otefin and the 6 consecutive histidines of the vector. *Escherichia coli* BL21 (DE3) cells were transformed with the otefin construct. Expression and purification of otefin were performed as previously described for lamin Dm_0 (52).

pGEXotef800 is a fusion construct between the glutathione *S*-transferase (GST) gene in pGEX-1 (42) and the *Bgl*II-*Eco*RII fragment of otefin. It encodes a fusion protein between the GST protein and amino acids 192 to 406 of otefin.

Expression and purification of this fusion protein were performed as described by Smith and Johnson (42).

In vivo phosphorylation and immunoprecipitation of otefin. *Drosophila* Kc cells were labeled in vivo essentially as described previously (44). Briefly, they were grown to 1.9×10^6 cells/ml, collected by centrifugation at $5,000 \times g$ for 5 min at room temperature, washed once with PBS, and resuspended to a concentration of about 1×10^7 cells/ml in Schneider cell medium containing 3×10^{-4} M KH_2PO_4 and $^{32}P_i$ (50 μCi/ml). Cells were grown for 40 h on a slow orbital shaker, centrifuged as described above, washed three times with PBS, and Dounce homogenized. Immunoprecipitation was performed by incubating homogenate equivalent to 3.5×10^6 cells with 40 μl of polyclonal antiotefin antibodies (cross-linked with glutaraldehyde to 10 μl of protein A–Sepharose CL-4B beads) for 3 h at room temperature. The beads were then washed several times with buffer IP, and proteins were eluted from the beads with 0.1 M triethanolamine (pH 11.3). The eluted proteins were subjected to SDS–10% PAGE and blotted on nitrocellulose filters. The filters were autoradiographed and then subjected to immunoreaction with an antiotefin MAb.

In vitro phosphorylation by cdc2 kinase. $p13^{suc1}$ (7) was overexpressed in *E. coli* cells. One milliliter of clear bacterial supernatant was loaded on a 50-cm-long Sepharose CL-6B column (Pharmacia) and eluted in a combination of 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 10% glycerol, 10 μg of aprotonin per ml, 1 mM phenylmethylsulfonyl fluoride, and 10 μg of pepstatin A per ml; the fraction containing $p13^{suc1}$ was dialyzed against MDBN (40 mM β-glycerophosphate [pH 7.2], 2 mM $MgCl_2$, 500 mM NaCl). Between 10 and 15 mg of $p13^{suc1}$ was conjugated to 1 ml of CNBr-activated Sepharose beads (Pharmacia). The beads were stored in MDBN at 4°C. Prior to their use, the $p13^{suc1}$ beads (100 μl) were washed with MDB buffer (MDBN without NaCl) and incubated with an equivalent amount of membrane-free *Drosophila* embryo extract (43) for 2 h at 4°C with constant agitation. The beads were then washed (three times each with MDBN, with MDBN containing 1 M NaCl, and with MDBN containing 1.5 M NaCl) in order to remove proteins bound nonspecifically. The cdc2 kinase-containing beads were stored up to a few days at 4°C in MDB containing 1 M NaCl.

The cdc2 kinase-containing beads (~10 μl) were incubated for 30 min at room temperature with 3 to 6 μg of histone H1 (calf thymus type III-S; Sigma, St. Louis, Mo.) or with 3 μg of purified wild-type or mutated otefin or 3 μg of pGEXotef800 in a 22-μl buffer containing 50 mM $MgCl_2$, 0.5 mM ATP, 1 μCi of [γ-^{32}P]ATP (10 mCi/ml), 1 mM dithiothreitol, 50 mM NaCl, and 40 mM β-glycerophosphate (pH 7.2). The supernatant was subjected to SDS–10% PAGE followed by autoradiography. In some experiments, the cdc2 kinase activity was analyzed in the presence of a cdc2 kinase substrate peptide (ADAQHATPPKK KRKVEDPKDF; Santa Cruz Biotechnology). The SPKK sequence of otefin was mutated to APKK by overlap extension PCR (21), and the mutations in the sequence (CTAGTCC to CTGCTCC) were determined by DNA sequencing.

Cyclic AMP-dependent PKA catalytic subunit was purchased from Promega (Madison, Wis.). Purified otefin (5 μg) or histone H1 was incubated for 10 min at 30°C with 50 U of PKA in a buffer containing 40 mM Tris-HCl (pH 7.4), 20 mM $(CH_3COO)_2Mg$, 0.2 mM ATP, and 5 pmol of [γ-^{33}P]ATP (1,000 to 3,000 Ci/mmol). The proteins were subjected to SDS–10% PAGE, stained with Coomassie brilliant blue, dried, and autoradiographed.

RESULTS

Otefin is essential for nuclear envelope formation. To analyze otefin function, 0- to 6-h-old *Drosophila* embryo extracts, in which interphase-like nuclei can be assembled from sperm chromatin (47, 48), were incubated with 100 to 300 μg of polyclonal anti-*Drosophila* otefin antibodies. Extracts incubated under the same conditions with 100 to 300 μg of preimmune rabbit sera (IgG fraction) or with normal rabbit IgG served as controls. No membrane assembly was observed when otefin activity was inhibited. Electron microscope (Fig. 1) and light microscope (data not shown) analyses revealed that while chromatin went through the characteristic decondensation process, membrane vesicles did not attach to its surface, and nuclear envelope did not assemble around it (Fig. 1B). Incubation of the extract with preimmune sera (IgG fraction) or with commercially available normal rabbit IgG fraction had no effect on nuclear assembly; the presence of membranes around the chromatin was observed (Fig. 1A).

Otefin localization by in situ analysis during egg chamber development. MAb 618A2O7, directed against *Drosophila* otefin (20, 30), was used to localize otefin in situ during different stages of egg chamber development. As with lamin Dm_0, a "rim" pattern of nuclear immunofluorescence was always observed in nurse cells, follicle cells, and oocytes, consistent with the presence of otefin in the nuclear envelope (Fig. 2). From stage 4 onwards, otefin staining of the oocyte nuclear envelope was more intense than that of the nurse cell nuclear envelope. Until stages 6 to 7, the cytoplasmic background in the developing oocyte was similar to that in nurse cells, indicating that the accumulation of large amounts of cytoplasmic otefin had not commenced. Examples of immunostained whole mount and cryosections of stage 5 egg chambers are shown in Fig. 2A and B.

After stages 6 to 7, the amount of cytoplasmic otefin in the oocyte increased significantly, reflecting formation of a pool in the egg. Intensified staining of the nuclear envelope of the oocyte nucleus was also observed. Examples of stage 7 egg chambers immunostained with antiotefin antibody are shown in Fig. 2C and D. Analysis of cryosections derived from egg chambers indicated that like lamin Dm_0 (44), otefin is present within the oocyte nucleus. Indeed, confocal microscopy of oo-

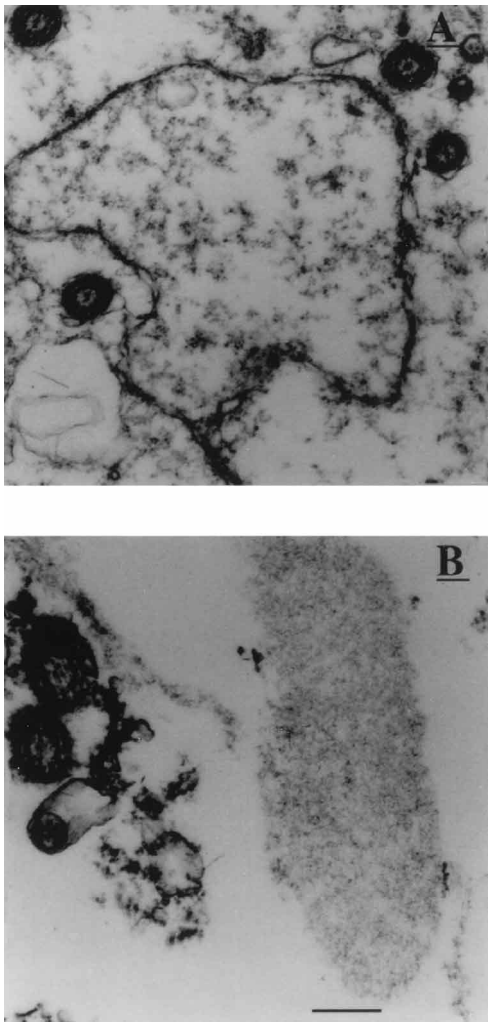
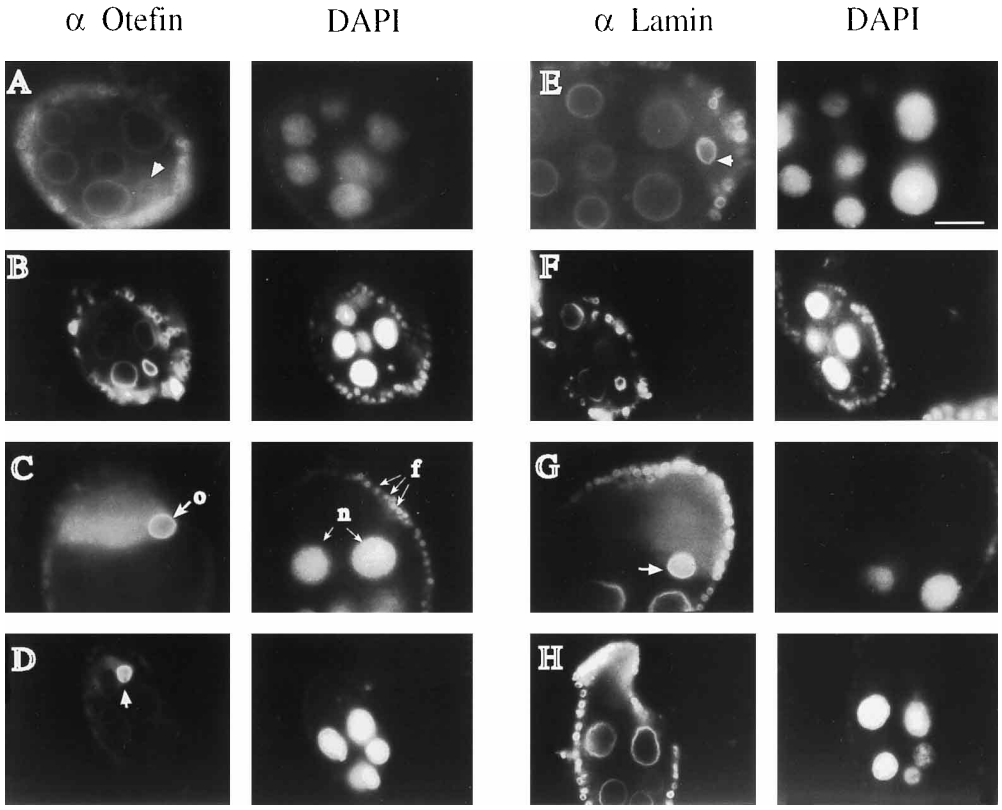


FIG. 1. Inhibition of otefin activity prevents in vitro nuclear envelope assembly in embryonic extracts. Twenty microliters of embryonic extracts was preincubated for 90 min with either 100 μg of polyclonal antiotefin antibodies (B) or with 100 μg of preimmune serum antibodies (IgG fraction) (A). Sperm chromatin was added, and the incubation proceeded for an additional 90 min. Samples from the two experimental systems were viewed with a standard transmission electron microscope. Decondensed chromatin was enveloped with nuclear membranes in preimmune antibody-treated extracts (A) but not in antiotefin antibody-treated extracts (B). Bar, 1 μm.



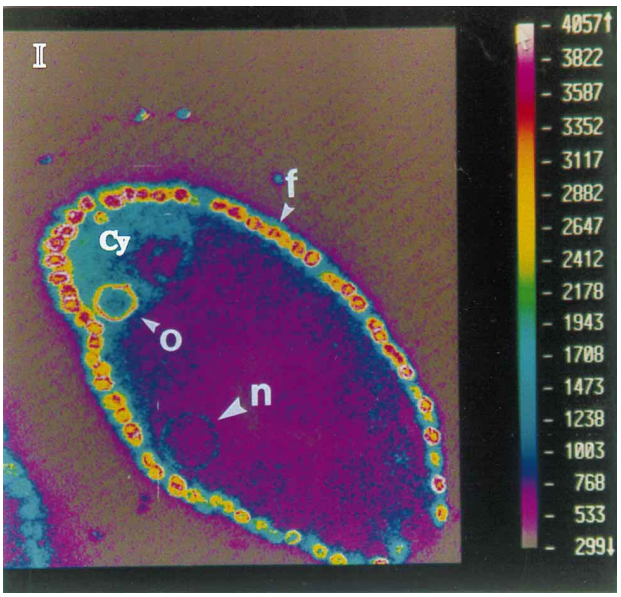


FIG. 2. In situ localization of otefin and lamin during *Drosophila* egg chamber development. The antiotefin MAb 618A2O7 (A to D and I) and antilamin MAb 611A3A6 (E to H) were used as primary antibodies. Cy_3-conjugated rabbit anti-mouse IgG was used as the secondary antibody. (A to H) Antibody staining (left) and DAPI staining of the DNA (right). *Drosophila* (Canton S) egg chambers were from stages 5 (A, B, E, and F), 7 (C, D, G, and H), and 10 (I). Whole-mount immunostaining is shown in panels A, C, E, and G, and immunostaining of cryosections is shown in panels B, D, F, and H. Arrows in panel C mark the positions of the oocyte nucleus (o), nurse cell nuclei (n), and follicle cell nuclei (f). Unlabeled arrowheads in other panels designate the oocyte nucleus. The bar represents 5.6 μm for A, B, E, and F and 10 μm for C, D, G, and H. The egg chamber in panel I was viewed with an ACAS 570 (Meridian Instruments, Okemos, Mich.) confocal stage scanning microscope with a 100×/NA = 1.3 oil immersion objective. The positions of the oocyte nucleus (o), oocyte cytoplasm (Cy), nurse cell nucleus (n), and follicle cell nuclei (f) are indicated. The color scale on the left of the confocal microscope (panel I) shows the relative intensity of the Cy_3 labeling.

cytes at stage 10 (Fig. 2I) or at stages 7 to 9 and 11 to 12 (data not shown) confirmed this observation by revealing that the intensity of otefin immunostaining within the nucleus is comparable to that seen in the cytoplasm of the oocyte. At stage 14, the oocyte is arrested at the first meiotic metaphase and the oocyte nucleus is disassembled. Analysis of stage 14 egg chambers demonstrated intense cytoplasmic staining of otefin, consistent with the presence of a large nonnuclear pool of otefin (data not shown).

The immunofluorescence pattern of whole-mount egg chambers was similar to that reported previously for cryosections stained with polyclonal antilamin antibodies (44). Immunostaining of whole egg chambers with MAb 611A3A6 (antilamin) revealed that lamin localization in these stages is similar to that of otefin. From stage 4 onwards, the nuclear periphery of all cell types was intensely stained. From stages 6 to 7 onwards, intense lamin staining was also observed in the cytoplasm and in the interior of the oocyte nucleus. Examples of stage 5 and stage 7 egg chambers are shown in Fig. 2E to H. Thus, there are similarities in the formation of the oocyte pool of these two peripheral proteins of the inner nuclear membrane.

To investigate further the cellular localization of otefin during egg chamber development, egg chambers were divided into two groups: g-10 and stages 11 through 14. These were homogenized and separated by centrifugation at 12,000 × *g* into nucleus-containing pellet and nonnuclear supernatant. The supernatant containing the egg chamber cytoplasm was then fur-

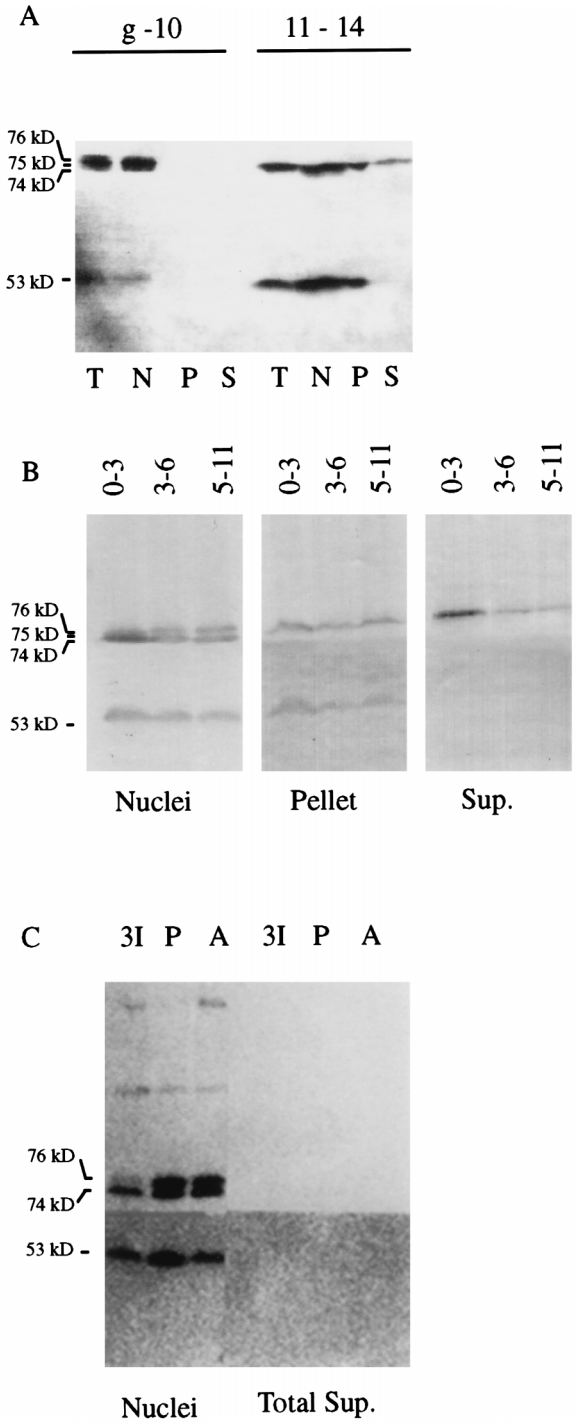


FIG. 3. Subcellular localization of otefin and lamin during development. (A) Otefin is associated with the cytoplasmic membrane vesicle fraction of the mature oocyte. Egg chambers from the g-10 stages or from stages 11 to 14 were homogenized and subjected to immunoblot analysis either directly (T) or after fractionation to nuclei (N), 100,000 × *g* membrane-enriched pellet (P), and membrane-free cytoplasm (S). The lack of otefin and lamin signals in the cytoplasmic fraction in the g-10 stages is probably due to the fact that the cytoplasmic fraction is mainly comprised of earlier stages, in which the majority of the cytoplasm is derived from follicle and nurse cells. (B) Analysis of otefin and lamin during early embryogenesis. *Drosophila* embryos were homogenized and fractionated into nuclei, 150,000 × *g* membrane-enriched pellet (Pellet), and membrane-free cytoplasm (Sup.). MAb 611A3A6 (antilamin) and MAb 618A2O7 (antiotefin) were the primary antibodies, and alkaline phosphatase-conjugated rabbit anti-mouse IgG was the secondary antibody. The age of the embryos in hours is specified on top of the corresponding lanes. (C) Immunoblot analysis of otefin and lamin in 3rd instar larvae (3I), pupae (P), and adult flies (A). MAb 611A3A6 (antilamin) and MAb 618A2O7 (antiotefin) were used as primary antibodies, and ^{125}I-labeled rabbit anti-mouse IgG was used as a secondary antibody. After homogenization of the animals, the homogenate was separated into nuclear (Nuclei) and postnuclear supernatant (Total Sup.) fractions. Each lane contains protein amounts equivalent to 5 to 10 μl of packed animals. The molecular masses of the lamin isoforms (74 to 76 kDa) and otefin (53 kDa) are indicated. To facilitate the detection of otefin in the cytoplasm, the otefin-containing parts of the blots in panels B and C were subjected to longer exposures. This is reflected in the higher background.

ther fractionated by centrifugation at 100,000 × *g* into a membrane-enriched pellet and a soluble membrane-free cytoplasm (48). Immunoblot analysis of the three different fractions with the MAb 618A2O7 (antiotefin) is shown in Fig. 3A. During the g-10 stages of oogenesis, otefin (53 kDa) was detected only in the nuclear fraction. The absence of a signal in the egg chamber cytoplasm in the g-10 stages of oogenesis (Fig. 3A, lanes P and S) can be explained by the relatively low concentration of cytoplasmic otefin in the egg chamber cytoplasm at these stages and the limited sensitivity of immunoblot detection. At stages 11 to 14 of egg chamber development, otefin was observed in both nuclear and cytoplasmic fractions. In the cytoplasm, otefin was associated exclusively with the 150,000 × *g* pellet fraction (Fig. 3A, lane P), while lamin Dm_{mit} (75 kDa) was detected in both the 150,000 × *g* pellet fraction and the membrane-free cytoplasmic fraction (Fig. 3A, lanes P and S).

Otefin is associated with both nuclei and nonnuclear membrane vesicles during embryonic development. Otefin and lamin Dm_0 derivatives were analyzed during various stages of embryonic development in order to determine their cellular localization and their relative amounts. Homogenates from embryos of different ages were separated into nuclear and cytoplasmic fractions, and the cytoplasmic fraction was then fractionated by 150,000 × *g* centrifugation into a membrane-enriched pellet and a membrane-free supernatant. For each fraction, proteins derived from 5 μl of packed embryos were subjected to immunoblot analysis with antiotefin and antilamin MAbs. The results of four independent experiments revealed that the ratio of otefin to lamin in nuclei is roughly constant (Fig. 3B, Nuclei). In the cytoplasm, however, otefin remained associated with the membrane pellet, while lamin was also present in the membrane-free supernatant (Fig. 3B, Pellet and Sup.). The amounts of both lamin and otefin in the cytoplasmic pool decreased with development, although small amounts of otefin and lamin were also detected in the cytoplasm of 9- to 12-h-old embryos (data not shown).

Otefin is present in most cells during larval and adult stages. During the larval, pupal, and adult stages, otefin was detected only in nuclei, similar to lamin Dm_0 (Fig. 3C). Although some otefin is presumably present in the cytoplasm of mitotic cells, this was below the level of detection in our protein blots.

Larval and adult tissues were stained with monoclonal and polyclonal antiotefin antibodies in order to study the distribution of otefin in the different tissues. Otefin was localized to the nuclear envelope in all of the tissues examined of 3rd instar larvae, including imaginal discs, brain, fat, gut, and salivary glands (Fig. 4A to C). However, in tissues with high levels of polytenization, such as salivary gland (Fig. 4C), fat cells, and some cells in the hindgut (not shown), the intensity of otefin immunofluorescence was four to six times lower than that in diploid nuclei or in nuclei which undergo only a few cycles of polytenization (Fig. 4B), as judged by quantitative analysis of images. The reduced ratio of otefin to lamin Dm_0 in salivary glands, compared to that in larval diploid cells, was verified by

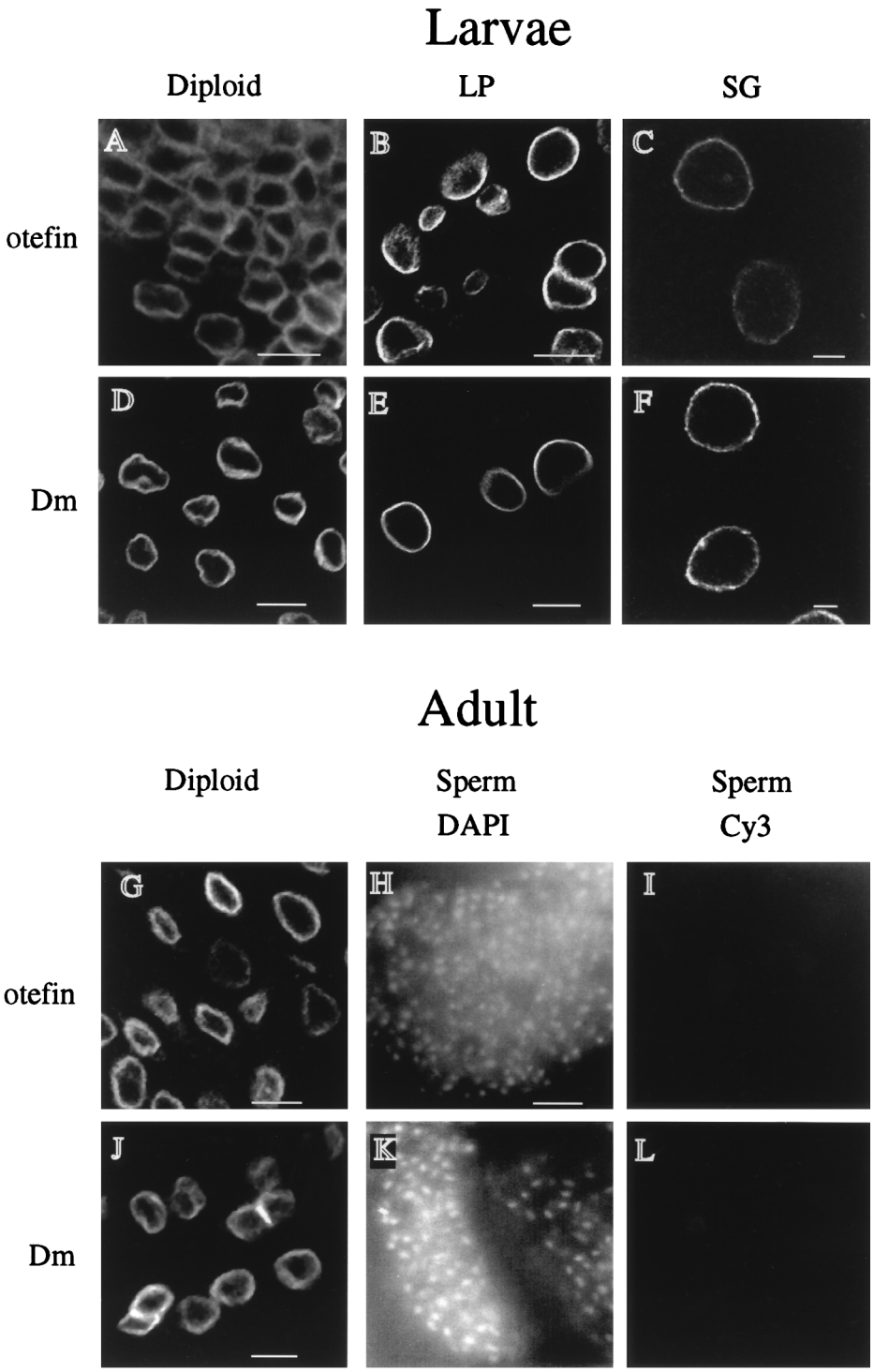


FIG. 4. In situ localization of the *Drosophila* nuclear envelope proteins otefin and lamin in *Drosophila* larvae and adult tissues. The larva cells include diploid cells from imaginal disc, midgut nuclei with a low level of polytenization (LP [as judged by the DAPI staining; data not shown]), and salivary glands (SG). Adult tissues include midgut cells (G and J) and testes (H to I and K to L). Polyclonal antiotefin antibodies (A to C, and I), antiotefin MAb 618A2O7 (G), and antilamin MAb 611A3A6 (D to F and L) were used as primary antibodies. Cy_3-conjugated rabbit anti-mouse IgG and Cy_3-conjugated goat anti-rabbit IgG were used as the secondary antibodies for the MAbs and polyclonal antibodies, respectively. The DAPI-stained samples in panels H and K correspond to the Cy_3-stained samples in panels I and L, respectively. The tissues were viewed with a Bio-Rad MRC-1024 confocal scanhead coupled to a Zeiss Axiovert 135M inverted microscope (A to G and J) or with a Leitz epifluorescence microscope with a 63×/NA = 1.4 Planapo objective lens (H to I and K to L). The bar represents 5 μm in A, D, G, and J and 10 μm in B, C, E, F, and H. The bar in panel H also applies to panels I, K, and L.

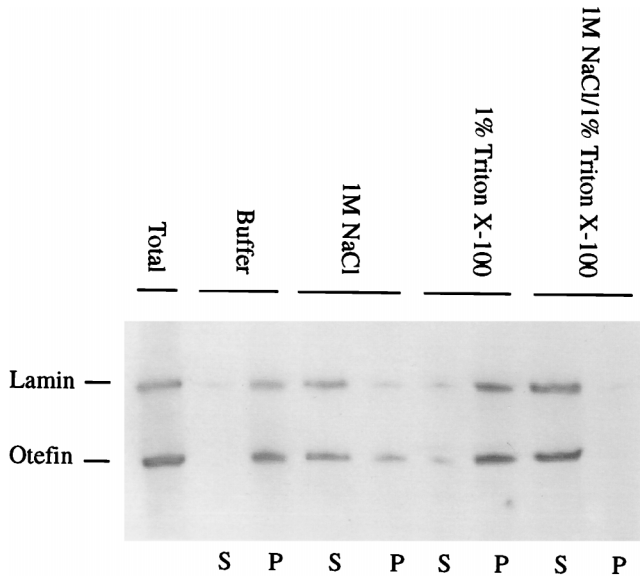


FIG. 5. Extraction of otefin and lamin Dm_0 from membrane vesicles. *Drosophila* embryos (0 to 6 h old) were dechorionated, homogenized, and fractionated into nuclei, 100,000 × *g* membrane-enriched pellet, and membrane-free cytoplasm. The 100,000 × *g* membrane vesicle pellet was collected and washed in 2 volumes of buffer NM and extracted with 1 volume of buffer, 1 M NaCl, 1% Triton X-100, or 1% Triton X-100 containing 1 M NaCl. Following extraction, the residual membrane vesicle pellet (P) was separated from the postmembrane supernatant (S) by centrifugation at 150,000 × *g*. The positions of lamin and otefin are indicated.

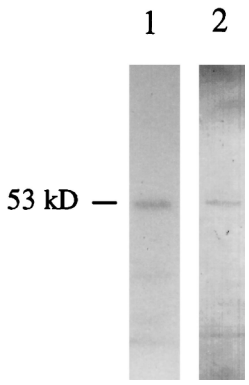


FIG. 6. Otefin is a phosphoprotein in vivo. Kc cells were metabolically labeled with $^{32}P_i$. The nuclear pellets were boiled in buffer containing 1% SDS, and otefin was precipitated with protein A–Sepharose CL-4B beads cross-linked by glutaraldehyde to polyclonal antiotefin antibodies. The precipitated proteins were subjected to SDS–10% PAGE, blotted on a nitrocellulose membrane, and exposed to an X-ray film (lane 1). The filter was then reacted with MAb 618A2O7 (antiotefin) as the primary antibody and alkaline phosphatase-conjugated rabbit anti-mouse IgG as the secondary antibody (lane 2). Alkaline phosphatase activity was detected according to the method in reference 28. The position of otefin (53 kDa) is marked on the left.

protein blot analysis with otefin- and lamin Dm_0-specific antibodies (data not shown). This phenomenon was observed whether monoclonal or polyclonal antiotefin antibodies were used. Addition of 1% sodium deoxycholate during the fixation of the tissues did not change the intensity of the otefin labeling (data not shown). In addition, salivary glands which were stained under similar conditions with the antilamin MAb showed levels of staining similar to or higher than those of lamin Dm_0 in diploid nuclei (Fig. 4D to F). Therefore, differences in staining intensities between salivary glands and diploid cells were not due to variations in the penetration of the antibodies into these tissues.

Most adult cells were labeled with both antiotefin and antilamin Dm_0 derivative antibodies (Fig. 4G and J). One exception were cells during stages 6 to 11 of spermiogenesis in testes, in which the nucleus is elongated and the chromatin is condensed (45). Neither antiotefin nor antilamin Dm_0 derivative antibodies reacted with these cells (Fig. 4H to I, K, and L).

The mode of otefin association with membrane vesicles. Extractions with salts and detergents were employed to study the mode of the association of otefin with membrane vesicles (41). The vesicle-enriched fraction was isolated from 0- to 6-h-old *Drosophila* embryos and extracted with NM buffer containing different reagents. The supernatant and pellet were then separated by centrifugation at 150,000 × *g* and subjected to immunoblot analysis with MAb 618A2O7 (antiotefin) and MAb 611A3A6 (anti-lamin Dm_0 derivatives). Incubation with 1 M NaCl (Fig. 5), 3 M NaBr, or 0.1 M NaOH (not shown) resulted in extraction of both otefin and lamin Dm_{mit}. These data show that the peripheral association of otefin with the membrane vesicles is weaker than that with the nuclear envelope, which is resistant to extraction with solutions containing high salt concentrations (1). Both otefin and lamin Dm_{mit} remained largely associated with the 150,000 × *g* pellet fraction after extraction with 1% Triton X-100 (Fig. 5), indicating that both otefin and lamin are present in a large protein complex that is Triton stable. Both proteins were completely solubilized with 1% Triton X-100 combined with 1 M NaCl (Fig. 5).

Otefin is a phosphoprotein in vivo. One possible explanation for the weaker association of otefin with membrane vesicles, compared to that with interphase nuclei, could be posttranslational modifications of the nonnuclear form of otefin. These modifications are most likely the result of phosphorylation of specific amino acid residues, since otefin is relatively rich in serine and threonine (35). To test these assumptions, *Drosophila* Kc cells were metabolically labeled in low-phosphate medium with $^{32}P_i$. The cells were Dounce homogenized, and the nuclei were separated and denatured in SDS. Otefin was immunoprecipitated from the homogenates with polyclonal antiotefin antibodies cross-linked by glutaraldehyde to Sepharose beads containing protein A, separated by SDS–10% PAGE, and blotted on nitrocellulose membranes. Exposure of the blot to X-ray film revealed a single major band of phosphoprotein (Fig. 6, lane 1), which comigrated with otefin (Fig. 6, lane 2). These results demonstrate that in vivo, otefin is a phosphoprotein.

In vitro phosphorylation of otefin by cdc2 kinase occurs on serine 36. Since cdc2 kinase and PKA are probably both involved in the phosphorylation of inner nuclear membrane proteins, they were tested for their ability to phosphorylate otefin (10, 12, 14, 25, 36, 49). The cdc2 kinase activity was affinity purified from a *Drosophila* early embryo extract with $p13^{suc1}$ conjugated to CNBr-activated Sepharose beads, and the cdc2 kinase-containing beads were incubated with purified otefin and [γ-^{32}P]ATP. Histone H1 served as a positive control for cdc2 kinase phosphorylation (6, 34). As shown in Fig. 7A, otefin and histone H1 were both phosphorylated by the *Drosophila* kinase. These reactions were specific, since they could be competed with excess amounts of a substrate peptide for cdc2 kinase (data not shown). A protein (pGEXotef800) created by fusion between GST and residues 193 to 406 of otefin could not be phosphorylated by the purified *Drosophila* cdc2 kinase (Fig. 7A). The best consensus site for phosphorylation by cdc2 kinase within the 1- to 192-amino-acid region of otefin

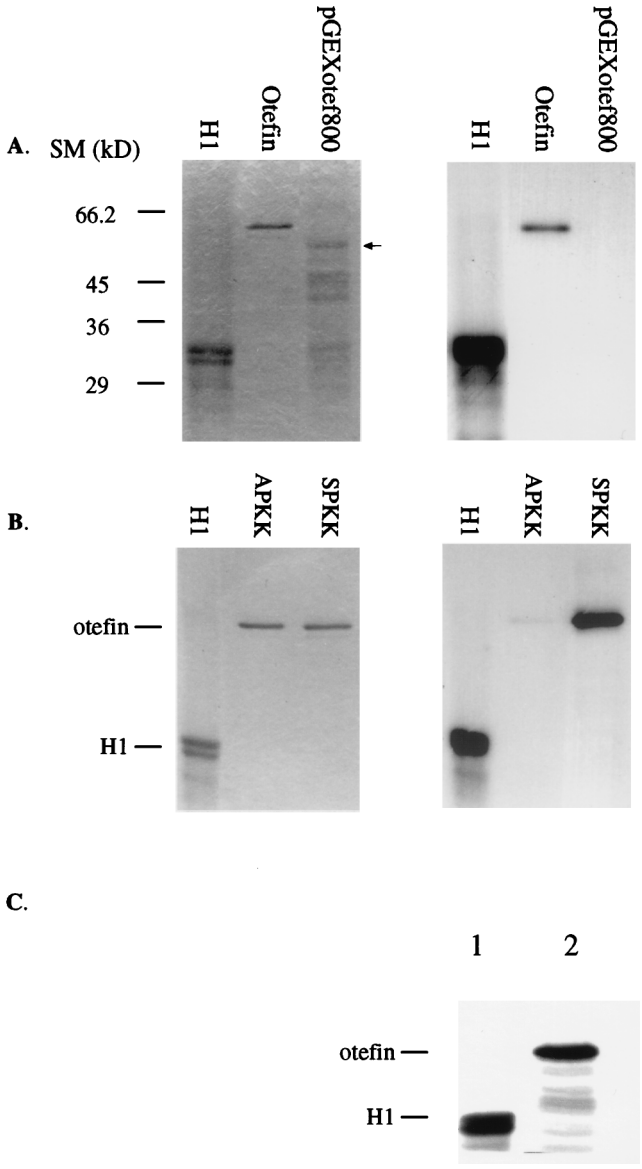


FIG. 7. In vitro phosphorylation of otefin by PKA and by cdc2 kinase. $p13^{suc1}$ conjugated to CNBr-Sepharose beads was used to purify the cdc2 kinase from *Drosophila* embryo extract. (A) The cdc2 kinase-containing beads were incubated with 6 μg of histone H1, 3 μg of wild-type otefin, or with 3 μg of pGEXotef800 (a fusion protein between pGEX and residues 193 to 406 of otefin). (B) The cdc2 kinase-containing beads were incubated with 3 μg of histone H1, 3 μg of otefin mutated at serine 36 (APKK), or with 3 μg of wild-type otefin (SPKK). (C) PKA was incubated with 3 μg of histone H1 (1) or with 3 μg of wild-type otefin (2). The proteins were separated by SDS–10% PAGE, stained with Coomassie brilliant blue (left panels), dried, and autoradiographed (right panels). The positions of the size markers (SM) are shown in panel A, and the positions of otefin and histone H1 are shown in panels B and C.

is the sequence SPKK (amino acids 36 to 39) (32, 36). The SPKK sequence in wild-type otefin was mutated by an overlap extension PCR to APKK in order to analyze whether this site is the target for in vitro phosphorylation by cdc2 kinase. The mutated otefin was overexpressed in *E. coli*, purified to near homogeneity, and subjected to phosphorylation with $p13^{suc1}$ beads bound to the *Drosophila* cdc2 kinase. Only background levels of ^{32}P labeling were observed with APKK-mutated otefin (Fig. 7B). In contrast, significant labeling was observed with wild-type otefin or with histone H1 in the absence (Fig. 7B) or presence (not shown) of the mutated otefin. These data show that the major site for in vitro phosphorylation of otefin by cdc2 kinase is at serine 36.

In vitro phosphorylation of otefin by PKA. The sequence of otefin also contains two putative sites for phosphorylation by PKA (40): RRET at positions 42 to 45 and RRSS at positions 131 to 134. The wild-type otefin was expressed in *E. coli*, purified to near homogeneity, and subjected to phosphorylation with purified PKA in the presence of [γ-^{33}P]ATP. Otefin was then subjected to analysis by SDS–10% PAGE and autoradiography. The results of this experiment demonstrate that otefin could be phosphorylated in vitro by PKA (Fig. 7C, lane 2). Histone H1 (Fig. 7C, lane 1), which served as a positive control, was also phosphorylated.

DISCUSSION

The role of otefin in nuclear envelope assembly. The present study shows that inhibition of otefin activity abolished membrane vesicle attachment to chromatin. Electron and light microscopy analyses revealed that extracts in which otefin activity was inhibited with antiotefin antibodies supported chromatin decondensation but not membrane vesicle attachment to the surface of chromatin. Inactivation of otefin in these experiments was specific, since (i) protein blots and immunofluorescence analyses revealed a specific interaction between these antibodies and the otefin protein present in the nuclear periphery, and (ii) preimmune sera or nonspecific antirabbit antibodies had no effect on nuclear envelope reconstitution. Experiments in which lamin Dm_0 activity was inhibited gave similar results and suggested a similar role for lamins (reviewed in references 31 and 50). The similar phenotype obtained when otefin or lamin Dm_0 activity is inhibited points to the possibility that otefin and lamin are present in the same protein complex. Study of the potential association between lamin Dm_0 and otefin is currently in progress. Sequence comparison between nuclear lamins, lamina-associated proteins, and the complete genome sequence of the yeast *Saccharomyces cerevisiae* reveals that with the exception of LBR, which is the yeast sterol C_{14} reductase, proteins homologous to either lamins or lamina-associated proteins are not present in significant homology in yeast. One possible explanation for this phenomenon is that since *S. cerevisiae* has a closed mitosis, it does not require these proteins for targeting nuclear membrane vesicles to chromatin.

Otefin expression during development. In this study, we also used antibodies to show that otefin is present in almost every cell in *Drosophila*. This was determined for egg chambers, syncytial embryos (this study and reference 20), embryos prior to hatching (data not shown), 3rd instar larvae, and adult flies. The distribution of otefin during development was similar to that of lamin Dm_0 derivatives, and both proteins always appeared in the same cells. In contrast, the distribution of otefin was different from that of *Drosophila* lamin C, which is expressed only in differentiating cells during late stages of embryogenesis (39). Otefin and lamin Dm_0 derivatives were not detected during spermiogenesis, from the stage when chromatin starts to condense (stages 6 to 11 [45]). In the mouse and in *Xenopus laevis*, there are sperm-specific lamins (3, 15, 16). Such sperm-specific lamin(s) and a sperm-specific otefin are yet to be discovered in *Drosophila.*

It is interesting to note that otefin was present within the nucleus (i.e., in addition to that found in the nuclear envelope) of the germinal vesicle, similar to lamin Dm_0 derivatives. This represents the only cell in *Drosophila* which contains significant amounts of otefin within its nucleoplasm. The staining within

the nucleus appeared to be diffuse without any visible substructures and was similar to that observed in the cytoplasm.

The labeling of otefin with either monoclonal or polyclonal antiotefin antibodies in salivary glands, fat, and hindgut nuclei was significantly reduced compared to that in nuclei which undergo fewer cycles of polytenization. This phenomenon was not observed with lamin Dm_0 derivatives (this study) or with lamin C (39). The reduction in the intensity of the otefin signal can be explained either by lower levels of otefin per unit area of the nuclear envelope or by modifications of the epitopes recognized by these antiotefin antibodies. It seems that whatever functions otefin has in these cells, they require modified or less otefin.

During egg chamber development, otefin accumulates in large amounts in a nonnuclear pool, similar to lamin Dm_{mit}. Otefin and lamin Dm_{mit} continue to be present in a nonnuclear pool for more than the first 6 h of embryogenesis. It is interesting that the cytoplasmic pool of two major nuclear envelope proteins is maintained following 6 to 8 h of embryonic development, which is a relatively long period after the rapid nuclear divisions occur. Although it is difficult to rationalize this phenomenon functionally, it may explain why in the *Drosophila* in vitro nuclear assembly system, the 0- to 6-h-old embryo cytoplasmic extract is capable of assembly of nuclei from naked DNA or sperm chromatin (4, 23, 47).

The association of otefin with membrane vesicles. The solubility studies showed that otefin is a peripheral protein attached to membrane vesicles in the maternal pool. Strong evidence for this classification is its extraction from vesicles with 1 M NaCl, 3 M NaBr, or 0.1 M NaOH (41). In other studies, we showed that otefin is a peripheral protein of the nuclear envelope, since in the nuclear envelope otefin can be extracted with urea, GuHCl, or 0.1 M NaOH, but not with solutions containing a high salt concentration (1). This difference in the solubilities of otefin in high-salt solutions between the nuclear envelope and the membrane vesicles indicates a different association of otefin with the two cell compartments. Otefin and the vesicle-bound fraction of lamin Dm_{mit} (48) remained associated with the insoluble $150{,}000 \times g$ pellet fraction of the cytoplasmic maternal pool following extraction with Triton X-100. This may indicate that otefin is present in a large protein complex that remains insoluble following removal of the lipids with detergent. This detergent-resistant complex is salt sensitive, since it could be solubilized with 1% Triton X-100 and 1 M NaCl. The properties of otefin and lamin Dm_{mit} in the maternal pool are different from those of vertebrate lamin B and p58 (LBR) in mitotic vesicles, since the latter proteins are solubilized by extraction with Triton X-100 alone (2, 8). One possible explanation is that the interaction between lamin Dm_{mit} and otefin in the maternal pool (meiotic) is different from that in the mitotic vesicles. The ways in which otefin is associated in the maternal pool and in mitotic vesicles remain to be compared.

In vivo and in vitro phosphorylation of otefin. The difference between the association of otefin with the membrane vesicle fraction and that with the nuclear envelope could result either from posttranslational modifications of otefin, from posttranslational modifications of proteins that are associated with otefin in a protein complex, or from both. Elucidating the ability of candidate protein kinases to phosphorylate otefin at specific sites in vitro is the first step in analyzing the role of these modifications in vivo. PKA and cdc2 kinase were chosen for further studies since (i) phosphorylation by cdc2 kinase is likely to be involved in regulating the interaction that occurs during mitosis between the nuclear lamina and the inner nuclear membrane (10, 12, 36, 49), (ii) PKA has additive effects to cdc2 kinase on the process of nuclear envelope breakdown (25), and (iii) otefin contains putative target sites for the activity of PKA and cdc2 kinase. The cdc2 kinase from *Drosophila* embryos efficiently phosphorylated otefin in vitro. The phosphorylation site was identified as serine 36 in the SPKK sequence, since a mutation of this serine residue eliminated the ability of otefin to serve as a substrate for cdc2 kinase. PKA also phosphorylated otefin in vitro, but the exact site(s) for PKA phosphorylation has yet to be characterized. Experiments aimed at determining whether the same sites in otefin are phosphorylated in vivo and whether these sites are cell cycle regulated still need to be performed.

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The first two authors contributed equally to this work.

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