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IMMUNOHISTOCHEMICAL LOCALIZATION OF β-TUBULIN IN THE UNINCUBATED AVIAN GERM AND IN THE PERI-, PARA- AND SUBGERMINAL OOPLASM: HOMOLOGY WITH MEROBLASTIC TELEOST EMBRYOS

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Abstract. β-Tubulin was immunohistochemically localized in the unincubated avian germ and in the peri-, para- and subgerminal ooplasm. Numerous immunostained threads were found surrounding the clusters of mitotic figures seen in the peripheral subgerminal ooplasm. In the Rauber's sickle this method enabled clear discrimination to be made between unstained ooplasmic areas and the immunostained surrounding blastoderm cells. A vast, superficial, extra-embryonic ring-shaped area of paragerminal ooplasm (forming a white halo, seen on the surface of the intact living yolk) presenting a dense immunoreactivity on sections, encircles the clear perigerminal ooplasm and the rim of the blastoderm at some distance.

Keywords: Tubulin, avian blastoderm, ooplasm, epiboly, Rauber's sickle, teleost embryo.

INTRODUCTION

In a recent study (CALLEBAUT, 1994), numerous giant clusters of mitotic figures in the subgerminal ooplasm (forming a syncytium) below the germ wall of unincubated avian blastoderms were described. By autohistoradiographic incorporation studies of ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine or ^{3}H-tyrosine during and after the period of establishment of bilateral symmetrization of the avian germ, we demonstrated that a pronounced nucleic acid and protein synthesis occurs in these clusters of mitotic figures and in the neighbouring intervitelline ooplasm. The role of these chromosome clusters in avian eggs, in which a meroblastic type of cleavage exists, is unknown but their localization suggest some morphological homology with the external part of the multinucleate yolk syncytial layer described in the also meroblastic eggs of teleosts (LENTZ & TRINKAUS, 1967).

This yolk syncytial layer, which is also localized between the yolk mass and the deep blastomeres provides, according to TRINKAUS (1984), most of the contractile force for epiboly, a phenomenon which also exists in the meroblastic eggs of birds. During the sliding movements of large units of ooplasm over one another and subsequent anchoring, microtubules have been shown to play an important role in amphibian eggs (GERHART et al.,1989). Recently in the zebrafish (*Danio rerio*) blastulas, it has been shown that microtubule arrays of the yolk cell play an important role during epiboly (SOLNICA-KREZEL & DRIEVER, 1994).

This stimulated us to study the β-tubulin distribution in unincubated avian germs and in the surrounding perigerminal, paragerminal and underlying subgerminal ooplasm.

MATERIAL AND METHODS

Fertilized unincubated quail eggs (stored at 15-20°C for no longer than 1-2 days) were opened. After removal of the egg white, their egg yolk balls were placed in Ringer's solution. Egg yolks with blastoderms presenting a sickle of RAUBER (RAUBER, 1876) (permitting an exact caudocephalic orientation), were used in this study. The blastoderms at that moment were in stage 1 (VAKAET, 1970). Charcoal marks were applied *in vivo* on the vitelline membrane above the white halo (paragerminal ooplasm) visible just peripherally to the clear perigerminal zone, surrounding the rim of the blastoderm. This charcoal labeling was also applicated over the white halo surrounding germs in the cleavage stage from eggs extracted from the uterus according to the technique of OLSEN & BYERLY (1932). These egg yolks with blastoderm *in situ* were then fixed *in toto* for 3h at 4°C in 2% paraformaldehyde and 0.1% glutaraldehyde in 10mM phosphate - buffered saline (PBS) at pH 7,2. After rinsing in PBS, the egg yolks with blastoderm still *in situ* were dehydrated during the following days. In 95% alcohol, the germs were excised with some surrounding ooplasm. Still adherent to their vitelline membrane, they were transferred into absolute alcohol. After a passage through xylene, they were embedded in paraffin. Serial 10 µm thick sections were made parallel with the caudocranial axis (medio- and parasagittal) of the germ. The blastoderms in the paraffin were placed vertically, peripendicular to the microtome knife edge to avoid dorsoventral compression during sectioning. The sections were mounted on glass slides coated with Para-Tissuer (Imtec Diagnostics n.v.,Uithoorn, The Netherlands) and airdried for 1h at 37°C.

Immunohistochemical staining of the deparaffinized sections was performed by the unlabelled antibody peroxidase-anti- peroxidase (PAP) technique as previously described (VAN NASSAUW *et al.*,1989). Briefly, endogenous peroxidase was blocked with 1% hydrogen peroxide in methanol, and non- specific binding was blocked with normal rabbit serum (DAKO A/S, Glostrup, Denmark), diluted 1:25 in PBS containing 5% bovine serum albumin (BSA) and 0.1% sodium azide.

Subsequently, the sections were incubated overnight at 4°C in the primary antiserum diluted in PBS containing 0.1% BSA and 0.1% sodium azide. We used a commercially avialable mouse monoclonal antibody directed against rat brain β-tubulin (Sigma Chemical Co., St. Louis, MO), diluted 1:400. After washing in PBS, the sections were incubated for 1h in rabbit anti-mouse IgG (DAKO A/S), diluted 1:25 in PBS*. The sections were washed in PBS, before incubation for 1h in mouse PAP complex (DAKO A/S), diluted 1:250 in PBS.

Peroxidase activity was revealed using the diaminobenzidine method (GRAHAM & KARNOVSKY, 1966). The sections were counterstained with 0.1% toluidine blue. Method specificity was controlled by omitting the incubation with the primary antibody in the regular staining procedure.

RESULTS AND DISCUSSION

Brown staining of the tissues, indicating tubulin immunoreactivity was found in the blastoderms of the unincubated quail eggs (Figs 1-2). Yolk granules in the blastoderm and in the subgerminal ooplasm (Fig. 2) presented no immunoreactivity but were stained by toluidine blue.

Consequently the contrast between the cellular borders of the blastoderm and the sub- and perigerminal ooplasm was very obvious. Moreover the fixative and immunostaining used also permitted a good general survey to be made of the main morphological details of the unincubated germ, even at the lowest magnification. By this means the 3 elementary tissues (endophyll, RAUBER's sickle and upper layer) of the unincubated avian blastoderm (CALLEBAUT *et al.*, 1996 a) are distinctly visible (Figs 1-3-5). Most obvious is the unequal caudocranial uptake of ooplasm by the blastoderm (CALLEBAUT, 1993 a, 1994). The endophyll was visible as a caudal sheet of flat cells clearly separated from the upper layer and continuous with RAUBER's sickle (the early avian gastrulation organizer: CALLEBAUT & VAN NUETEN, 1994) forming an axilla shaped groove (Fig. 3) in the caudal part of the subgerminal cavity.

The contact between RAUBER's sickle and the vegetal subgerminal ooplasm is very intimate and according to CALLEBAUT *et al.* (1996 b), RAUBER's sickle seems to be homologous with the vegetal dorsalizing cells or centre of NIEUWKOOP (1973) in amphibian blastulas. At higher magnification, in the RAUBER's sickle cell mass the distinction between the exclusively toluidine blue stained ooplasmic areas (so called «yolk islands») and the surrounding RAUBER's sickle cells (Fig. 4) is clearly seen. These results are in agreement with earlier observations. First, after appropriate fixation (calcium formalin) and Unna staining we were also able to visualize the massive capture of underlying ooplasm by the encircling movement or extensions of the blastomeres in the region of RAUBER's sickle (CALLEBAUT, 1993 a).

Second, after radioactive oocytal labeling we observed in the RAUBER's sickle of unincubated blastoderms, the presence of voluminous «islands» of ooplasm still presenting an intervitelline labeling, which demonstrates their direct uptake without previous lysis by secretion (CALLEBAUT, 1994).


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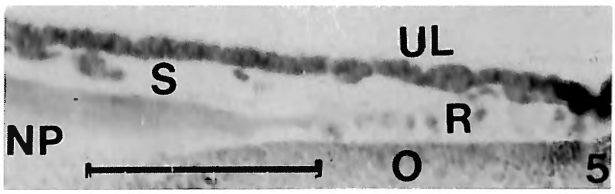
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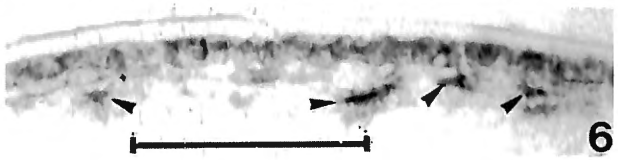
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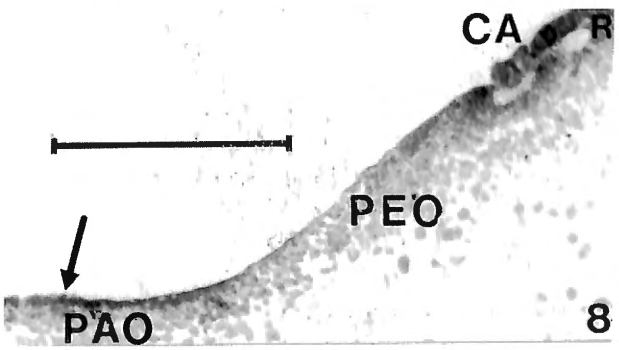
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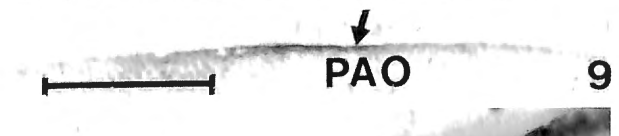
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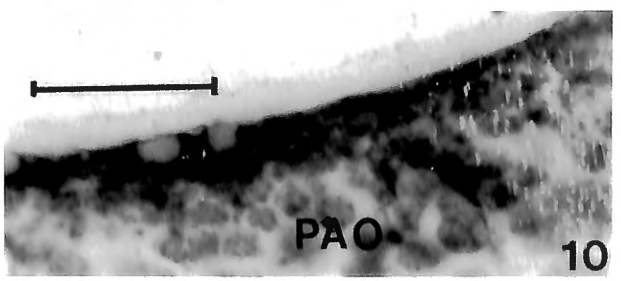
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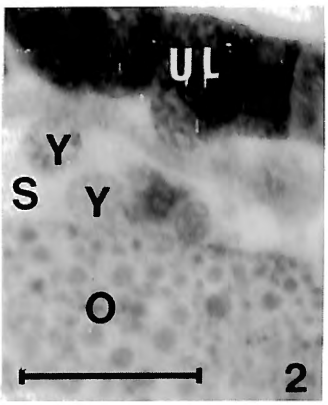
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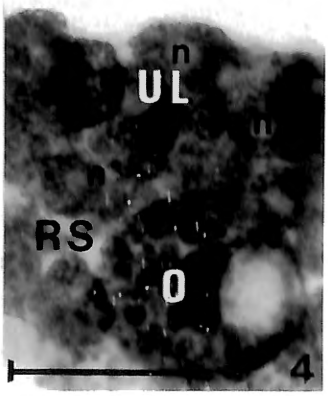
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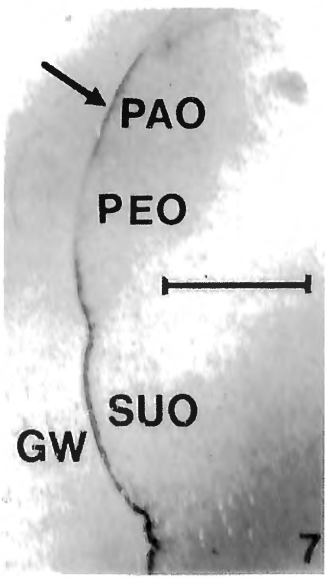
PAO
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In the anti-sickle region (CALLEBAUT, 1993 b, CALLEBAUT & VAN NUETEN, 1995) in the cranial part of the area pellucida (Figs 2-5) no endophyll is seen. No contact of the germ with the underlying ooplasm exists here because the subgerminal cavity extends in between. Thus In the anti-sickle region (CALLEBAUT, 1993 b, CALLEBAUT & VAN NUETEN, 1995) in the cra-

Fig. 1. – β-Tubulin immunoreactivity in a mediosagittal section through an unincubated quail blastoderm and neighbouring subgerminal ooplasm; in the central subgerminal ooplasm we see the nucleus of PANDER (NP) (PANDER, 1817).
O: ooplasm below germ wall; A: caudal,axilla shaped part of the subgerminal space; R: cranial recess of the subgerminal space (bar = 400 μm).

Fig. 2. – Section through the anti-sickle region of an unincubated quail blastoderm, immunostained for β-tubulin; below the upper layer (UL), in the subgerminal space (S) numerous loose yolk masses (Y) are seen; O: subgerminal ooplasm; Note the absence of a deep layer (endophyll): compare with Fig. 3 (bar = 30 μm).

Fig. 3. – Higher magnification of the caudal part of Fig. 1.
A: axilla shaped caudal part of the subgerminal space (S) the arrowheads indicate the endophyll (caudal deep layer) in continuity with RAUBER's sickle (RS);
UL: upper layer;
O: Subgerminal ooplasm artificially disrupted from RAUBER's sickle by sectioning (bar = 200 μm).

Fig. 4. – Mediosagittal section through RAUBER's sickle (RS) localized below the upper layer (UL) at the caudal part of an unincubated quail germ. Note the uptake of a large round part of subgerminal ooplasm (O) in RAUBER's sickle; n: nuclei of blastomeres; immunostaining for β-tubulin and counterstaining with toluidine blue (bar = 30 μm).

Fig. 5. – Higher magnification of the cranial part (anti-sickle region) of Fig. 1; R: cranial recess of the subgerminal space (S) below the upper layer (UL) of the anti-sickle region, containing loose yolk masses and some cells, as the result of the disruption of the blastoderm from the underlying ooplasm (CALLEBAUT, 1994); no endophyll is present in this region; O: subgerminal ooplasm below anti-sickle region; NP: Nucleus of PANDER (bar = 200 μm).

Fig. 6. – Mediosagittal section through the cranial germ wall of an unincubated quail blastoderm; Four clusters of grouped chromosomes surrounded by tubulin immunoreactive threads (arrowheads) are seen in the subgerminal ooplasm (bar = 100 μm).

Fig. 7. – Low power overview of a section through the cranial germ wall (GW) at the surface of the peripheral subgerminal ooplasm (SUO) and the surrounding peri- (PEO) and paragerminal ooplasm (PAO) after β-tubulin immunostaining. On the surface of the paragerminal ooplasm a vast saturnring-shaped area presenting immunoreactivity (indicated by an arrow) is seen (bar = 400 μm).

Fig. 8. – Mediosagittal section through the caudal rim (CA) expanding at a distance above the ooplasm behind RAUBER's sickle (R) of an unincubated quail blastoderm and its surrounding perigerminal (PEO) and paragerminal (PAO) ooplasm. On the surface of the latter paragerminal ooplasm, we see the saturn ring shaped area (arrow) presenting β-tubulin immunoreactivity (bar = 200 μm).

Fig. 9. – Mediosagittal section through the paragerminal ooplasm (PAO) showing the whole extent of the superficial immunoreative ring with maximum staining in its middle part (arrow) (bar=150 μm).

Fig. 10. – Higher magnification of the middle part of the saturn ring shaped area of Fig. 9; immunostaining is seen between the yolk granules; neither nuclei nor chromosomes are present; PAO: paragerminal ooplasm (bar = 30 μm).

nial part of the area pellucida (Figs 2-5) no endophyll is seen. No contact of the germ with the underlying ooplasm exists here because the subgerminal cavity extends in between. Thus in this anti-sickle region no selectively toluidine blue stained ooplasmic «islands» surrounded by immunoreactive parts of the blastoderm could be observed. In the subgerminal ooplasm below the germ wall (both caudally and cranially), numerous peripheral subblastodermic clusters of mitotic figures (CALLEBAUT, 1994) are seen. These clusters are surrounded by intervitelline ooplasm containing numerous tubulin immunoreactive threads (Fig. 6). Very obvious and unexpected (even visible at low magnification) is the existence of a vast superficial extra-embryonic ring shaped area (saturn ring-like) of paragerminal ooplasm presenting a dense tubulin immunoreactivity, encircling the rim of the blastoderm at a large distance (Figs 7-8). On mediosagittal sections this ring is approximately 300-400 μm large. Its borders are not sharply defined, but in its middle part it presents a maximum size of approximately 150 μm large extending somewhat in the depth (Figs 9-10). Peripheral to the saturn ring-like region of maximum tubulin reactivity, some feeble superficial tubulin reactivity persists over a long distance in the direction of the vegetal pole.

In all directions there is always a juxtagerminal or perigerminal zone, free of tubulin reactivity between the rim of the blastoderm and the saturn ring-like area. In the latter area also the tubulin reactive ooplasmic structures are localized between the yolk spheres (Fig. 10). The ring-shaped tubulin reactive area contains yolk spheres which are smaller than in the surrounding ooplasm. Neither nuclei nor chromosomes are present in this ring or in the peri- or paragerminal ooplasm. The charcoal marks applied *in vivo* indicate that its localization corresponds to the place where the white halo is visible in the intact living egg, already at the cleavage stage, just peripherally to the perigerminal clear zone at the surface of the avian egg yolk (CALLEBAUT & MEEUSSEN, 1988). Previous observations on the relationship of the avian blastoderm and its subgerminal ooplasm (CALLEBAUT, 1994) and the present study, suggest that there are some homologies with the tubulin distribution in extrablastodermic structures observed in zebra fish eggs at the onset of epiboly (SOLNICA-KREZEL & DRIEVER, 1994). The saturn ring-like tubulin reactive area in the avian paragerminal zone, seems to correspond to the circular region of organizer centers from which microtubules radiate in the anuclear yolk cytoplasmic layer in *Danio rerio* eggs. The feeble tubulin reactivity found more peripherally in avian eggs seems to correspond to the microtubules aligned along the animal-vegetal axis in *Danio rerio* eggs. It is in this acellular zone, far distal to the margin of overgrowth, that penetration of albumen-derived material occurs during early avian incubation (CALLEBAUT, 1983). The syncytium of giant clusters of mitotic figures in the subgerminal ooplasm, below the germ wall of the avian blastoderm seems to correspond to the external yolk syncytial layer in the teleost egg. The chromatin granules and intervitelline material in the avian nucleus of Pander, can perhaps be considered as homologous to the elements found in the internal yolk syncytial layer, below the animal pole of *Danio rerio* eggs. All the described avian extrablastodermic structures can also be considered as belonging to one giant syncytial yolk cell. There is also homology between the earliest manifestation of epiboly of the teleost blastoderm (TRINKAUS, 1996) and the expansion of the rim of the avian blastoderm, before oviposition (CALLEBAUT AND MEEUSSEN, 1988). The sliding of the marginal blastoderm cells over the underlying ooplasm (Fig. 8) and the formation of part of the area opaca can also been considered as epiboly. Thus in both vertebrate groups epiboly starts before gastrulation.

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