Cell-specific Accumulation of *Drosophila* Proteasomes (MCP) During Early Development

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Abstract. The proteasome (MCP) is a high relative molecular mass multicatalytic proteinase complex composed of nonidentical protein subunits. We have investigated the cellular distribution of the enzyme complex during *Drosophila* embryogenesis using the proteasome specific antibodies N19-35 and N19-28 for immunocytology. Antibody staining of whole-mount embryos shows that during embryogenesis proteasomes are present in proliferating cells and that their accumulation and turnover is differentially regulated. Our data suggest that the proteasome may serve different proteolytic processes and that the enzyme may be involved in cell-specific proteolytic events required for cell proliferation and morphogenesis during early *Drosophila* development.

THE proteasome (MCP) is a multicatalytic, nonlysosomal, high relative molecular mass proteinase complex (Wilk and Orlowski, 1980, 1983; Dahlmann et al., 1985; Rivett, 1985, 1989; Tanaka et al., 1986; Waxman et al., 1987). At neutral or slightly basic pH the enzyme cleaves peptides at the carboxy site and can be activated by SDS (Dahlmann et al., 1989*a*; Falkenburg and Kloetzel, 1989). By biochemical, morphological, and immunological criteria, the proteasome was shown to be identical with the 19S cylinder type RNP or prosome (Falkenburg et al., 1988; Arrigo et al., 1988). The proteasome has a molecular mass of ~605 kD and is composed of 15–20 nonidentical protein subunits with molecular masses between 35 and 22 kD (Kloetzel et al., 1987; Haass and Kloetzel, 1989; Tanaka et al., 1988), which are encoded by an evolutionarily conserved gene family (Haass et al., 1990*a*; Tanaka et al., 1990). The proteasome has the appearance of a tripartite reel-shaped structure (Baumeister et al., 1988) and has been isolated from organisms as distant in evolution as archaebacteria, yeast, *Drosophila*, *Xenopus*, and man (Dahlmann et al., 1989*b*; Kremp et al., 1986; Kleinschmidt et al., 1988; Schuldt and Kloetzel, 1985; Arrigo et al., 1985; Akhayat et al., 1987; Kleinschmidt et al., 1983; Schmid et al., 1984; Tanaka et al., 1988). The enzyme is therefore thought to play an essential role in intracellular protein metabolism.

The importance of proteinases in *Drosophila* embryo development is best demonstrated for the establishment of the dorsal-ventral polarity in the *Drosophila* embryo, where two serine proteinases, snake (Delotto and Spierer, 1986) and easter (Chasan and Anderson, 1989; Jin and Anderson, 1990), were shown to play a decisive role. The first evidence that proteasomal enzyme activities may be under developmental control was obtained by showing that the proteasome subunit composition varies considerably during fly development (Haass and Kloetzel, 1989). In addition, the observation that large amounts of proteasomes are accumulated in the oocyte of sea urchin (Akhayat et al., 1987) and *Xenopus* (Kleinschmidt et al., 1983) and that in *Drosophila* proteasome can be isolated from transcriptionally inactive early embryos, suggested that proteasomes may be important for early stages of embryogenesis (Haass and Kloetzel, 1989). So far the actual in vivo substrates and function(s) of the proteasome are unknown. Therefore information concerning the distribution of the proteasome in the living organism may help to correlate sites of accumulation with potential function(s) of the enzyme complex.

In this report we demonstrate for the first time a regulated, cell-specific accumulation of the proteasome and present evidence suggesting that this multicatalytic proteinase complex may be involved in processes correlated with nuclear functions, cell proliferation, and morphogenetic events during *Drosophila* embryogenesis.

Materials and Methods

Fly Stocks

Flies of the Oregon-R stock of *Drosophila melanogaster* were raised at 23°C in a 12-h light-dark cycle. Embryos were collected on food trays spread with yeast and removed from the food trays with Ringer's solution. Care was taken to remove yeast adhering to the embryos. Embryos were staged according to Campos-Ortega and Hartenstein (1985).

Immunohistochemistry

Whole-mount staining of *Drosophila* embryos was performed according to Rubin (1986) and as described by Haass et al. (1990*b*). The previously described affinity-purified proteasome-specific antibodies N19-35 and N19-28, which have been successfully used in expression library screening (Haass et al., 1989, 1990*a*), were used at 1:50 dilution. For the detection of first antibodies biotin-conjugated goat anti-rabbit secondary antibody was preabsorbed on embryos for 1 h at room temperature. For peroxidase staining reactions the HRP-Avidin complex (Vectastain; ABC-Kit; Vector

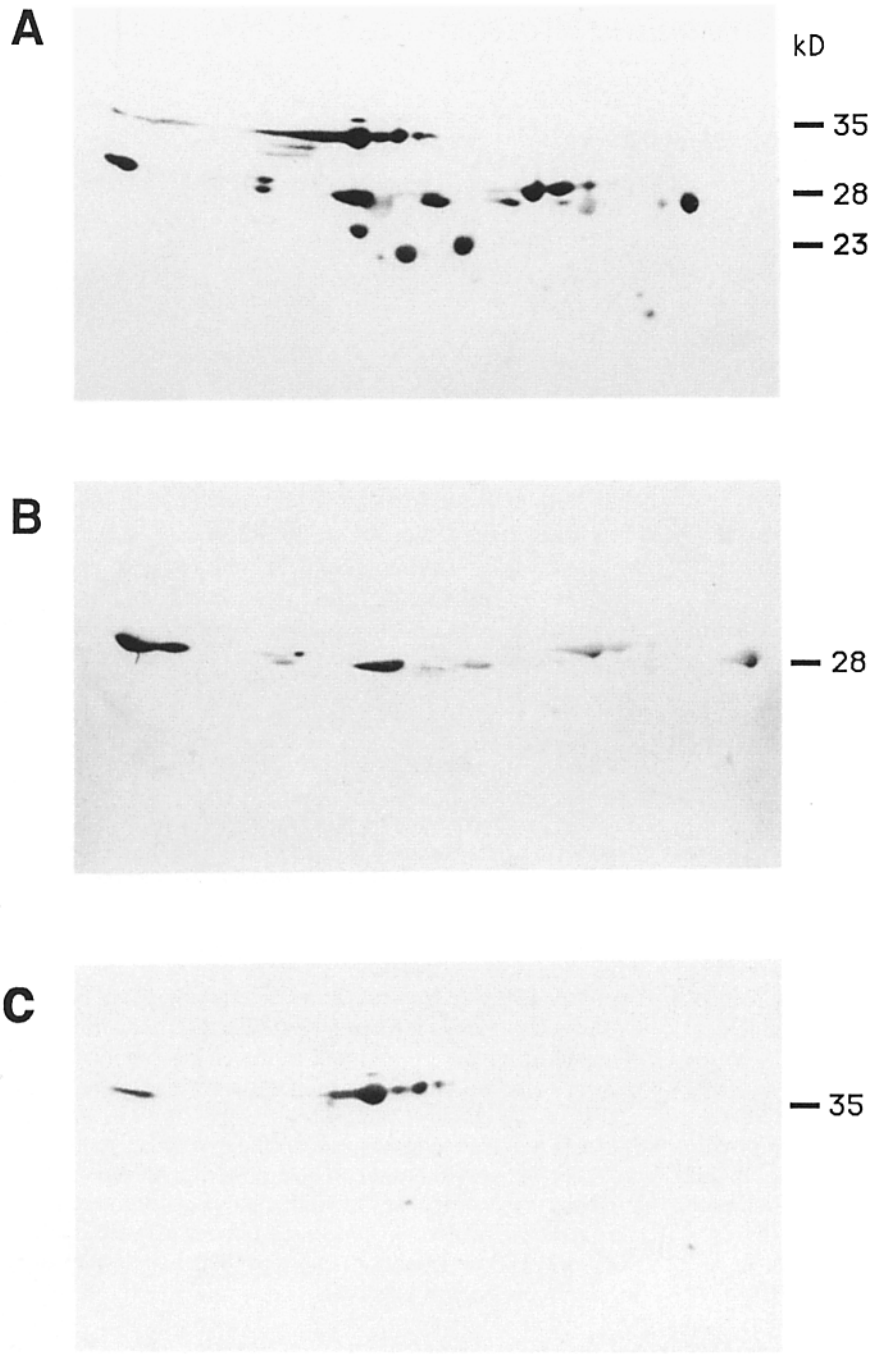


Figure 1. Characterization of antibodies N19-35 and N19-28. The affinity-purified antibodies N19, N19-35, and N19-28 were tested by immunoblotting on proteasomal subunit proteins which were separated by two-dimensional gel electrophoresis. (*A*) Immunostaining with N19. The antibody which was raised against the total, undenatured proteasome complex recognizes a large number proteasome subunits. (*B*) Immunostaining with affinity-purified antibody N19-28. The antiserum reacts strongly with several of the 28-kD subunits. (*C*) Immunostaining with affinity-purified antibody N19-35. The antiserum detects only the 35-kD proteasome subunits. There is no cross-reaction with any of the other proteasome proteins.

Laboratories Inc., Burlingame, CA) was used according to the manufacturer's protocol. Whole-mounts were viewed under a Zeiss Axiophot microscope using DIC optics. In whole-mounts identical staining patterns were obtained independent of the antibody used. The embryos were photographed using a Kodak Ektachrome 160 color film. For immunohistochemistry on frozen tissue-thin sections, embryos were dechorionated and fixed with 4% paraformaldehyde before embedding. All further steps were essentially as described by Siwicki et al. (1988) and Haass et al. (1990*b*).

Electrophoresis and Immunoblotting

SDS-PAGE was performed according to Laemmli (1970). Two-dimensional gel electrophoresis was performed as described by O'Farrell (1975). For immunoblotting experiments the procedure as described by Schuldt and Kloetzel (1985) was used.

Results

Proteasome-specific Antibodies N19-35 and N19-28

To probe for proteasome accumulation and distribution during embryogenesis the proteasome-specific antisera N19-35 and N19-28 were used (Haass et al., 1989, 1990*a*). In immunoblots N19-35 identifies the 35-kD *Drosophila* proteasome subunits while N19-28 reacts with several of the subunits in the 28-kD molecular mass range (Fig. 1). All subunits that are recognized by the two antibodies are constitutive protein components of the proteasome (Haass and Kloetzel, 1989). Since there is no evidence for free uncomplexed proteasome subunits and both antibodies immunoprecipitate the protea-

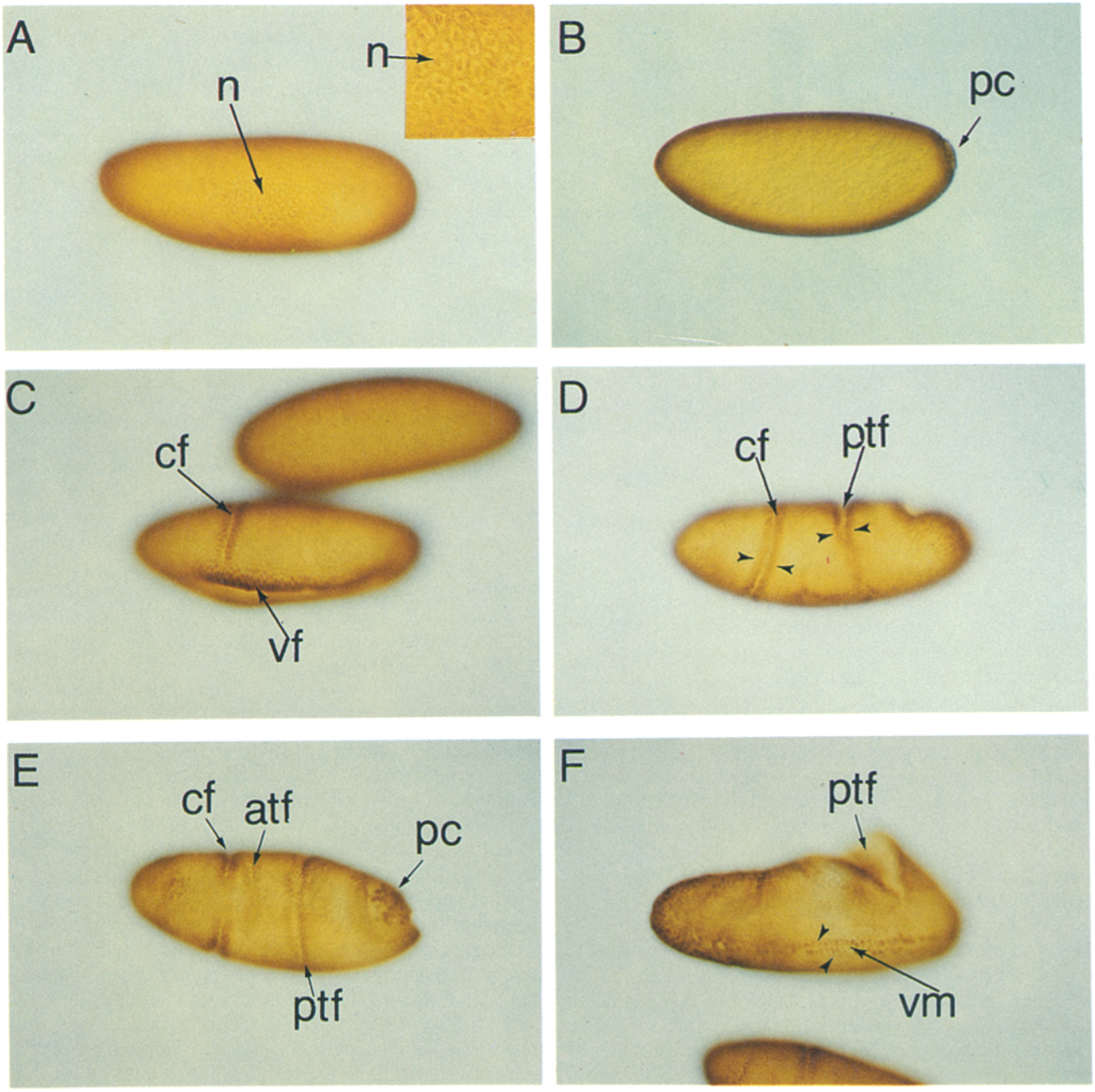


Figure 2. Proteasome distribution during *Drosophila* embryogenesis. Proteasome distribution was analyzed by immunostaining using the antisera N19-35 (*A*, *B*, *C*, and *F*) and N19-28 (*D* and *E*). In all cases identical staining patterns were obtained. The anterior end of the embryo is to the left, the posterior end is to the right. (*A*) Stage 3 embryo. In syncytial blastoderm proteasomes are located in the cytoplasm and in nuclei where they are found to be associated with the dividing chromosomes. (*B*) Stage 4 embryo. During cellularization proteasome levels become strongly reduced. Some proteasome accumulation is detectable in pole cells (*pc*). (*C*) Stage 5 embryo. During early gastrulation cell-specific proteasome accumulation becomes visible in cells of the cephalic (*cf*) and ventral furrows (*vf*). (*D* and *E*) Stage 6 embryo. Proteasomes are accumulated in cells anterior and posterior of the morphogenetic folds. (*F*) Stage 7 embryo. After ventral furrow closure proteasomes are found to be accumulated in a single row of cells on either side of the ventral midline (*vm*). *atf*, anterior transversal fold; *cf*, cephalic furrow; *n*, nucleus; *pc*, pole cells; *ptf*, posterior transversal fold; *vf*, ventral furrow; *vm*, ventral midline.

some from crude cellular extracts, the distribution of the 35- and 28-kD subunits is representative for the whole proteinase complex (Arrigo et al., 1985; Haass et al., 1989, 1990*a*).

Proteasome Distribution During Embryogenesis

For immunocytological analysis of proteasome distribution in whole-mount embryos the above described antibodies N19-35 and N19-28 were used. Immunoreaction in whole-mounts was completely blocked when the two antisera were preincubated with excess amount of isolated proteasome. Identical results were obtained with N19-35 and N19-28 as well as with the independently derived antibodies Dm35K1 and Dm25K2 (Schuldt and Kloetzel, 1985) (data not shown). During the initial, transcriptionally inactive stages of *Drosophila* embryogenesis proteasomes are localized in

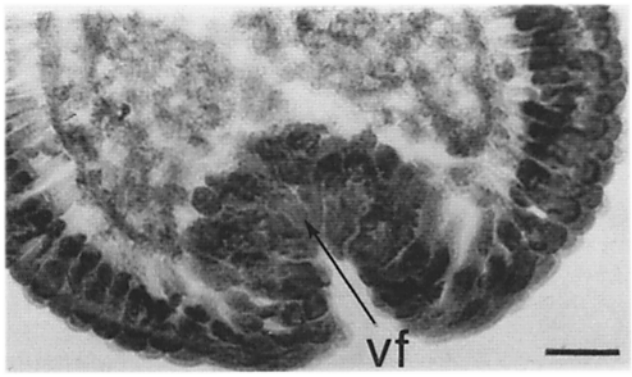


Figure 3. Immunolocalization of proteasomes in frozen tissue-thin sections of early *Drosophila* embryos. Transversal section through the ventral furrow. The proteasome is strongly accumulated in cells of the invaginating ventral furrow (*vf*). Antibody N19-35 was used. Bar, 13.24 μm.

the cytoplasm as well as in the nucleus (Fig. 2 *A*). In the nuclei the nucleoplasm exhibits only low proteasome levels, while the proteinase complex appears to be intimately associated with chromosomes. Due to the metasynchronous nuclear divisions in syncytial/blastoderm different stages of nuclear division can be identified. Cellular blastoderm proteasome levels seem to be reduced, although the cortex always reveals a weak immunosignal (Fig. 2 *B*). At this stage of embryo development proteasomes become detectable in the pole cells at the posterior tip of the embryo (proteasome accumulation in pole cells is discussed in a separate paragraph). Strong proteasome accumulation can first be visualized during the early phases of gastrulation in cells of the morphogenetic folds (Fig. 2, *C* and *D*). Concomitant with the establishment of the cephalic and ventral furrows proteasomes appear accumulated in cells undergoing morphogenetic movements anterior and posterior of the cephalic furrow and in a row of three to four elongated cells on both sides of the ventral furrow (Fig. 2 *C*). Similarly, proteasomes are found accumulated in a row of cells on both sides along the anterior and posterior transversal folds (Fig. 2, *D* and *E*). In fact, freeze sections through the ventral furrow show that proteasomes are strongly accumulated in cells of the invaginating cell layers (Fig. 3). When the posterior dorsal fold penetrates further into the dorsal side of the embryo and after ventral furrow closure, proteasomes can be visualized in a single row of cells on either side of the ventral midline (Fig. 2 *F*).

As the germ band rapidly elongates proteasomes are accumulated in patches of large cells within the extending germ band (Fig. 4, *A* and *B*). Within the precephalic neuroblasts proteasomes are present in the cytoplasm and in the nuclei. As in nuclei of the syncytial blastoderm stage proteasomes are closely associated with the chromosomes. Before the onset of germ band contraction proteasomes can be detected in a repeated row of large cells from which the segmental organization of the embryo can be anticipated (Fig. 4, *C* and *D*). Concomitant with the appearance of the parasegmental furrows, strong proteasome accumulation is observed within the developing segments. When the individual segments become clearly distinguishable cells bordering the intersegmental furrows reveal a higher proteasome level than the cells in the inner part of each segment (Fig. 4 *E*). Independent of focus no proteasome staining is observed within the furrows. As the germ band shortens proteasomes are detectable in a large number of ectodermal cells, the ventral cord, and the ventral, proliferating part of the central nervous system (Fig. 4, *F* and *G*).

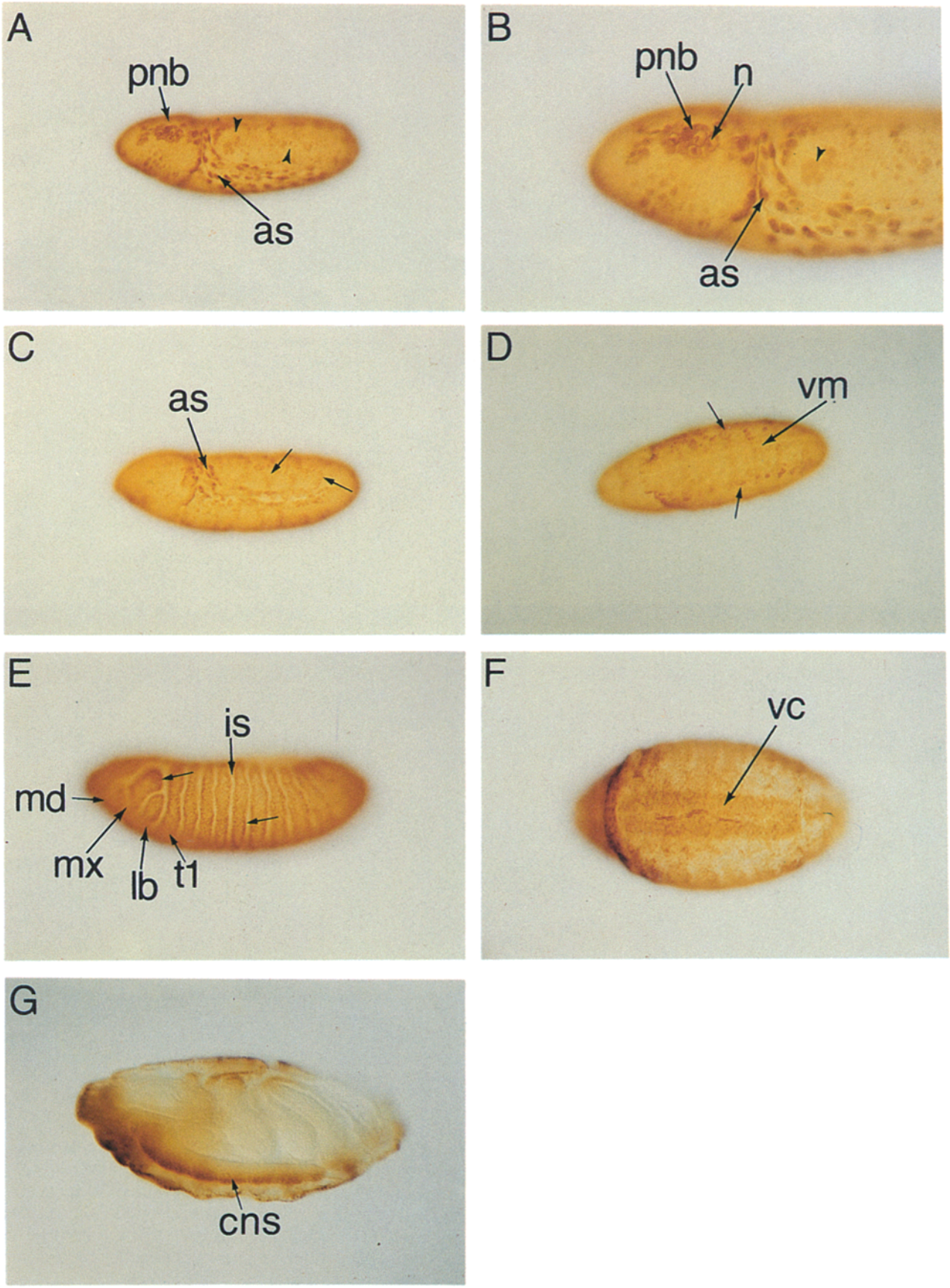
Proteasome Expression in Pole Cells

During early embryogenesis proteasome levels in pole cells are extremely low and in most cases not detectable. Proteasome synthesis and accumulation are first clearly apparent at cellular blastoderm when pole cells have approached a horizontal orientation (Fig. 5 *B*). In the majority of pole cells high proteasome levels persist through stage 7 when the amnioproctodeal invagination begins and the pole cells start to move to the inside of the embryo. However, some of the pole cells show already at this stage a reduced proteasome level (fig. 5, *C–E*). When the pole cells have reached the pocket of the posterior midgut primordium, no proteasomes are detectable anymore (Fig. 5 *F*). Interestingly, proteasome synthesis is not resumed in pole cells and gonads during further embryo development.

Discussion

Our immunocytological analysis of proteasome distribution demonstrates for the first time that during *Drosophila* embryogenesis proteasome accumulation, and possibly also proteasome synthesis, is cell specific and that cellular levels and synthesis of this high relative molecular mass multicatalytic proteinase complex are differentially regulated events during early development. Proteinase complex accumulation is most obvious during early gastrulation in cells of the various folds that are involved in morphogenetic movements. While it is difficult to identify the onset and turnoff of proteasome synthesis once a large number of cells have started to accumulate, the proteinase complex, the tight regime of proteasome accumulation, and turnover is best demonstrated in pole cells, the precursor cells of the gonads. Pole cells reveal a high proteasome content once they have reached the posterior tip of the embryo. Although proteasome levels remain high in the majority of pole cells when they move to the interior of the embryo, a larger number of pole cells reveal already a reduced proteasome level. When the pole cells have reached the posterior midgut primordium proteasome synthesis has ceased completely and is not resumed during further embryonic gonad formation. Whether the nonsynchronous proteasome turnover in pole cells simply reflects differences in their developmental state or whether this is correlated with programmed cell death of pole cells is at present not clear.

Figure 4. Proteasome distribution during *Drosophila* embryogenesis. Antiserum N19-35 was used in *A*, *B*, *C*, and *G*. Antiserum N19-28 was used in *D*, *E*, and *F*. (*A*) Stage 9 embryo. Proteasome synthesis is most prominent in the precephalic neuroblasts (*pnb*) and the amnioserosa (*as*). Patches of large cells in the elongating germ band also reveal proteasome accumulations. (*B*) Blow up of embryo shown



in *A*. (*C* and *D*) Stage 9/10 embryos. Proteasome synthesis is detectable in patches of large cells from which the segmental organization of the embryo can be anticipated. (*E*) Stage 13 embryo. Strong proteasome accumulation is seen within the segments. No proteasomes are present in the intersegmental furrows (*is*). *as*, amnioserosa; *is*, intersegmental furrow; *pnb*, precephalic neuroblast.

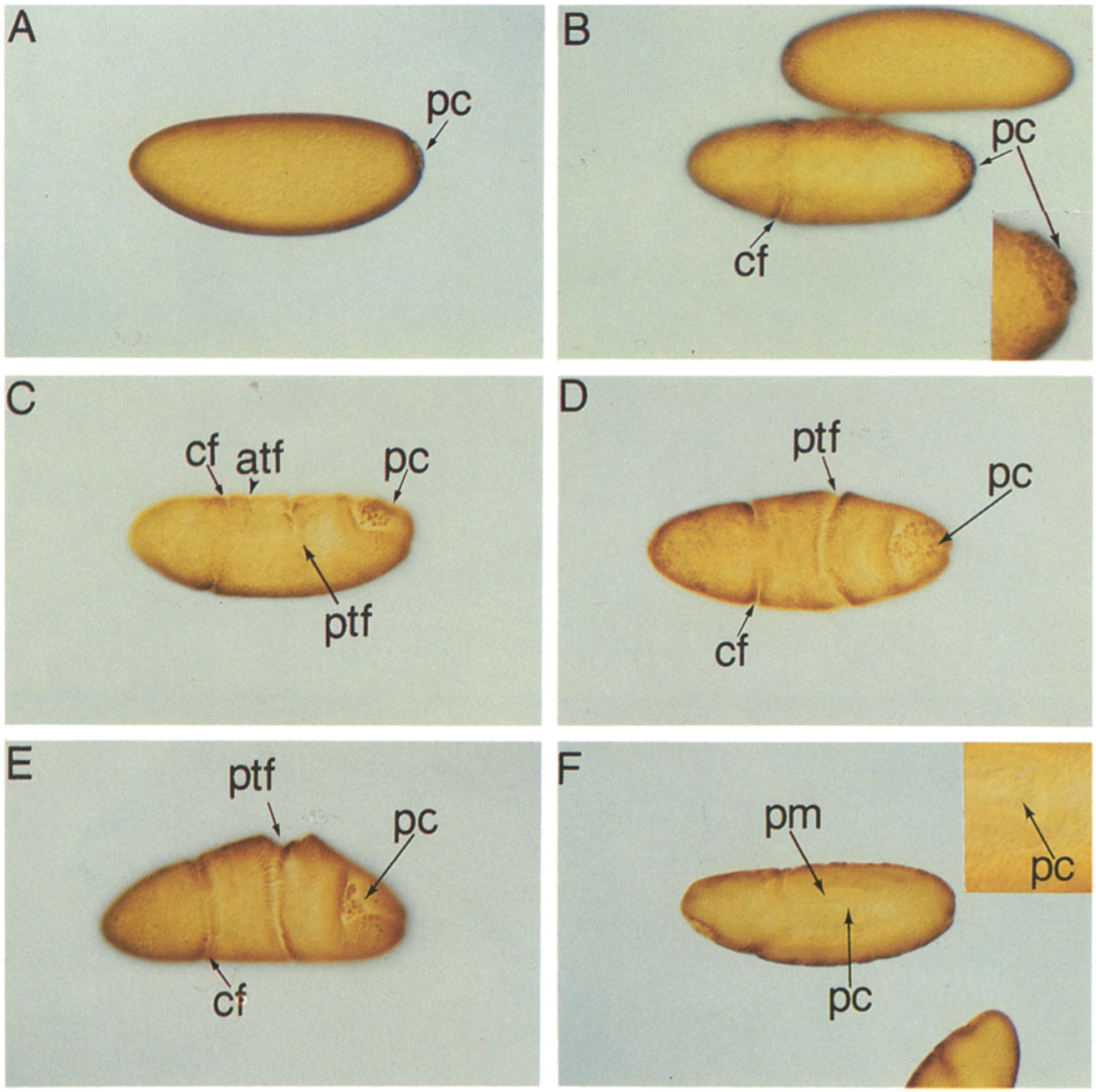


Figure 5. Analysis of proteasome accumulation in pole cells. (*A*) Stage 5 embryo. Proteasome accumulation becomes detectable in pole cells. (*B*) Early stage 6 embryo. Strong proteasome accumulation takes place in all pole cells. (*C–E*) Late stage 6 and early stage 7 embryos. Aminoproctodeal invagination takes place. The pole cells move to the interior of the embryo. Proteasome levels slowly decrease. Different pole cells show different levels of proteasomes. (*F*) Stage 9 embryo. Pole cells have reached the pocket of the posterior midgut primordium. Proteasome accumulation is not detectable anymore. Antibody N19-35 was used in *A*, *B*, and *D*. Antibody N19-28 was used in *C*, *E*, and *F*.

These data not only demonstrate that proteasome accumulation and turnover are tightly regulated events but also suggest that proteasomal enzyme activity in certain cell types or tissues is only required for a limited developmental period, whereby some cells do not reveal any proteasome synthesis at all (see also Haass et al., 1989). Interestingly accumulation of proteasomes is not resumed during embryonic gonad formation. In this respect it appears noteworthy that during terminal differentiation of embryonic red cells in chicken, the proteasome level gradually ceases and end-differentiated cells do not contain the proteasome anymore (Scherrer et al., 1988).

Proteasome accumulation seems to follow a distinct pattern during the early stages of embryogenesis. The enzyme complex is first accumulated in cells that are involved in morphogenetic movements and the presence of proteasome often is concomitant with the establishment of mitotic domains as defined by Foe (1989). The accumulation of the proteinase complex in dividing cells and the physical interaction with chromosomes as observed in dividing nuclei during

syncytial blastoderm and in nuclei of proliferating precephalic neuroblasts is intriguing and may suggest a proteolytic function during embryo development connected with either nuclear or cellular divisions. However, nuclear localization of proteasomes is also found in nondividing cells such as fat cells and tall cells in adult flies (Haass et al., 1989), as well as in salivary glands and elongating spermatids (Glätzer, K. H., and P.-M. Kloetzel, unpublished observation). Furthermore, cells of the amnioserosa, which will in part form the pericardial wall in adult flies (Technau and Campos-Ortega, 1986), reveal high proteasome levels despite the fact that they remain arrested in G1 of the 14th mitotic division. The latter observations indicate that proteasome function is not restricted to proliferating cells and that the enzyme is important for nuclear metabolism in quite different cell types. Nuclear location of the proteasome is not specific for *Drosophila* but is a general phenomenon that has been found in a variety of species (Kleinschmidt et al., 1983; Arrigo et al., 1988; Seelig, A., M. Gernold, and P.-M. Kloetzel, manuscript in preparation).

Proteolysis can serve as an important regulatory mechanism and selective, rapid turnover often is a characteristic feature of key enzymes and regulatory proteins such as transcription factors (Rogers et al., 1986). Thus proteasomes may be involved in a development- and cell-specific activation or inactivation (turnover) of short-lived nuclear factors and cytoplasmic proteins. The differential expression of the enzyme suggests that the proteasome may serve very distinct proteolytic processes during early embryo development in the cytoplasm as well as in nuclei. However, at present any discussion about potential in vivo substrates and biological function(s) of this multicatalytic proteinase complex during *Drosophila* embryo development has to remain speculative. Since expression, cellular distribution, turnover, as well as subunit composition of the proteasome vary according to cell type, tissue, or developmental state, it is difficult to imagine that proteasomes possess only a single type of proteolytic function that is common to all cell types or cellular compartments. The observation that individual proteasome subunits undergo posttranslational modification such as phosphorylation and glycosylation (Haass and Kloetzel, 1989; Kremp et al., 1986) and that constitutive subunits possess regulatory motifs such as a nuclear targeting signal and potential phosphorylation sites (Haass et al., 1989, 1990*a*; Fujiwara et al., 1989; Tanaka et al., 1990) suggest that not only the synthesis of the proteasome but also its substrate specificities may be tightly regulated in a cell- and development-specific manner.

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