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Original Article

Differential synthesis and cytolocalization of prosomes in chick embryos during development

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ABSTRACT Prosomes, also called "multicatalytic proteinase" or proteasomes, were purified from chick embryos of different developmental stages by a simple, single-step procedure. These were characterized by their characteristic protein patterns determined by SDS polyacrylamide gel electrophoresis (SDS PAGE) and immunoblotting with four monoclonal antibodies, namely, anti-p27, -p28, -p29 and -p31, prepared against duck prosomes. *In vitro* labeling of embryos with ^{35}S-methionine followed by SDS PAGE and fluorography of the purified prosomes revealed that their polypeptides are differentially synthesized at various stages during development. Among 12 polypeptides (p21 to p56), p21 is synthesized at the beginning of gastrulation (stage 2) followed by the synthesis of p24 at stage 3. Nine other polypeptides (p25 to p35) are synthesized at the head-fold stage (stage 6), while the synthesis of polypeptide p56 is only detected at stage 10 (10-somite stage). Indirect immunofluorescence studies, with the 4 monoclonal antibodies, demonstrated 3 distinct, developmental stage-specific patterns of cytodistribution of these four prosome polypeptides in the embryos. During early embryogenesis, these are uniformly nuclear in location, while at later stages (stage 4 onwards) they are also present in the cytoplasm. Interestingly, one of the antigens (p 28), although found uniformly in all types of tissues in the embryos up to the gastrulation stage, is undetectable in the neural tissues and nonuniformly distributed in other tissues of stage-10 embryos. These data suggest that there are subcomponents of prosomes which are synthesized as well as distributed in an independent manner during development, possibly reflecting subcomponent-specific multiple functions of these particles.

KEY WORDS: *prosomes, MCP, proteasomes, biosynthesis, development, immunolocalization, tissue-specificity*

Introduction

Prosomes, identical to the high molecular weight (20S) multicatalytic proteinase or proteasomes (Arrigo *et al.*, 1988; Falkenburg *et al.*, 1988; Pal and Murakami, 1988; Nothwang *et al.*, 1992a), are composed of protein and small RNA. Prior to the finding that these particles have protease activity, they were characterized as facultative RNP particles (prosomes) associated with the ribosome-free repressed population of globin and other mRNPs from duck and mouse erythroblasts (Schmid *et al.*, 1984; Martins de Sa *et al.*, 1986; Nothwang *et al.*, 1992c) and subsequently from a variety of organisms and cell types (see for review, Arrigo *et al.*, 1987; Scherrer, 1990; Scherrer and Bey, 1994).

Prosomes have a characteristic raspberry- or cylinder shaped structure (Schmid *et al.*, 1984; Martins de Sa *et al.*, 1986). Detailed analyses revealed that they have about 26-28 polypeptides (detected by 2-dimensional gel electrophoresis) of MW ranging between 21 kDa and 35 kDa (with an additional 56 kDa polypeptide in avian species), and 2 to 12 small RNAs of 60 to 120 nucleotides (Martins de Sa *et al.*, 1986; Coux *et al.*, 1992). The major RNA of prosomes in human and duck was found to be tRNA^{lys,3}, the reverse primer of HIV (Nothwang *et al.*, 1992c,b). However, the presence of RNA in prosomes has not been uniformly detected (Kleinschmidt *et al.*, 1983; Castano *et al.*, 1986). Prosomes are resistant to dissociation by salts of high ionic strength and detergents such as sarkosyl (Schmid *et al.*, 1984), but are dissociable instantaneously in 10^{-4} M Cu^{++} and Zn^{++}, losing protease activity (Nothwang *et al.*, 1992b). According to MW, isoelectric point, immunological determinants and sequence, their polypeptides are evolutionarily highly conserved (Grossi de Sa *et al.*, 1988; Tanaka *et al.*, 1992; Bey *et al.*, 1993), and are indistinguishable from the proteasomes in the archeobacteria, *Thermoplasma acidophilum* (Dahlmann *et al.*, 1992; Puhler *et al.*, 1992).

Abbreviations used in this paper: SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; p21-p35, polypeptides of 21 kDa-35 kDa; free mRNP, ribosome-free messenger ribonucleic acid-protein complex; A_{254}, absorbance at 254 nm.

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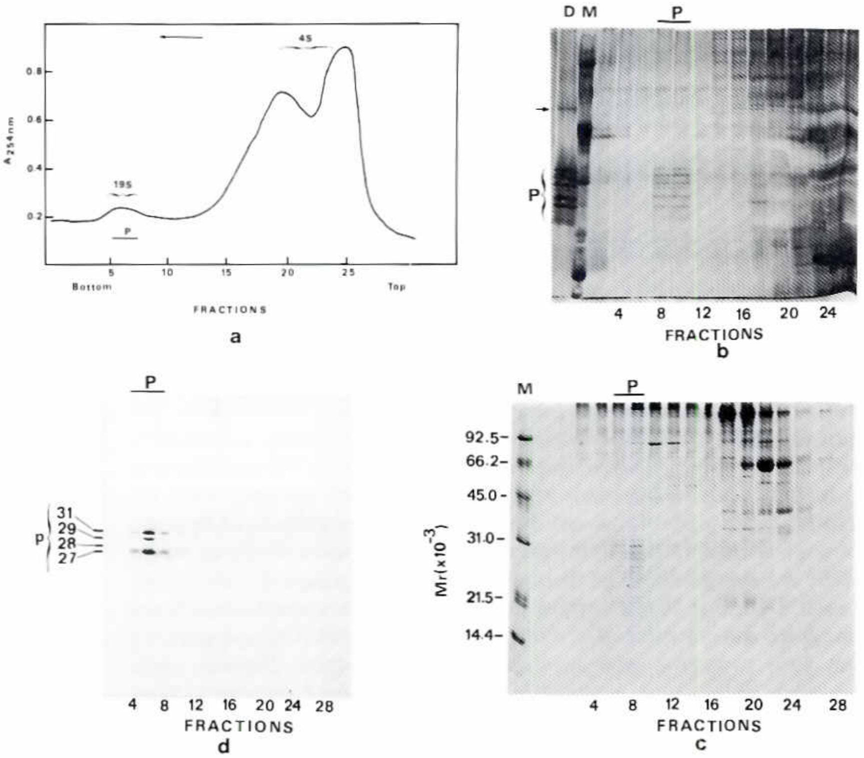

Fig. 1. Preparation of prosomes from the chick embryos (stage 1) and their characterization. (a) *Sucrose gradient profile of the sarkosyl-treated embryo extract centrifuged through a sucrose gradient containing 0.5% sarkosyl; the 19S position in the profile was determined by an 18S rRNA marker run in a parallel gradient.* **(b)** *Protein analysis of each two subsequent fractions (pooled) from the sucrose gradient by SDS PAGE and silver nitrate staining.* **(c)** *Same as* **(b)** *except that the purified free mRNPs from the embryos of stage 4 was fractionated by sucrose gradient centrifugation. P, corresponds to the prosome fraction; Lane M contains MW marker proteins and D, purified duck prosomes (the arrow indicates the position of the polypeptide, p56).* **(d)** *Western blot analysis by duck anti-prosome monoclonal antibodies (p27, p28, p29 and p31) of the proteins from a homologous gel as that shown in (b).*

Proteasomes, homologous to prosomes, also have an RNA component (Rivett, 1993). In *Xenopus laevis* ovary cells, these particles co-purify with a pre-tRNA 5' processing (endonuclease) activity (Castano *et al.*, 1986). Proteasomes have recently been shown to also exist as still higher molecular size complexes sedimenting as 26S particles (Pal and Murakami, 1988; for review see Goldberg and Rock, 1992). This complex contains the core component (20S) and other additional components, such as ubiquitin (Li and Etlinger, 1992; Richter-Ruoff *et al.*, 1992). Therefore, the 26S proteasome is possibly involved in ubiquitin-mediated protein degradation pathway (Seufert and Jentsch, 1992; Hilt *et al.*, 1993). Interestingly, two of the prosome polypeptides are encoded within the MHC class II gene cluster (Goldberg and Rock, 1992; Heinemeyer *et al.*, 1993). Due to their association with many

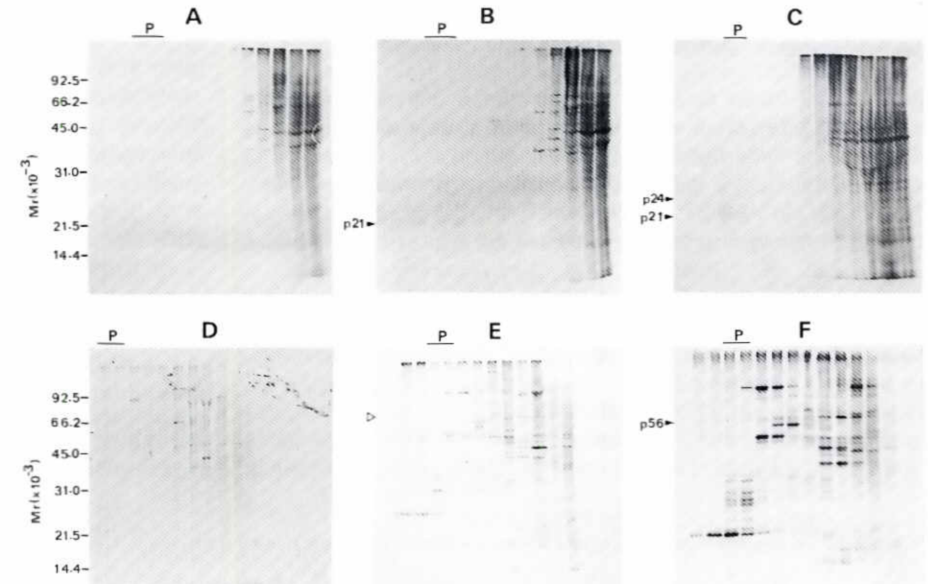

Fig. 2. Synthesis of prosome proteins during development. *Embryos of different developmental stages (Hamburger and Hamilton, 1951) were in vitro labeled with ^{35}S-methionine for 1 h. Prosomes were purified as described in Materials and Methods. Proteins were analysed by SDS PAGE and fluorographed.* **(A-F)** *Fluorograms of the protein gels of the gradient fractions from either the sarkosyl-treated extracts* **(A-C)** *or the free mRNPs (D-F) of different stages:* **(A)** *stage 1 (0 h);* **(B)** *stage 2 (6 h);* **(C)** *stage 3 (12 h);* **(D)** *stage 4 (18 h);* **(E)** *stage 6 (24 h),* **(F)** *stage 10 (36 h). P corresponds to the fractions containing prosomes. Positions of the p21, p24, and p56 are labeled in* **B,C** *and* **F**, *respectively. Absence of the p56 in stage 6 embryo protein synthetic pattern* **(E)** *is indicated by a blank arrow.*

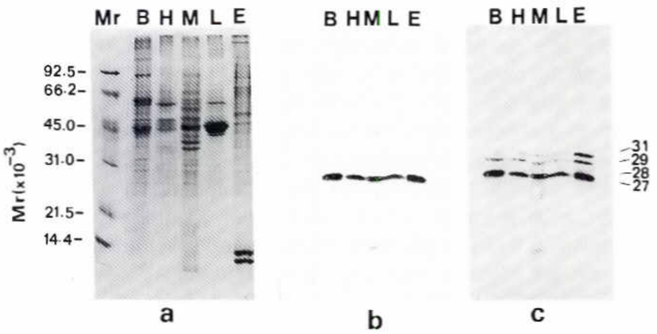

Fig. 3. SDS PAGE and western blot analysis of the free mRNPs prepared from different organs (B, H, M, L) of I9-day-old chick embryos, and extra-embryonic membranes (E) of 4-day-old chick embryos, by anti-prosome antibodies. (a) *Coomassie blue stained gel,* **(b)** *immunoblot with anti-p27,* **(c)** *the same blot redeveloped with a mixture of 3 other antibodies (p28, p29 and p31). Mr, molecular weight marker proteins; B, H, L,M and E represent brain, heart, liver, muscle and extra-embryonic membranes, respectively. The MW of the proteins recognized by the antibodies are labeled in (c).*

such functionally unrelated structures and their ubiquitous nature, a number of investigations have been undertaken by various laboratories to determine the functions of these particles.

In our laboratories we have undertaken several investigations on prosomes during oogenesis and embryonic development in different eukaryotic systems. We have previously demonstrated that eggs are rich sources of prosomes and that in embryonic and somatic cells, cytolocalization of prosomes changes as a function of development and differentiation (Akhayat *et al.*, 1987; Gautier *et al.*, 1988; Grossi de Sa *et al.*, 1988; Pal *et al.*,1988; Briane *et al.*, 1992). In the present communication, we describe a simple, one-step procedure for purification of prosomes from embryonic materials, and we present data on the synthesis and cytolocalization of these particles during development in the chick embryos. During early embryogenesis, prosome polypeptides are not only synthesized asynchronously at various stages of development but are also differentially distributed among tissues and cell types.

Results

Isolation and characterization of prosomes from embryos

To purify and analyse prosomes in chick embryos, we took advantage of the property of their high resistance to the detergent sarkosyl, which dissolves most of the cellular components including the ribosomes. Thus, for characterizing prosomes from the embryos of early developmental stages (stages 1 to 3 of Hamburger and Hamilton, 1951), a single step method of purification was devised (Materials and Methods). Prosomes were also purified from ribosome-free mRNPs (free mRNPs) isolated by the conventional multi-step procedure (Schmid *et al.*, 1984). In the former case, lysing of embryos and sucrose gradient centrifugation were carried out in a wide range of sarkosyl concentrations (0.3 to 1.0%); 0.5% sarkosyl concentration was found to be the optimum. The resulting 19S fractions in the sucrose gradients of both the sarkosyl-soluble supernatant of whole embryos (Fig. 1a) and the purified free mRNP preparations, were found to contain typical prosome protein profiles in SDS PAGE, as seen after silver nitrate and Coomassie blue staining, respectively (compare Fig. 1b and 1c). On sucrose gradients, the sarkosyl-treated extracts of the embryos fractionated into 3 zones of A_{254} absorbing material, one in the 19S region and the other two forming major peaks in the 4S region (Fig. 1a). In similar conditions (data not shown), the 20S free mRNP fraction also dissociated into 3 sub-fractions in the same regions. Protein analysis of each two pooled fractions in the gradient, fractions 5 to 10 (Fig. 1a) revealed protein bands (MW 21 kDa to 35 kDa) characteristic of prosomes/proteasomes (Fig. 1b,1c) as described earlier (Schmid *et al.*, 1984; Martins de Sa *et al.*, 1986; Arrigo *et al.*, 1988; Falkenburg *et al.*, 1988; Pal and Murakami, 1988). However, the 56 kDa protein band, characteris-

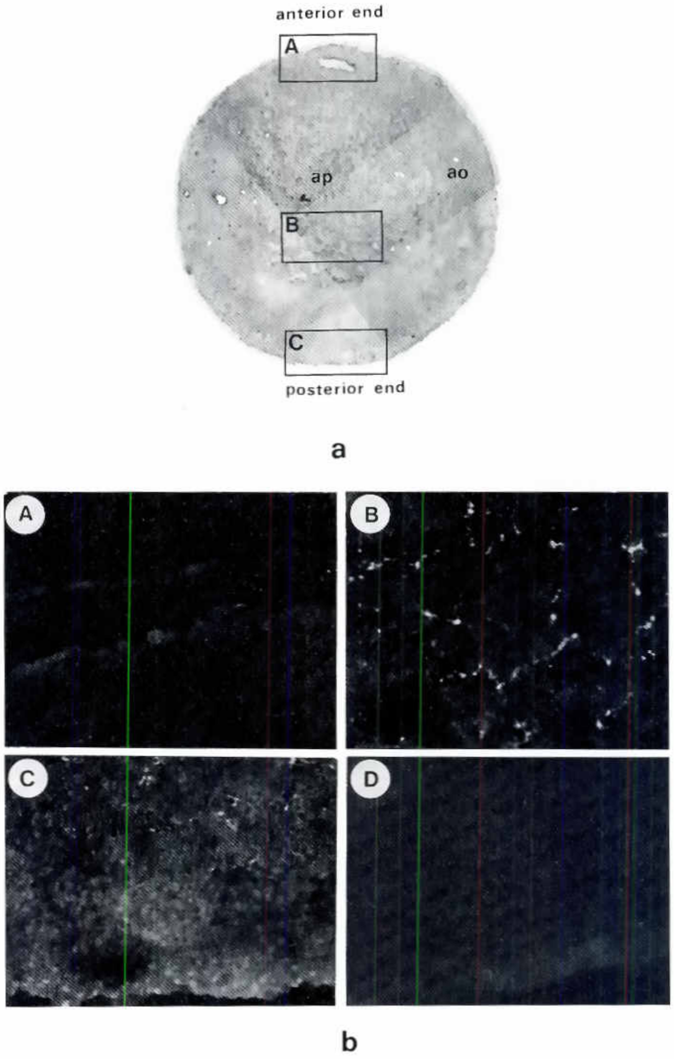

Fig. 4. Localization of the prosome antigens by indirect immunofluorescence on chick blastoderm (entire) with anti-prosome antibodies. (a) *Phase contrast micrograph (reconstituted) of the entire blastodisc (x 24), of stage XI (Eyal-Giladi and Kochav, 1976); a.p., area pellucida, a.o., area opaca. Boxes marked A, B and C correspond to the micrographs, A,B and C, respectively in Fig. 4b.* **(b)** *Immunofluorescence micrographs, obtained with anti-p27 antibody (A,B,C, x190) showing gradual enhancement of fluorescence intensity from anterior to posterior end. D, control (x190).*

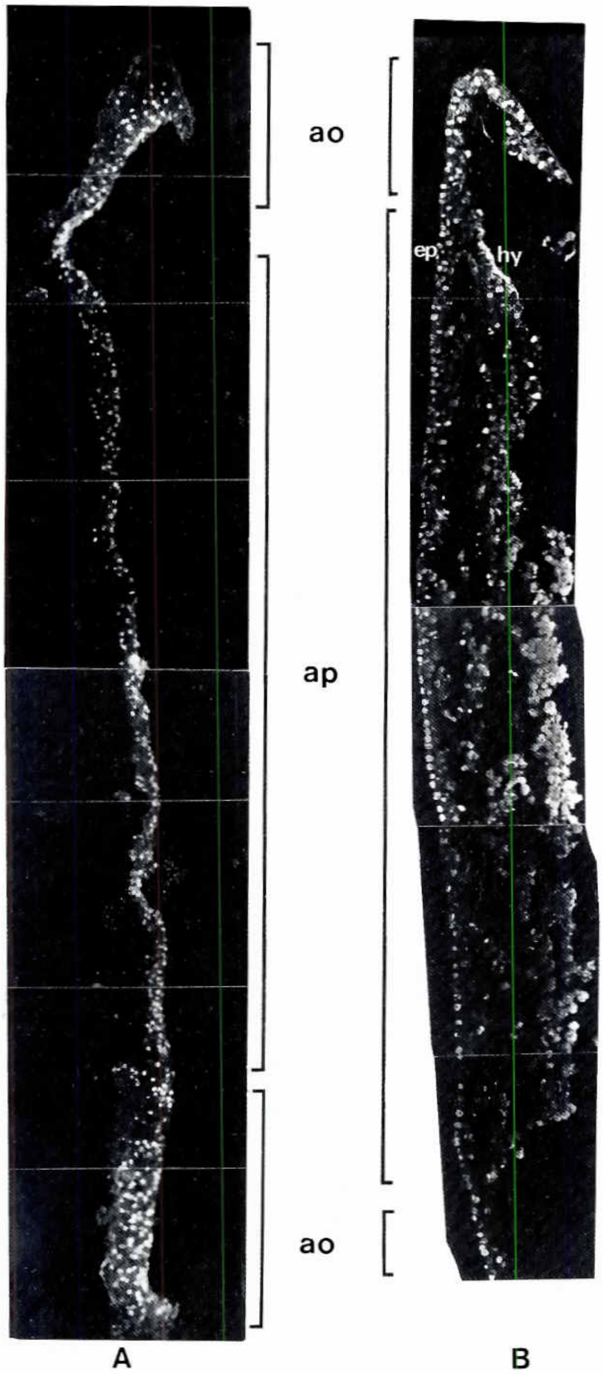

Fig. 5. Immunofluorescence (anti-p27) on chick embryo longitudinal sections (reconstituted). (A) *Stage XI (x100) and* **(B)** *Stage 1 (x150); ao, area opaca, ap, area pellucida, ep, epiblast, hy, hypoblast.*

tic of avian species, could not be detected by Coomassie blue staining in any of the stages of embryos investigated. As seen clearly in Fig. 1b, p56 polypeptide previously observed in the duck is not detectable in chick embryos (position shown by an arrow). Furthermore, these fractions also contained some additional protein bands of higher molecular weight, possibly as contaminants.

In order to further characterize and confirm that these proteins actually are prosome subunits, they were transferred from a duplicate gel containing all the 28 fractions (each two subsequent fractions pooled) of the gradient onto nitrocellulose membranes and immunoreacted with 4 monoclonal antibodies to duck prosomes that were previously characterized (Grossi de Sa *et al.*, 1988). As seen in the immunoblot (Fig. 1d), the protein bands of MW 27 kDa, 28 kDa, 29 kDa and 31 kDa showed positive reaction localized in fractions 4 to 8 of the gradient; protein band p28 was rather faint. Thus, in view of these results and the fact that the prosomes resist 0.5% sarkosyl which dissolves most other cellular structures, this group of proteins may be regarded as belonging to the prosome particles. Furthermore, since there was no positive immunoreaction of these 4 antibodies in any other region of the gradient, and in particular, not detectable in the soluble protein fractions, the prosome proteins are thus present in the cell in the assembled complex form only.

Synthesis of prosome polypeptides during development

Since the prosomes could be purified in a reasonably high quantity already from the chick blastoderms obtained from the freshly-laid eggs, it appeared interesting to study the synthesis of prosome proteins and its possible correlation with various important events during morphogenesis. For this analysis, blastoderms of stage 1 and embryos of early gastrula (stage 2), mid-gastrula (stage 3), full primitive streak (stage 4), headfold (stage 6) and 10-somite (stage 10) stages, were labeled with ^{35}S-methionine *in vitro*. The prosome proteins purified from the labeled embryos of different stages were subjected to SDS PAGE and fluorography (Fig. 2). For all stages, material of equivalent radioactivity (prepared from the comparable subcellular fractions) was used for prosome purification by sucrose gradient analysis, and for fluorography; the gels were exposed for the same period of time. For a given stage, the profile of ^{35}S-methionine labeled protein bands of the prosomes prepared from the sarkosyl-soluble supernatant, was not different from that prepared from the purified 19S free mRNP fraction (data not shown). As seen in Fig. 2A (slots 'P'), no incorporation of radiolabeled amino acid into prosome proteins could be detected in the blastoderms of stage 1. The first prosome polypeptide which is synthesized at stage 2 (beginning of gastrulation, 6 h of incubation) is p21 (Fig. 2B), followed by another polypeptide, p24 which is synthesized at stage 3 (12 h incubation) embryos (Fig. 2C); this is more clearly seen (Fig. 2D) in the embryos of full primitive streak stage (18 h incubation). The profiles of protein synthesis remained unchanged even after longer exposure to fluorography. Interestingly, in stage-6 embryos (the head-fold stage, 24 h incubation), almost all other prosome polypeptides began to be synthesized (Fig. 2E). Furthermore, synthesis of the p56 polypeptide, characteristic of avian species, could only be detected in stage-10 (36 h incubation) embryos (Fig. 2F). However, it still could not be detected by Coomassie blue staining even at this stage of embryogenesis.

Differential distribution of prosome polypeptides in different organs of chick embryos

In order to determine the presence and distribution of prosome polypeptides in different organs, total free mRNP proteins prepared from different organs of 19-day-old chick embryos (brain, heart, muscle and liver) were analysed by immunoblotting (Fig. 3a-c). Similarly, extra-embryonic membranes (yolk sac, chorion, amnion and allantois, extracted together) of 4-day-old embryos were also analysed (Fig. 3a-c). As seen in Fig. 3a, in all organs tested, faint protein bands in the 21 kDa to 35 kDa region could be observed by Coomassie blue staining. However, Western blot analyses of equal quantities of total mRNP proteins revealed different intensities of various prosome polypeptides in these organs (Fig. 3b,c). The p27 band was most intense in brain as well as in the extra-embryonic membranes (Fig. 3b), whereas the p28 polypeptide was not very distinct in the organs except in the extra-embryonic membranes (Fig. 3c). Furthermore, both the p29 and p31 bands were much more intense in the extra-embryonic membranes than in other organs (Fig. 3c). Among these organs, the p29 polypeptide was again most intense in the brain. Thus, in addition to the quantitative variation in the total prosome content, there exists a differential distribution of distinct prosome polypeptides in different organs.

In situ localization of prosome antigens in the embryos

Embryos of four morphogenetically significant stages (stage XI of Eyal-Giladi and Kochav, 1976; and stages 1, 4 and 10 of Hamburger and Hamilton, 1951) were used for immunolocalization of prosome antigens by indirect immunofluorescence, in order to investigate the correlation between prosome distribution and the differentiation of distinct cell lineages during embryogenesis.

For the earliest developmental stage studied (stage XI, Eyal-Giladi and Kochav, 1976), in addition to thin sections, the entire blastoderms were used for immunofluorescence. At this developmental stage, the blastoderm is essentially composed of a single layer of cells (epiblast) except at the periphery, the area opaca (Figs. 4a, 5A). It is to be noted that the general pattern of fluorescence for the embryos of up to stage 4 remained similar for all the 4 antibodies used (anti-p27, -p28, -p29 and -p31). Henceforth, we present immunofluorescence data obtained with anti-p27 antibody, unless stated otherwise.

At stage XI, fluorescence could be seen in the nuclei (Fig. 4b,C) and possibly in extracellular spaces or on plasma membranes (Fig. 4b,B), depending on the sectors of the blastoderm (Fig. 4b,A-C). Indeed, the pattern of fluorescence was in the form of a gradient: its intensity being strongest near the posterior marginal zone (Fig. 4b,C) and weakest, barely detectable, in the area opaca at the anterior end (Fig. 4b,A). This result was consistent irrespective of the surface (dorsal or ventral) of the blastoderm exposed for immunoreaction.

To further verify the fluorescence pattern obtained on the entire blastoderm, 5 µm sections of blastoderms were immunostained. In this case, however, at stage-XI embryo sections, the gradient of intensity in fluorescence was more subtly distributed (Fig. 5A). The main observation was that the nuclei of cells from the anterior to the posterior end of a longitudinal section (both the area opaca and the area pellucida) were fluorescent although to different degrees, whereas the cell cytoplasm seemed to be free of prosome antigens (Fig. 4b,A).

When a second type of tissue layer (the hypoblast) is well formed the fluorescence pattern in the stage-1 embryo did not change; cell nuclei in both epiblast and hypoblast were fluorescent (Figs. 5B and 6). At higher magnifications, nuclear fluorescence was observed to be strongest at the nuclear membrane and in patches, possibly corresponding to the nucleolus (Fig. 6C). Furthermore, intensity of nuclear fluorescence seems to vary from cell to cell (Fig. 6C).

The fluorescence pattern of transverse sections of the stage-4 (18 h incubation) embryo is presented in Fig. 7. The fluorescence could be observed in most cells (compare Fig. 7A,B, Hoechst stain vs. A',B', immunofluorescence). The antigen, although very strong in the nucleus, was now also detected in the cytoplasm and on the plasma membrane (Fig. 7A',B'); plasma membrane fluorescence was more intense at the periphery of the embryo (extra-embryonic region, Fig. 7A') than at the center (Fig. 7B'). At this stage of

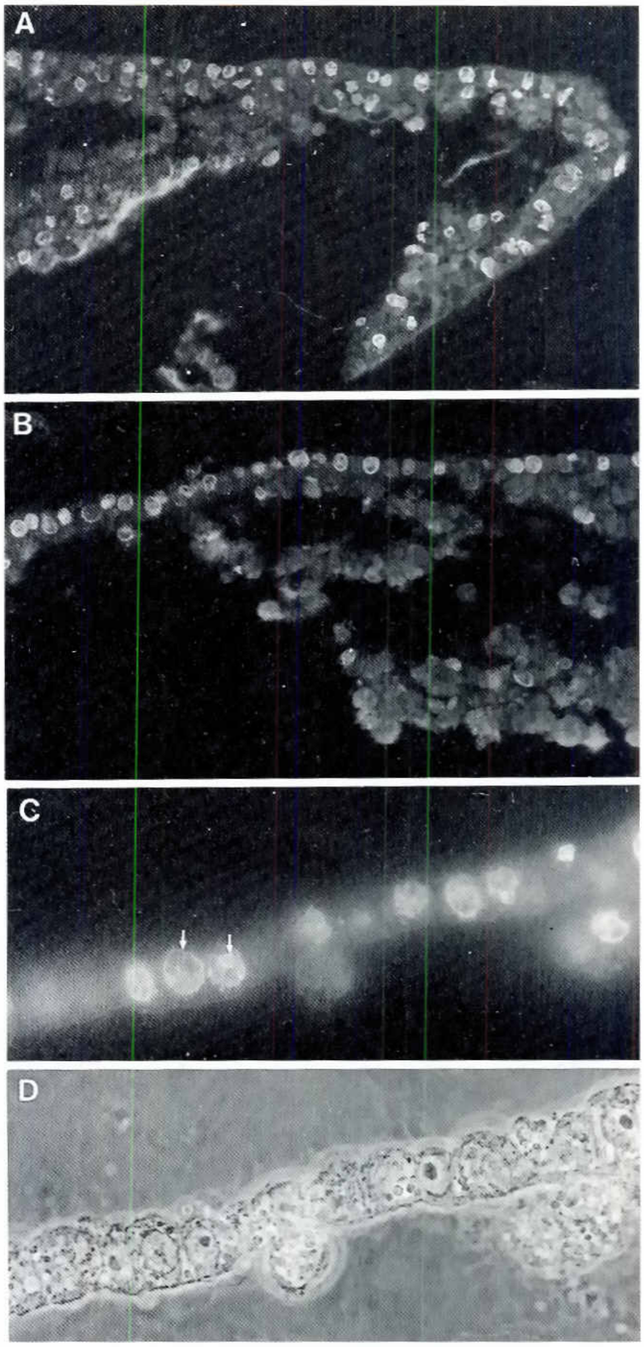

Fig. 6. Higher magnification immunofluorescence micrographs of some regions in Fig. 5B. (A,B) *x300* **(C)** *x750, fluorescence micrographs;* **(D)** *phase contrast micrograph of C; fluorescence in the nuclear membrane and nucleous is indicated by arrows* **(C)**.

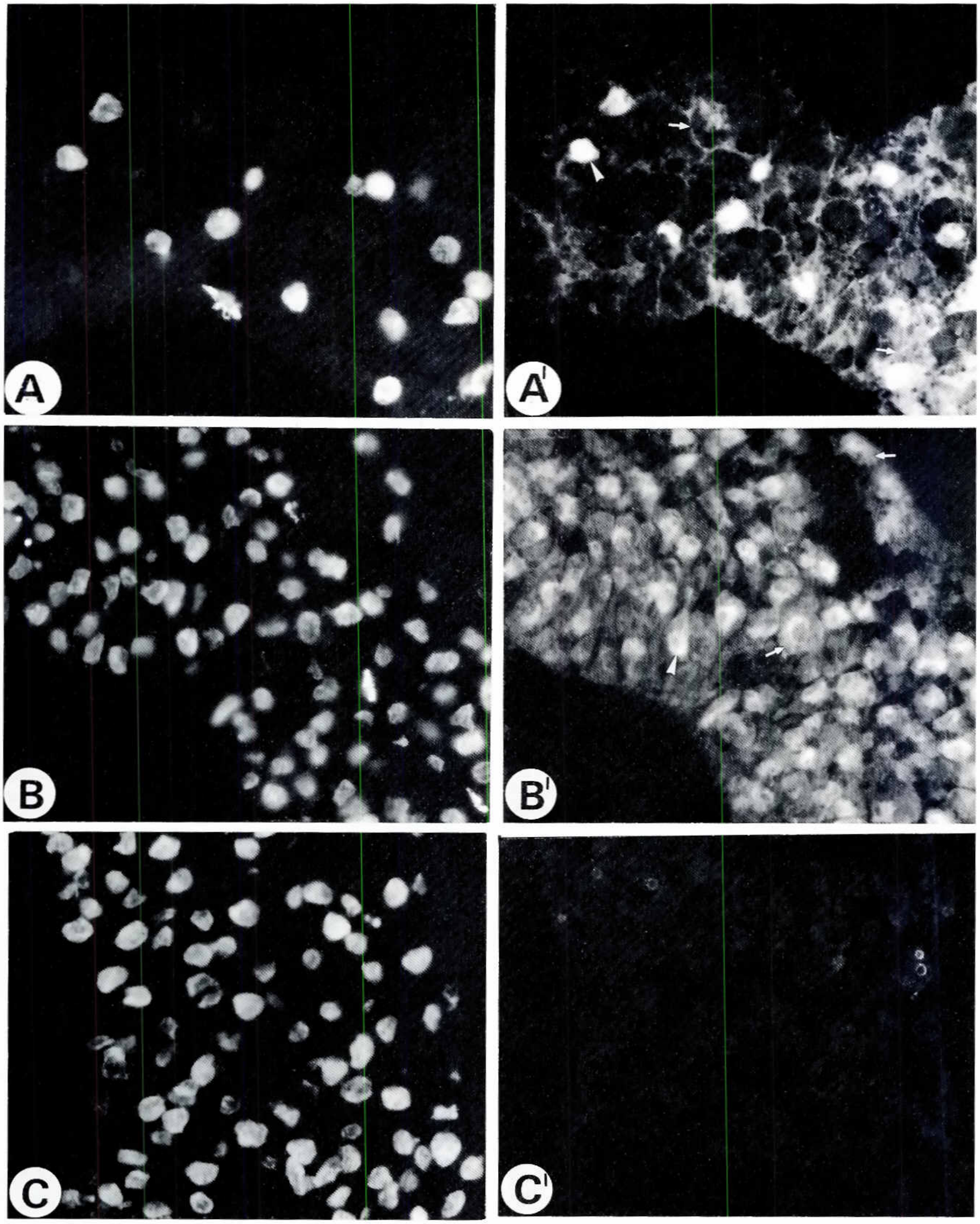

Fig. 7. Immunofluorescence on stage-4 embryo transverse sections through posterior primitive streak region. (A' and B') *Fluorescence micrograhps (X480) of extra-embryonic and embryonic regions, respectively;* **(C')** *control. Fluorescence on the plasma membrane, in the cytoplasm and nucleus ise indicated by arrows.* **(A,B and C)** *Hoechst-stained micrographs of A',B' and C', respectively.*

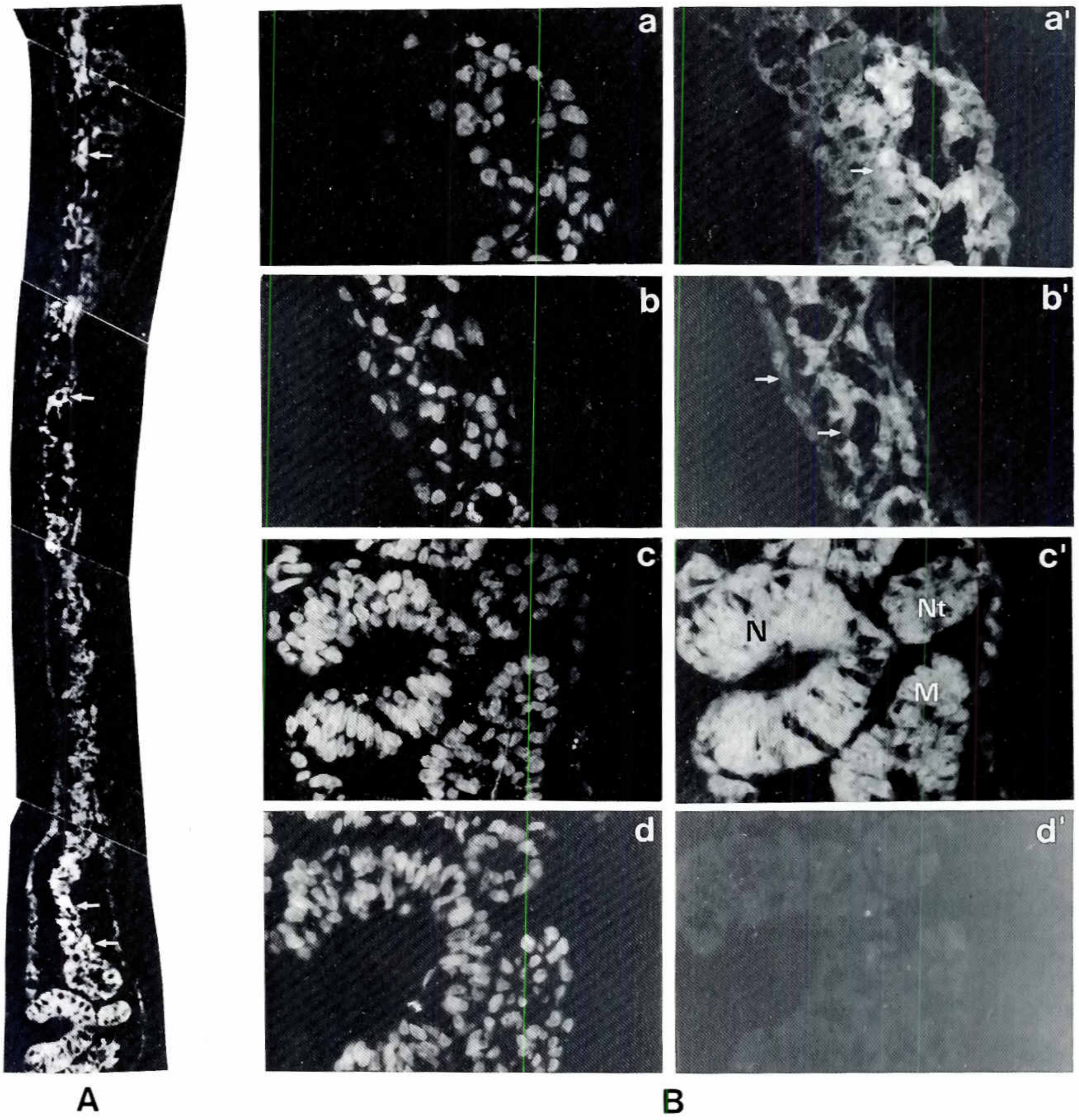

Fig 8. Immunofluorescence (anti-p27) on stage-10 embryo transverse section through the trunk region. (A) *Reconstituted one-half of the entire section (x100); arrows indicate stronger fluorescence in the mesodermal tissues.* **(B)** *Different regions in* **A**, *under higher magnifications (x240). (a',b' and c') Regions showing extraembryonic vascular region, extraembryonic coelom region and central part of the embryo proper, respectively; cytoplasmic fluorescence is indicated by arrows in a' and b'. Neural tube (N), notochord (Nt) and unsegmented mesoderm (M) are labeled in c', d', control; (a,b,c,d,) Hoechst-stained photomicrographs of a',b',c' and d', respectively.*

development also, the fluorescence patterns with all 4 antibodies remained identical.

The fluorescence pattern of stage-10 (36 h incubation) embryos (transverse section through the trunk region) is presented in Figs. 8 and 9. Two very interesting observations were made: 1) With all antibodies (except anti-p28) the tissue sections showed quite uniform fluorescence, but apparently, the mesodermal tissues were slightly more intense (Fig. 8A, arrows). The cytoplasm, in addition to the nuclei, in most of the cells was strongly positive for the prosome antigens (Fig. 8B, a',b',c'). As in stage-4 embryos, cytoplasmic fluorescence was more intense and clear in the extra embryonic regions, namely, vascular region (Fig. 8B,a', arrow) and coelomic region (Fig. 8B,b' , arrow) than in the embryo itself (Fig. 8B,c'). 2) The anti-p28 antibody, however, did not label the neural

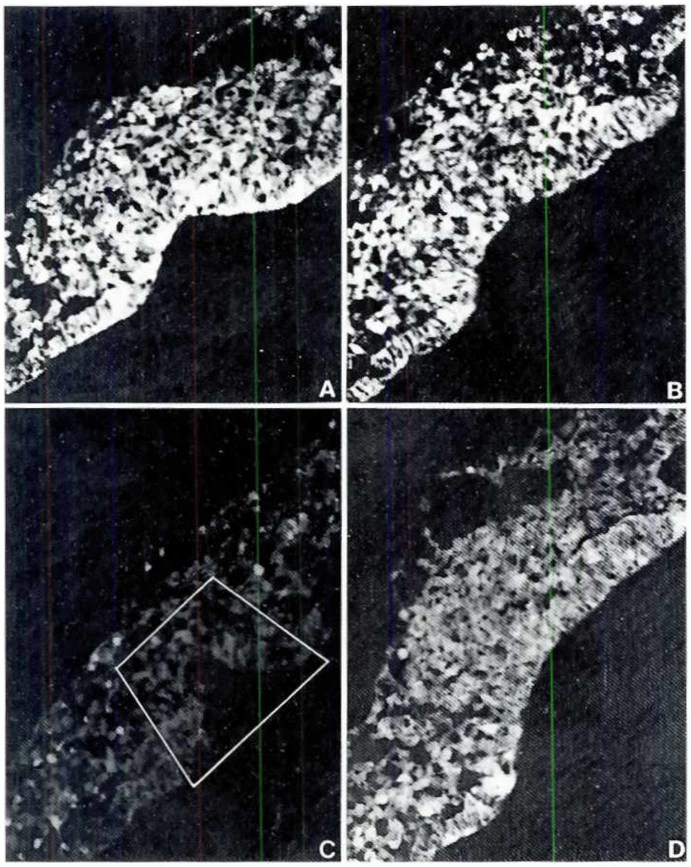

Fig. 9. Immunofluorescence on stage-10 embryo transverse sections (x190) through the posterior trunk (primitive streak) region with 4 monoclonal antibodies, anti-p31 (A), anti-p29 (B), anti-p28 (C) and anti-p27 (D). *Note the absence of anti-p28 staining in the neural tissue (marked by a box) and nonuniform staining of cells of other tissues in* **(C)**.

tissue, and labeled the other tissues nonuniformly (Fig. 9C), as opposed to the other three antibodies which uniformly labeled all the tissues (Fig. 9A,B,D). Thus the p28 antigen was apparently cell- and tissue specific. These results have been reproduced in three separate experiments.

Discussion

Our previous studies demonstrating that prosomes are minor cellular constituents and that they are also present in the nucleus, particularly during early stages of cellular differentiation (Akhayat *et al.*, 1987; Grossi de Sa *et al.*, 1988; Pal *et al.*, 1988), prompted us to investigate prosomes during early embryogenesis in chick. First, a simple, single-step method of prosome preparation from the total embryo extracts was devised (see Results). Immunological characterization by western blot analysis (Fig. 1d) and electron microscopy (data not shown) revealed that prosomes purified by this method were authentic; they were identical to those isolated by others (Nothwang *et al.*, 1992a). This analysis also indicated that the chick embryos are a relatively rich source of prosomes, and that the prosomes are in the characteristic form of a 19S sarkosyl-resistant complex.

After characterization of the prosomes from embryos, the prosome polypeptide synthesis during early embryogenesis was investigated. A very interesting pattern of prosome protein synthesis was observed, indicating the possibility that the prosomes present in the stage-1 blastoderms are of maternal origin and their *de novo* synthesis follows a development-related pattern. No incorporation of ^{35}S-methionine in the prosome proteins could be observed prior to gastrulation. Two polypeptides, p21 and p24, were synthesized during gastrulation (see Fig. 2B,C), while most of the other polypeptides were synthesized at the onset of neurulation (24 h incubation) only. In sea urchin also, no synthesis of prosome proteins could be detected prior to 48 h gastrula stage (Akhayat *et al.*, 1987). Prosome protein synthesis starts therefore with individual peptides, possibly indicating changes in the subunit composition of the particles, and resumes fully in late blastulation and gastrulation when zygotic transcription begins. During morphogenesis, new genetic programmes must be triggered by gastrulation, prior to the establishment of the major tissue-specific cell lineages. Thus, synthesis of the majority of prosome polypeptides followed by their association to form prosome particles, *per se*, after gastrulation, might have a significant bearing in terms of gene regulation.

The stage-specific synthesis of different prosome polypeptides leads to the further proposition that these different proteins may be the constituents, in variable sets, of more than one type of prosome. Thus, the p21, p24, and p56 polypeptides may belong to different types of individual prosome particles. This assumption gains support from previous observations that in fetal rat liver, specific types of prosomes including the p31 antigen are distributed along the bile canaliculi in a developmentally regulated fashion, while prosomes stained by a polyclonal antibody were distributed all over the cells (Briane *et al.*, 1992). There may thus exist cell lineage-specific prosome types; the synthesis of these prosomes might then be dependent on the establishment of the corresponding cell/tissue lineages during development.

The question of the exact time period when the individual prosome subunits (which are already present in the stage 1 blastoderms) are synthesized, still remains to be answered. As mentioned, the studies on sea urchin (Akhayat *et al.*, 1987), the chicken (the present study) and urodelan amphibians (Pal *et al.*, 1988) suggest that the prosomes present abundantly in the early embryos must have been synthesized during oogenesis and thus belong to the maternal components of the embryo. Incidentally, it was observed (the present study) that the extra-embryonic membranes contain relatively higher amounts of the prosome polypeptides than the embryonic organs (Fig. 3c).

In situ immunofluorescence analysis with all four monoclonal antibodies revealed similar gross fluorescence patterns in embryos up to the primitive streak stage (stage 4). At the onset of organogenesis (stage 10), however, one of the antigens (p28) was uniformly absent in the neural tissue and in some cells of other tissue types (Fig. 9C). Hugle *et al.* (1983) also observed a tissue-specificity for a given antigen of the "cylinder particles", which resemble prosomes, both in their protein composition and ultrastructure, in *Xenopus laevis*. This tissue-specificity of p28 and perhaps some other prosome antigens, which are not yet studied, suggests the possibility that the prosome particles are composed of a set of common protein polypeptides/subunits and a set of variant ones depending on the type of tissues they belong to.

The immunofluorescence results obtained on the entire blastoderm of stage XI appeared interesting. The prosome antigens were localized mainly near the posterior marginal zone, being abundant in the intercellular spaces and in the nuclei. It is known that the highest metabolic activity, in correlation with the first morphogenetic process in chick development, is localized at the posterior marginal zone (Eyal-Giladi, 1984). Thus, it is tempting to speculate that the accumulation of prosomes in this region is physiologically significant and that they may be involved in the regulation of cell cycle, thereby facilitating the process of early morphogenesis.

Immunofluorescence analysis also demonstrated the presence of the prosome antigens in the nuclei in early blastoderms and a shift in their cytolocalization from the nucleus to the cytoplasm during the course of development, although they remained localized mainly in the nucleus. Similar observations on the dramatic changes in the cytolocalization of prosomes have also been reported earlier in *Pleurodeles* during development (Pal *et* al., 1988) and in axolotl during oogenesis (Gautier *et al.*, 1988) as well as more recently in *Caenorhabditis elegans* (Schnabel and Scherrer, unpublished observations) and in fetal rat liver (Briane *et al.*, 1992). In light of the more recent findings that the proteasomes are involved in the regulation of cell cycle in the ovarian granulosa cells, possibly by regulating cyclin degradation (Amsterdam *et al.*, 1993) and in the ascidian embryos (Kawahara and Yokosawa, 1992), our observations suggest a cell cycle regulatory role of prosomes/proteasomes in the chick embryos during development.

Materials and Methods

In vitro embryo culture and ^{35}S-methionine labeling

Freshly-laid chicken eggs (obtained from Ferme Avicole, Strasbourg, France) were incubated at 37.5°C to the desired stages of development. Unincubated blastoderms and embryos were staged according to Eyal-Giladi and Kochav (1976) and Hamburger and Hamilton (1951), respectively. Embryos were isolated and cultured *in vitro* according to Olszanska and Lassota (1980). A small filter paper (Whatman no. 1) with a central hole of 8 mm in diameter was placed on the yolk surface in such a way that the embryo was just within the hole. The vitelline membrane was cut along the outer margin of the support, and the filter paper with the embryo attached was slowly removed by pulling with forceps avoiding much of the yolk. Five embryos were cleaned of yolk and placed in a Petri dish (3.5 cm diameter) containing 3 ml of thin albumen with 30 μCi/ml ^{35}S-methionine (specific activity, 1360 Ci/mmol, Amersham, London). The Petri dish containing the embryos was then transferred to a large Petri dish (9 cm in diameter) moistened with wet paper towel and incubated at 37.5°C for 1 h. Five batches containing 25 embryos for each stage were used. The kinetics of methionine incorporation was studied and its optimum dosage and labeling time were determined and standardized. After radio labeling, the embryos were detached from the vitelline membranes, immediately washed twice in phosphate buffered saline (PBS, 7 mM Na_2HP0_4, 1.5 mM KH_2P0_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and processed further.

Preparation of ribosome-free mRNPs and prosomes

Free mRNPs from the postmitochondrial supernatants of embryos of different stages of development as well as from different organs of 19-day-old chick embryos, and of extra-embryonic membranes (chorion, amnion and allantois) of 4-day-old embryos were prepared as described earlier (Martins de Sa *et al.*, 1986). For the prosome preparation, free mRNPs were centrifuged (Beckman rotor SW41, 37,000 rpm, 17 h) through 5 to 28% sucrose gradients containing either 0.5 M KCl or 0.5% sarkosyl. Sucrose gradients were fractionated and the absorbance of fractions was monitored at 254 nm. Each two successive fractions were pooled and analysed by SDS PAGE. For the embryos of the early stages (stages 1 to 3), due to lack of sufficient material, a simpler, one-step method of prosome preparation was essential. The labeled embryos were directly lysed in 25 mM TEA (Triethanolamine) buffer (pH 7.4) containing 5 mM 2-mercaptoethanol and 0.5% sarkosyl. The extract was centrifuged (10,000 rpm, 20 min, 4°C) and the supernatant was treated with DNAase 1 (30 min, 4°C), and subjected to sucrose gradient (0.5% sarkosyl) centrifugation, as described above. The advantage of this method for the preparation of prosomes from the embryos is described in detail in the Results section.

SDS PAGE and fluorography

Sucrose gradient fractions were precipitated with 10% TCA (2 h, 4°C). The protein precipitates were washed with cold acetone and analysed by SDS PAGE (13%) according to Laemmli (1970). The protein contents of free mRNPs of different organs were determined by the method of Bradford (1976), and the mRNPs were analysed by SDS PAGE. The following proteins (Bio-Rad) were used as molecular weight markers: phosphorylase b (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lactalbumin (14.4 kDa). Gels were stained with either Coomassie brilliant blue R-250 or silver nitrate (Wray *et al.*, 1981), treated with enhance, dried and exposed (Fuji X-ray film) for fluorography (-70°C).

Immunoblotting

Proteins separated by SDS PAGE were electrophoretically transferred onto nitrocellulose membrane (0.45 μm, Schleicher and Schull, Germany) according to Towbin *et al.* (1979). The protein blot was saturated overnight (4°C) with either 3% bovine serum albumin (BSA) or 5% solution of dry, non-fat milk in PBS to prevent non-specific adsorption, followed by incubation with monoclonal anti-prosome antibodies (either culture supernatants or ascites fluids) overnight at 4°C. After washing with PBS for 30 min with 4 changes, the blot was incubated with the peroxidase-labeled second antibody (goat anti-mouse IgG-peroxidase) diluted in PBS (1:1,000) containing 10% goat normal serum, for 4 h. The blot was washed in PBS (30 min), and the color was developed with H_20_2 and 4-chloro-1-naphthol.

Indirect immunofluorescence

Entire blastoderms isolated from freshly-laid eggs (stage XI, Eyal-Giladi and Kochav, 1976), were permeabilized in 0.5%Triton X-100 in PBS and transferred to drops of PBS on poly-L-lysine-coated slides. Blastoderms were covered with silanized coverslips and further permeabilized by freezing on dry ice (5 min). After removing coverslips, blastoderms were fixed in methanol (4°C, 20 min) and air dried (Strome and Wood, 1982). Preparations of entire embryos (blastoderms) were directly used for immunofluorescence as described below.

For preparing sections, the embryos were fixed in 4% paraformaldehyde in PBS (1 h, room temperature), washed successively in PBS containing 25 mM glycine and in PBS and then processed for cryosectioning following sucrose (20%) infiltration. Sections of 5 μm were cut and attached onto poly-L-lysine-coated slides. The sections and the entire blastoderms, prepared as described above, were incubated with anti-prosome antibodies diluted in PBS containing 0.1% BSA, 0.02% Tween 20 and 0.02% sodium azide, for 1 h (room temperature). Nonimmune mouse serum was used as negative control. After washing in PBS containing 0.02% Tween 20 (PBST) for 30 min with three changes, they were further incubated with FITC-goat anti-mouse IgG (30 min, room temperature). For labeling the nuclei, Hoechst (H 33258) was used along with the second antibody in all the experiments. After PBST wash (30 min), the sections were mounted in Mowiol and were observed under a Zeiss fluorescence microscope equipped with epifluorescent illumination. Photomicrographs were taken on Ilford HP5 film (400 ASA).

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