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Ubiquitin–protein conjugates and αB crystallin are selectively present in cells undergoing major cytomorphological reorganisation in early chicken embryos

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Ubiquitin–protein conjugates and αB crystallin are detected immunohistochemically in cells undergoing extensive morphological reorganisation in early chicken embryos. Cytoplasmic ubiquitinated proteins and αB crystallin are coordinately found in cells of the lens, notochord and myotome. The antigens appear in the myotome cells precisely at the point at which the cells begin to migrate from the dorsomedial lip of the dermamyotome. The findings indicate that ubiquitin and αB crystallin may have a coordinate role in the extensive architectural remodeling which occurs in these developing tissues in the early chick embryo. Some form of functional association between protein ubiquitination and αB crystallin in cells may explain why αB crystallin is found with ubiquitin–protein deposits in some neurodegenerative diseases.

Ubiquitin; αB Crystallin; Chicken embryo; Lens; Notochord; Myotome; Development

1. INTRODUCTION

Intracellular ubiquitin-protein deposits are a commonality in the major human idiopathic neurodegenerative diseases [1] as well as in the transmissible human [2] and animal encephalopathies [3] and some viral diseases, e.g. [4]. The observation of ubiquitin–protein conjugate immunoreactivity in large membrane-bound vacuoles in areas of granulovacuolar degeneration in hippocampal neurones in Alzheimer's disease [5] led to the discovery by immunogold electron microscopy that ubiquitin-protein conjugates are selectively enriched in the lysosomes of fibroblasts [6,7], polymorphonuclear neutrophils [8] and lymphoblastoid cells [4].

Protein ubiquitination has been clearly implicated in non-lysosomal proteolysis [9] and it may therefore be a unifying signal for both extralysosomal ATP-dependent proteolysis and lysosomal proteolysis [1,10]. Protein ubiquitination also seems to have a key role in programmed neuromuscular cell death during eclosion in the silk moth *Manduca sexta* [11].

The small heat-shock related protein, αB crystallin, is found in some normal tissues and in some intraneuronal inclusions in human neurodegenerative diseases [12,13]. The functions of αB crystallin in inclusion body biogenesis remain unclear but the protein may be involved with the ubiquitin system in the isolation or elimination of unwanted proteins in degenerating neurones. We have investigated immunohistochemically the distribution of ubiquitin–protein conjugates and αB crystallin in the developing chicken embryo with the notion that the findings would assist in the interpretation of our immunocytological observations in chronic degenerative diseases (reviewed in [10]).

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2. MATERIALS AND METHODS

A polyclonal antiserum against αB crystallin was prepared by immunizing rabbits with a synthetic peptide–haemocyanin conjugate. The peptide, corresponding to human αB crystallin residues 1-10 (MDIAIHHPWI), was synthesised on a model 431A peptide synthesiser (Applied Biosystems, Warrington, UK) using Fmoc chemistry and purified by reverse-phase HPLC prior to conjugation. A carboxy-terminal cysteine was incorporated into the peptide for linkage to the carrier protein using the heterobifunctional reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. The antiserum detects αB crystallin in Western blots of heart and brain and immunostains αB crystallin in the lens and a variety of normal and diseased non-lenticular human tissues [13]. The antiserum to ubiquitin-protein conjugates has previously been characterised and used in several studies on human degenerative diseases (reviewed in [10]). Ross broiler chick embryos (Ross Poultry) were incubated at 39°C until they reached the required stage of development as described by [14]. Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline and processed for wax embedding.

4 μm sections were stained using anti-αB crystallin and anti-ubiquitin-protein conjugate antisera by a standard streptavidin biotinylated peroxidase complex (Dako, High Wycombe, UK) method. Briefly, after dewaxing in xylene, sections were treated with 3% H_2O_2 in methanol, rehydrated to water, and incubated in the

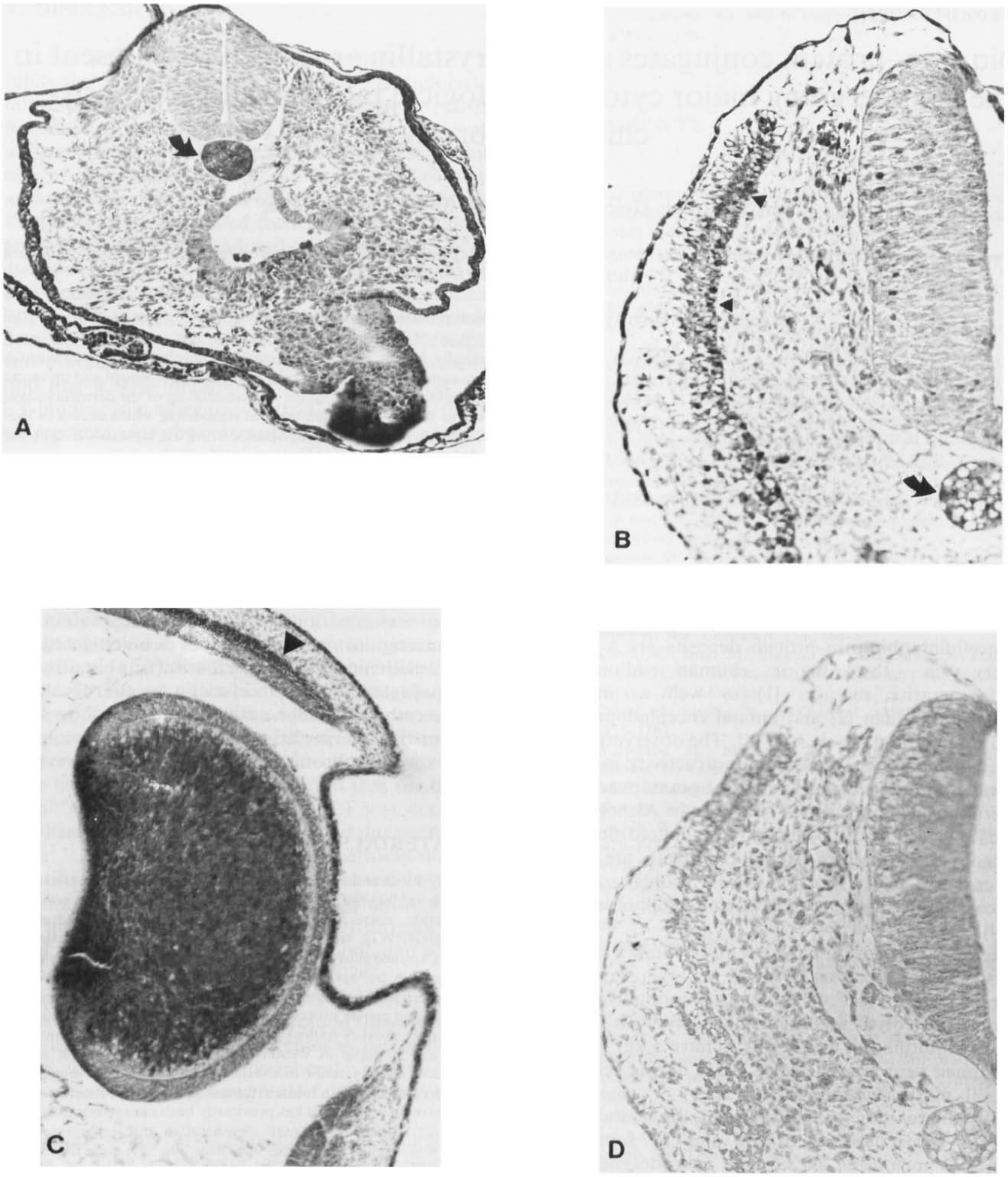
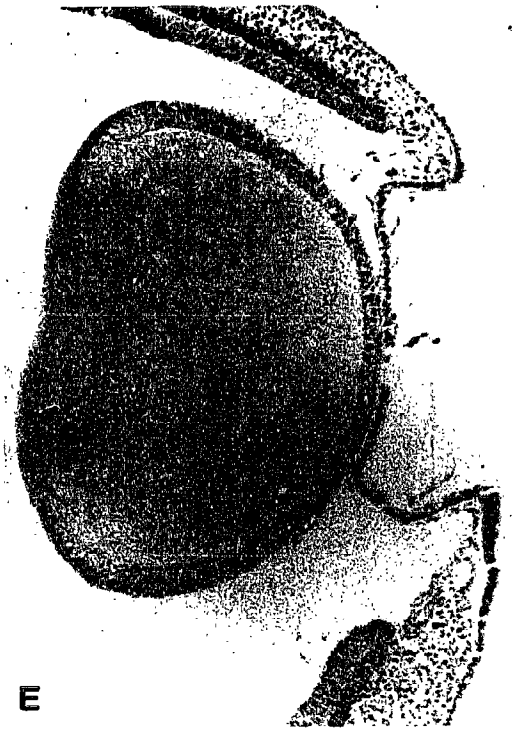


Fig. 1. Immunohistochemical demonstration of ubiquitin-protein conjugates in early chicken embryos. (A) Transverse section of posterior (caudal) region of 2.5 day embryo. Arrow indicates the ubiquitin-protein immunoreactivity. (B) Transverse section of thoracic region of 2.5 day embryo. Arrow indicates the notochord. Arrowhead points to myotome containing ubiquitin-protein conjugates. (C) Oblique transverse section of the lens of a 5 day embryo. Arrowhead indicates pigmented retina. The lens fibres and the overlying epithelium show intense ubiquitin-protein conjugate immunoreactivity. (D) Transverse section of thoracic region without immunostaining with primary antiserum to ubiquitin-protein conjugates. (E) Oblique transverse section of the lens without immunostaining with the primary antiserum to ubiquitin-protein conjugates. Magnification: (a) × 170; (b) and (d) × 230; (c) and (e) × 110.



following: (i) normal swine serum (20%) in 0.05 M Tris-saline, pH 7.6 (TS) for 20 min; (ii) either anti-ubiquitin-protein conjugate (1:250) in TS for 30 min or anti-αB crystallin (1:1000) in TS for 30 min; (iii) swine anti-rabbit immunoglobulin-horseradish peroxidase (1:50) in TS for 30 min; (iv) peroxidase substrate (0.05% diaminobenzidine, 0.07% imidazole, 0.01% H_2O_2 in TS) for 10 min; (v) enhancer (0.5% $CuSO_4$, 0.9% NaCl, in TS) for 10 min; (vi) except where indicated in the legends sections were counterstained with haematoxylin.

Negative controls were performed for each type of antigen by omission of the primary antiserum. In addition absorption controls for the αB crystallin antiserum were carried out by preincubating the anti-αB crystallin antiserum with synthetic peptide-haemocyanin conjugate overnight at 4°C. Absorption controls for the antiserum to ubiquitin-protein conjugates have been performed previously [5].

3. RESULTS

3.1. *Immunostaining for ubiquitin conjugates*

Embryos of approximately 2 or 3 days of development (Hamburger-Hamilton stage 14 and 20 respectively) were fixed, sectioned and immunostained with antiserum to ubiquitin-protein conjugates. In all embryos (10 examined) the pattern of ubiquitin-protein conjugate distribution was alike. Transverse sections illustrated a developmental progression of ubiquitin immunoreactivity consistent with the developmental progress of the embryo from anterior to posterior (rostral-caudal). At the posterior, more immature, end of the embryo in regions where the segmental plate of the paraxial mesoderm was unsegmented the only positive tissue was the epidermis overlying the surface of the embryo (data not presented). More anteriorly, near the region where the somites begin to appear, the level of ubiquitin-protein conjugates in the cytoplasm of notochord cells gradually increased until, adjacent to epithelial somites, the level was striking in comparison to other tissues (Fig. 1A). At this stage the notochord is non-vacuolated and in direct contact with the floor plate of the neural tube, staining persists as the notochord matures (Fig. 1B).

More mature somites dissociate into dermamyotome (epithelial in structure) and sclerotome. In this region the only significant levels of ubiquitin-protein conjugates are still in the epidermis and notochord. Soon after dissociation into dermamyotome and sclerotome cells migrate from the dorsomedial edge of the dermamyotome and move under it to form the myotome (the precursor of the muscle of the axial skeleton). As soon as this migration is seen, those cells forming the myotome also begin to exhibit high levels of ubiquitin-protein conjugates in their cytoplasm. What appeared to be nuclear staining was also observed in the nuclei of some myotome cells (Fig. 1B). A few cells scattered throughout the other tissues of the embryo, the identity of which has yet to be established, were also stained positive.

Immunoreactivity in the myotome and notochord is observed along the entire length of the embryo until both myotomes and notochord end. The head and developing nervous system do not appear to contain significant levels of ubiquitin-protein conjugates at this stage of development. The only other exception to this lack of staining is in the eye. The lens of the eye shows a striking pattern of staining. As the lens forms from a sphere of cells, derived from the ectoderm, the inner part of the sphere expands, the cells elongating to form lens fibres. Ubiquitin-protein conjugates appear at elevated levels in all of the cells of the lens. As the lens matures further the lens fibres retain their staining while the anterior lens epithelium appears negative, as is seen by 5 days of development (Fig. 1C).

Longitudinal and sagittal sections support out interpretation of the distribution of ubiquitin-protein conjugates described above. The repeated myotomes of the somites are clearly visible in longitudinal sections. Interestingly the immunostaining in this plane appears to begin at the anterior edge of the somite and the cells appear to spread posteriorly, a migration pattern which has been reported [15] for the myotome. In longitudinal sections the lens is again the only obviously stained tissue in the head region and the notochord is clearly evident. As suggested from the transverse sections, staining of the notochord is not evident posterior to the epithelial somites. The heart and epidermis also appeared to contain ubiquitin conjugates.

3.2. *Immunostaining of αB crystallin*

Adjacent embryonic sections to those stained for ubiquitin-protein conjugates were taken from several of

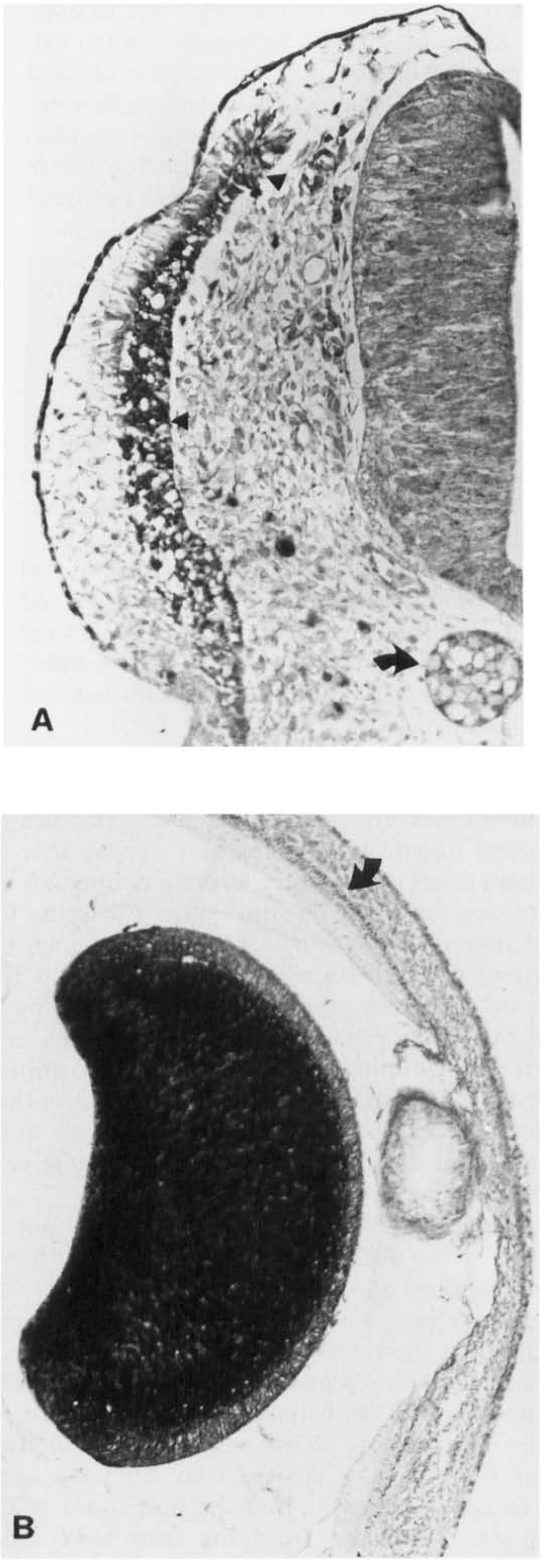


Fig. 2. Immunohistochemical demonstration of αB crystallin in early chicken embryos. (A) Transverse section of thoracic region of 2.5 day embryo. Arrow indicates the notocnord. Arrowhead points to myotome. (B) Oblique transverse section of the lens of a 5 day embryo. Arrow indicates pigmented retina. The lens fibers show intense αB crystallin immunoreactivity. Magnification: (A) × 170; (B) × 110.

the embryos studied and incubated with an antiserum specific for αB crystallin. The pattern of immunoreactivity observed was identical to that seen with the antiserum raised against ubiquitin–protein conjugates (Fig. 2). In order to rule out cross-reactivity of the antisera, both antisera were preadsorbed with either ubiquitin or αB crystallin. In each case the immunoreactivity was lost only when the serum was incubated with the antigen against which it was raised.

4. DISCUSSION

We have shown that antisera specific for ubiquitin–protein conjugates and αB crystallin react with an identical subset of tissues in young chick embryos. Immunoreactivity appears in the cytoplasm of the notochord and the lens of the eye and in the cells of the myotome as soon as they migrate from the anterodorsal lip of the dermamyotome. In each case the cells are undergoing extensive morphological reorganisation at the times when staining is observed. Staining was also seen in the epidermis (Fig. 1) and in the heart. The proteins in these tissues in chick embryos at the age studied by immunohistochemistry were examined by Western blotting with the antiserum to ubiquitin–protein conjugates. The analysis reveals smears of ubiquitin conjugates (data not presented) indicating a range of different sized ubiquitinated proteins or protein fragments in the tissues.

The full significance of the immunohistochemical observations is yet to be completely evaluated but must be set against the developmental transitions in each tissue. The notochord plays an important role in early embryogenesis. It is the notochord which induces formation of the neural plate and recent data suggest that it also plays a major part in organising cell specialization in the more mature neural tube [16,17]. The notochord also appears to influence somatogenesis [18]. Later it becomes vacuolated and finally contributes to the structure of the spinal column. Thus the major embryonic events involving the notochord are exceptionally early during embryogenesis, after which time it undergoes major morphological reorganisation (vacuolisation). Ubiquitin–protein conjugate and αB crystallin immunoreactivity are seen in the notochord some time before vacuolization is apparent, during the period when it is involved in the inductive events discussed above. Protein ubiquitination and αB crystallin may therefore be involved in some way in this inductive activity, or alternatively in the origination of the vacuolation process, which only later becomes overtly visible.

In the lens the presence of ubiquitin and αB crystallin has been known for some years, although studies on ubiquitin have not been carried out on tissue from animals younger than a few months post-partum [19]. These data have shown that the degree of ubiquitina-

tion of cellular proteins appears to decrease as the lens develops and ages in the adult. Our data suggest that ubiquitin-protein conjugates are present at high levels in the very early embryonic lens, when the cells are changing shape drastically, and accumulating crystallins. It is known that during this period the cells also lose many of their cellular components in order to form an optically useful structure. We therefore believe that ubiquitin plays a role in this cellular reorganization, as a cofactor in the non-lysosomal [9] and lysosomal pathways [6,7,10] of degradation of cell organelles, though this in no way excludes a protective role for protein ubiquitination in animals after birth.

The presence of ubiquitin-protein conjugates in myotome cells is of particular interest since these cells go on to form the skeletal muscle of the trunk of the embryo. Ubiquitin has been shown to be associated with mature adult muscle [20] so it might be expected to find ubiquitin-protein conjugates in muscle precursors (indeed, we do find that muscles in much older chicks show significant levels of ubiquitin-protein conjugate immunoreactivity). What is surprising and interesting is the timing of appearance of ubiquitin-protein conjugates. Ubiquitin conjugate immunoreactivity coincides with cells initiating their migation. Ubiquitin might therefore be involved in migration itself or in the cytological reorganisation at the onset of myotome development. The co-appearance of αB crystallin in these cells was unexpected and its possible function is unknown. The data presented here suggest that ubiquitin conjugation and αB crystallin are coordinately regulated in cells undergoing major architectural reorganisation. This may explain our observations in human chronic degenerative disorders which show the coincident appearance of ubiquitin-protein conjugates and αB crystallin in intracellular inclusions containing intermediate filaments which arise presumably by extensive cytoskeletal and organellar rearrangements during disease progression (reviewed in [10]).

Our results are consistent with the hypothesis that protein ubiquitination not only triggers the removal of modified or damaged proteins [9] but also is part of the mechanism which changes the internal architecture of cells during development. Specifically we find the level of ubiquitin-protein conjugates to be sharply increased in the notochord, as it becomes vacuolated; in the myotome, as it is first formed by migration of the cells from the epithelial dermamyotome; and in the developing lens as the cells elongate, later to lose most of their cytoplasmic structures. The coincident presence of αB crystallin in these tissues suggests that it is probably involved in the same processes in a way which is at present unclear.

An interesting question now to be addressed is the nature of the molecular mechanisms which control the coincident appearance of ubiquitinated proteins and αB crystallin. One clue may lie in the promoter sequences of the two genes each of which contain heat shock control elements [21,22]. Alternatively polyubiquitin and αB crystallin expression may be controlled through other promoters, e.g. which are hormone or growth factor dependent. Recent studies have indicated that polyubiquitin gene expression during programmed neuromuscular cell death is cell stress promoter independent and apparently hormonally regulated [11]. Studies at the level of transcription should indicate how the expression of ubiquitin and αB crystallin is regulated in early chicken development.

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