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The expression of *Stra6* and *Rdh10* in the avian embryo and their contribution to the generation of retinoid signatures

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ABSTRACT Two new components of the retinoic acid (RA) synthetic pathway, the cell surface receptor for retinol, *Stra6*, and the enzyme converting retinol into retinal, *Rdh10*, have recently been described. To understand how different tissues of the chick embryo generate different retinoid signatures, we describe the expression patterns of these two genes and ask whether they are altered by RA levels. We performed wholemount *in situ* hybridisation and altered RA levels by applying RA soaked beads and used vitamin A-deficient quail embryos. In some areas of the embryo, these two genes co-localised with a retinaldehyde dehydrogenase *(Raldh)*, as might be expected allowing retinol to be taken into the cell and converted into RA. In other areas of the embryo, the domain of expression of *Rdh10* was much smaller than that of the corresponding *Raldh*, suggesting that retinal is transferred between cells. In yet other areas, only one of the cytochrome P450 enzymes co-localises with *Stra6*. In the case of co-localisation with *Cyp1B1* in the hindbrain mesenchyme, this reveals that retinol is taken up into the cells for conversion to RA by *Cyp1B1* and used in establishing ventral progenitor domains in the hindbrain. In the case of co-localisation with a *Cyp26*, it suggests that other retinol dehydrogenases (Rdhs) have yet to be discovered. We propose that in certain regions of the embryo, there are new *Rdhs* and *Raldhs* yet to be discovered and that RA is not a major regulator of its synthetic enzymes.

KEY WORDS: *Stra6, Rdh10, chick embryo, retinoic acid, VAD embryo*

Introduction

Regulation of the appropriate level of retinoic acid (RA), an important developmental signaling molecule, is crucial for its precise action in the embryo. This regulation is tissue specific because different tissues of the embryo have different levels and types of retinoids present, or 'retinoid signatures' as measured by HPLC (Horton and Maden 1995; Maden *et al*. 1998). Thus when RA is applied to the embryo in excess, the retinoid signature of individual organ systems is disturbed and characteristic defects arise in the central nervous system, heart, urogenital system, limbs, pharyngeal arches, lung, eye and craniofacial structures (Fantel *et al*. 1977; Shenfelt 1972; McCaffery *et al*. 2003). Similarly when retinoid levels are lowered by a deficiency of precursors, the same organ systems are adversely affected (Clagett-Dame and DeLuca 2002; Kalter and Warkany 1959; Maden 2002; Dersch and Zile 1993; Zile 2004). Since RA controls the pattern of organ specific gene transcription the understanding of how RA levels are regulated in the embryo is of considerable significance.

There are multiple points at which the endogenous levels of RA could be controlled during embryogenesis. These include: retinol release into the blood from maternal stores (mammals) or from yolk stores in the egg (other vertebrates); uptake of retinol through the cell membrane into embryonic cells; enzymatic conversion of retinol into retinal and RA in the cell cytoplasm; activation of the retinoic acid receptors (RARs) by RA in the nucleus; and removal of RA so that gene activation no longer occurs. We know a good deal about many of these steps. For example, the distributions of the enzymes which convert retinal into RA, namely, RALDH1,

Abbreviations used in this paper: ADH, alcohol dehydrogenase; Cyp, cytochrome P450; HPLC, high pressure liquid chromatography; RA, retinoic acid; RALDH, retinaldehyde dehydrogenase; RAR, retinoic acid receptor; RDH, retinol dehydrogenase; SDR, short chain dehydrogenase/reductase; VAD, vitamin A-deficient.

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RALDH2 and RALDH3 are well characterized (Berggren *et al.* 1999; Blentic *et al.* 2003; Grun *et al*. 2000; McCaffery *et al*. 1999; Mic *et al*. 2000; Niederreither *et al*. 1997; Swindell *et al.* 1999; Molotkova *et al.* 2007) as are those of the enzymes which further metabolize RA, namely CYP26A1, CYP26B1 and CYP26C1 (Abu-Abed *et al*. 2002; Blentic *et al.* 2003; de Roos *et al.* 1999; Hollemann *et al*. 1998; MacLean *et al*. 2001; Reijntjes *et al*. 2003; Reijntjes *et al*. 2004; Swindell *et al*. 1999; Tahayato *et al*. 2003). The spatial relationships between these two classes of enzymes have been carefully mapped in many vertebrate embryos giving valuable insights into the action of RA as a paracrine signaling molecule (Reijntjes *et al.* 2004; White and Schilling 2008) which acts in the form of a gradient within, for example, the developing hindbrain (White *et al*. 2007). The distribution and function of the individual RARs has also been extensively documented (Dolle *et al*. 1989; Dolle *et al*. 1990; Ruberte *et al*. 1990; Ruberte *et al*. 1991)

Until recently, however, little was known about how retinol is taken up into the cell or which enzyme converts retinol into retinal and so knowledge of this upstream part of the pathway within the embryo is severely lacking. *Sra6* was recently identified as the cell surface receptor for retinol (Kawaguchi *et al*. 2007) and a mutation in this gene in humans causes anophthalmia, heart defects, diaphragmatic hernia, lung hypoplasia and mental retardation (Pasutto *et al*. 2007), a spectrum known as Matthew-Wood syndrome and a spectrum which is characteristic of vitamin A deficiency. *Stra6* seems to be widely distributed in the mouse embryo (Bouillet *et al*. 1997), but little is known about its precise relationship to the other components of the RA synthetic pathway and here we describe this in the early chick embryo and ask whether it is regulated by RA.

Following the uptake of retinol into the cell by STRA6, its conversion to retinal was originally though to be performed by alcohol dehydrogenases (ADHs) or short-chain dehydrogenase/reductases (SDRs). But these enzymes are ubiquitous in their embryonic distribution and null mutants for the ADHs generate no embryonic phenotypes (Duester 2001). Recently, a short chain dehydrogenase, RDH10, which converts all-*trans*-retinol into all-*trans*-retinaldehyde was identified in a mouse mutagenesis screen (Sandell *et al.* 2007). The mutant has defects in the limbs, craniofacial structures, branchial arches, eyes, ears, cranial ganglia, lungs, urogenital system, all of which are characteristic of vitamin A deficiency. Its distribution in the mouse embryo (Cammas *et al.* 2007; Romand *et al.* 2008; Sandell *et al.* 2007) is very

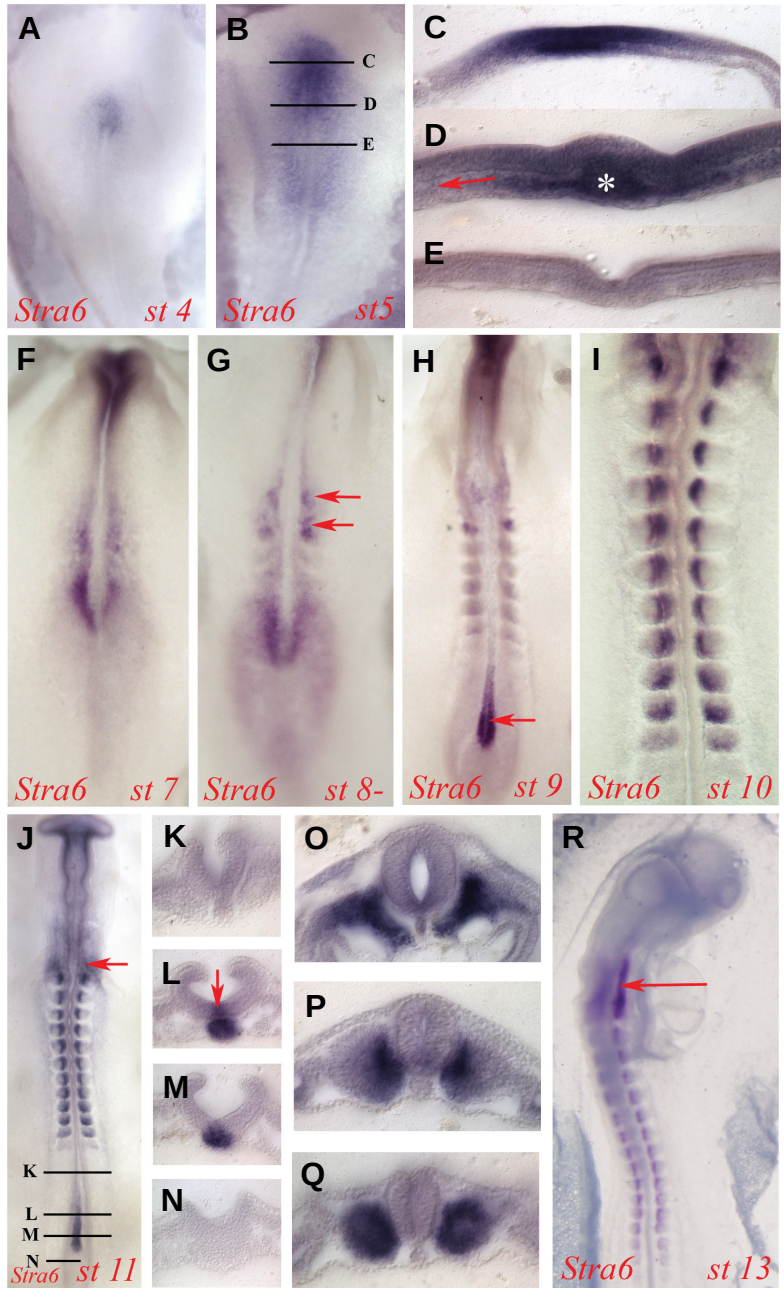

Fig. 1. Expression domains of *Stra6* in the stage 4 – 13 chick embryo. (A) *Stage 4 showing expression in and around Hensen's node.* **(B)** *Stage 5, lines mark the plane of section in (C,D,E).* **(C)** *Section anterior to the node.* **(D)** *Section through the node. Star marks expression in the mesoderm of the node, red arrow shows the direction of movement of ingressing mesodermal cells which cease expression.* **(E)** *Section posterior to the node across the primitive streak showing lack of expression.* **(F)** *Stage 7 showing expression in a cup shape around the regressing node.* **(G)** *Stage 8-, red arrows mark the first formed somites and the posterior cup shape persists.* **(H)** *Stage 9 showing expression now within the regressing node at the posterior of the embryo (arrow) and most strongly in the first formed somite.* **(I)** *Stage 10, close-up of the somite expression showing the initial expression throughout the first 1 or 2 somites and then it narrows down to the medial edge in the more mature, anterior somites.* **(J)** *Stage 11, showing expression in the somites, regressing node and beginning in the cranial mesenchyme (arrow). Lines mark the plane of section in (K,L,M,N).* **(K)** *Section anterior to the node showing no expression.* **(L)** *Section at the anterior end of the node showing expression in the forming notochord and floorplate (arrow) of the neural tube.* **(M)** *Section through the node showing expression ventral to the neural tube.* **(N)** *Section at the posterior end of the node showing no expression.* **(O-Q)** *Expression through the somites at different AP levels.* **(O)** *Anterior somites near the head show sclerotomal expression.* **(P)** *Mid-level somites show medial expression.* **(Q)** *Posterior somites show expression throughout.* **(R)** *At stage 13 the hindbrain mesenchyme expression has become intense (arrow) and eclipses that in the somites.*

precise and overlaps with many sites of RALDH expression but also has some unique localisations such as the floorplate of the neural tube and the zone of polarizing activity in the forelimb bud, sites of no known Raldh expression. *Rdh10* has also been described in the *Xenopus* embryo (Strate *et al*. 2009) where its expression shows differences from the mouse and its levels are regulated by excess RA and reduced RA concentrations. Here we describe the expression and regulation by RA of *Rdh10* in the early chick embryo and vitamin A-deficient (VAD) quail embryo along with that of *Stra6* and ask whether they are coordinated together with the *Raldhs* in RA-responsive developing fields. Thus we can put together all the components of the metabolic pathway of RA in the different regions of the avian embryo and reveal how different retinoid signatures come about in different embryonic fields.

Results

Stra6 expression

Expression of *Stra6* began at stage 4 around and within Hensen's node and in a zone spreading anterior to the node (Fig. 1A). This zone enlarged over the next stage as the node started to regress (Fig 1B). Transverse sections through the embryo showed that *Stra6* was expressed in both the epiblast and mesoderm layers anterior to the node (Fig. 1C). It was expressed in the mesoderm of the node itself (star in Fig. 1D) and was then down-regulated in laterally migrating mesodermal cells after a certain distance from the node (arrow in Fig. 1D). There was no expression posterior to the node or in the primitive streak at this stage (Fig. 1E). This is a different pattern from the other RA pathways enzymes that we have seen before at gastrulation stages (Maden 2004). It is co-expressed with *Cyp26A1* in the epiblast anterior to the node and in the mesoderm of the node; with *Cyp26C1* in the mesoderm anterior to the node; but it is not co-expressed with *Raldh3* which is in the epiblast of the node nor *Raldh2* or *Rdh10* in the mesoderm posterior to the node (summary in Fig. 5A). Therefore it is not co-expressed with a *Rdh* or a *Raldh*.

The expression of *Stra6* continued in a cup shape around the node as it regressed (Fig. 1F, G) and then entered the node itself by stage 9 (arrow in Fig. 1H) where it remained (Fig. 1J). Sections through this region at stage 11 showed a very precise expression in the anterior part of the node and forming notochord (Fig. 1N, M) and also in the floor plate of the neural tube for a brief period (Fig. L arrow, K). This expression domain of *Stra6* is very unusual from the point of view of other RA metabolising enzymes and the only co-expression in this region is with *Cyp26A1* (Blentic *et al*. 2003).

As the first somites formed they expressed *Stra6* (Fig. 1G, arrows) and it remained most strongly expressed in the anterior somites which will disperse to become cranial mesenchyme (Fig. 1H). From stage 10 onwards there was a developmental progres-

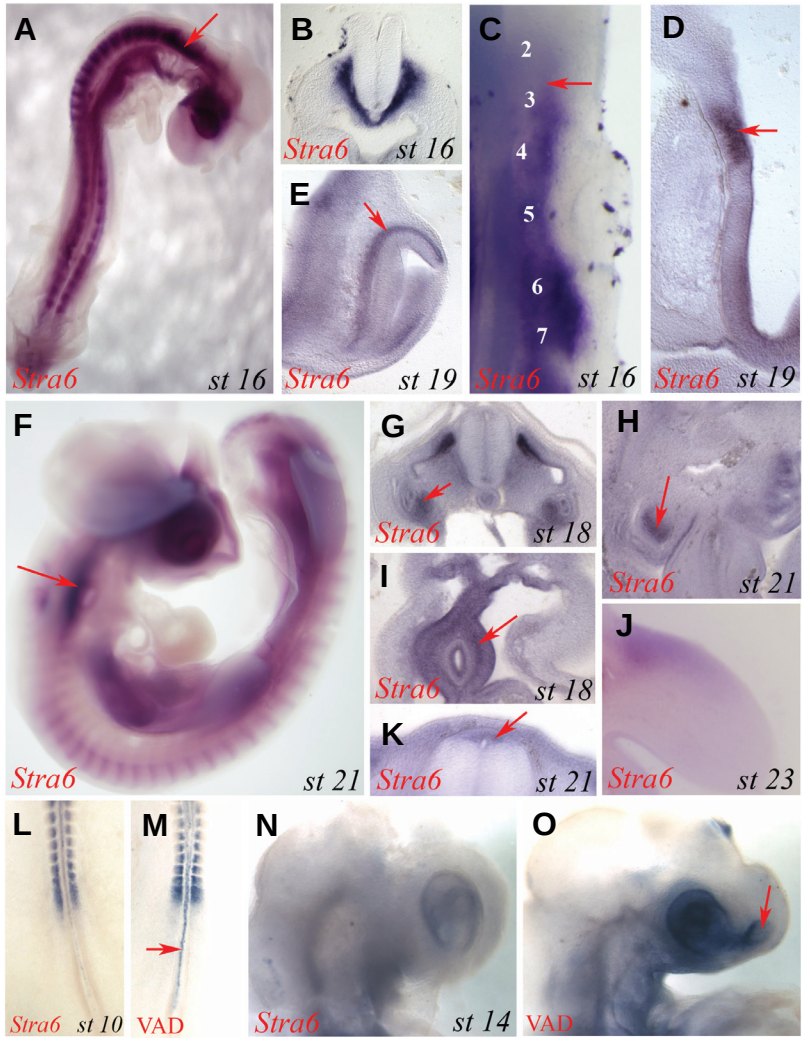

Fig. 2. Expression domains of *Stra6* in the stage 16 – 23 chick embryo and their alteration in VAD quails. (A) *Stage 16 chick. Arrow points to the hindbrain mesenchymal expression.* **(B)** *Section through the posterior hindbrain of a stage 16 chick showing intense expression in the mesenchyme surrounding the hindbrain.* **(C)** *Close-up of the stage 16 hindbrain showing the AP extent of the mesenchymal expression. White numbers mark the rhombomeres and the arrow marks the anterior extent of the expression in the mesenchyme. Just posterior to rhombomere 7 expression ceases. The otic vesicle is to the right of the image.* **(D)** *Section through the diencephalon of a stage 19 embryo showing a stripe of* Stra6 *expression in the mid-region (arrow) and weaker expression ventrally.* **(E)** *Section through the stage 19 eye showing expression in the retinal pigmented epithelium (arrow).* **(F)** *Stage 21 chick embryo. Arrow points to the hindbrain mesenchymal expression.* **(G)** *Section through a stage 18 embryo showing expression in the medial edge of the somite and weaker expression in the mesonephric mesenchyme (arrow).* **(H)** *Expression in the mesonephros of the stage 21 embryo (arrow).* **(I)** *Section showing expression in the hindgut mesenchyme (arrow) and coelomic epithelium.* **(J)** *Section through a stage 23 limb bud with a domain of expression on the proximodorsal region.* **(K)** *Section through the dorsal neural tube showing expression in the roof plate (arrow).* **(L)** *Expression in a normal stage 10 quail embryo.* **(M)** *Up-regulation of* Stra6 *expression in the newly-formed notochord of a stage 10 VAD quail embryo. The arrow marks the ectopic expression.* **(N)** *The head of a normal stage 14 quail embryo showing the retinal pigmented epithelium expression.* **(O)** *The head region of a VAD quail embryo showing ectopic expression in the nasal pit (red arrow) and much stronger expression in the eye and surrounding tissue.*

sion in the somitic expression of *Stra6* whereby the first one or two newly formed somites expressed it throughout compared to the older somites where expression narrowed down to the medial half which can be seen in Fig. 1I. Sections showed expression throughout the somite at the posterior end of the stage 11 embryo (Fig. 1Q) which localised to the medial half in more anterior somites (Fig. 1P). Later, in more mature somites, for example at stage 16 in rostral somites (Fig. 2A) and by stage 20 in all somites (Fig. 2F) the expression became restricted to the posterior halves of the medial edge of the somites. The somites are a site of several RA metabolising genes in addition to *Stra6*, namely *Cyp1B1*, *Rdh10* and *Raldh2*, but each is in slightly different domains within the somite (see Discussion).

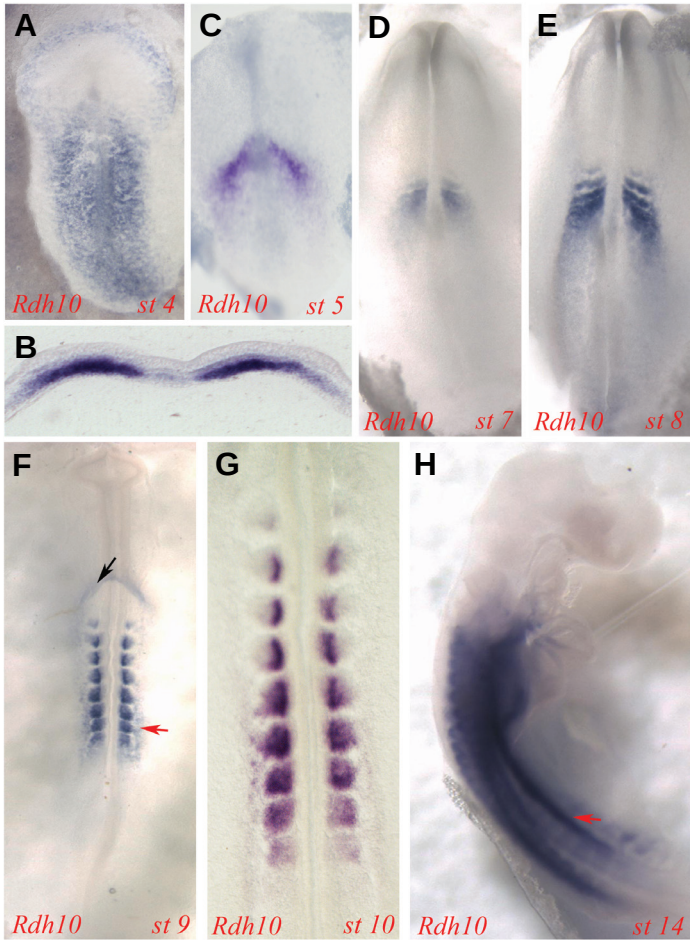

Fig. 3. Expression domains of *Rdh10* in the stage 4 – 14 chick embryo. **(A)** *Stage 4 showing expression posterior to Hensen's node.* **(B)** *Section through a stage 4 embryo as in A showing expression in the mesoderm.* **(C)** *Stage 5 showing expression has narrowed down to a stripe with the same anterior border.* **(D)** *Stage 7 showing expression in the mesoderm of the first forming somites.* **(E)** *Stage 8 showing continuing expression as the somites form.* **(F)** *Stage 9 embryo showing somite expression and two new domains. One in the endocardial tubes (black arrow) and the other in the intermediate mesoderm (red arrow).* **(G)** *Close-up of the stage 10 somite expression. In the first three newly formed somites, expression is throughout but it soon narrows down in the remaining, older somites to just the medial edge.* **(H)** *Stage 14 embryo. Anterior to the first somite there is no expression. Posterior to this level expression is seen in the somites and most strongly in the intermediate mesoderm (red arrow).*

Anterior to the somites *Stra6* started to become expressed in the cranial mesenchyme (Fig. 1J red arrow) and by stage 13 it had become the strongest domain of expression, eclipsing the somites (Fig. 1R arrow). It remained expressed in the cranial mesenchyme throughout the subsequent period of observations (Fig. 2A arrow, Fig. 2F arrow). Sections through the posterior hindbrain showed its intense expression in the mesenchyme surrounding the ventral two thirds of the hindbrain neuroepithelium (Fig. 2B). The expression domain extended from the level of rhombomere 3 posteriorly to rhombomere 7 between the neuroepithelium and the otic vesicle (Fig. 2C). This is another very unusual region of RA metabolising gene expression and the only other gene expressed there is *Cyp1B1* (Chambers *et al.* 2007) which can generate RA from retinol. We have previously proposed that RA generated in this location is involved in dorsoventral patterning of the chick hindbrain (Chambers *et al.* 2007) and now we can see that retinol is taken up by these mesenchyme cells.

As development continued from stage 16 to stage 18 several new domains of *Stra6* expression appeared. At the anterior end of the embryo (Fig. 2A) two regions around the eye began to express *Stra6*. One region was in the mid-diencephalon between the eyes (Fig. 2D arrow) where a clear stripe appeared and the ventral diencephalon showed a low level of expression. This region seems to be unique to *Stra6* in terms of RA enzyme expression. The second region was the eye itself where *Stra6* was expressed throughout the pigmented retinal epithelium (Fig. 2E arrow).

The other regions of expression which appeared between stages 16 and 18 were in the posterior of the embryo, one of which was the mesonephros (Fig 2G & H, arrows) and expression continued there throughout the remaining period of observation. Like the somites, the mesonephros is a site of several RA enzyme expressions, namely *Rdh10* (Fig. 4C), *Raldh1* and *Raldh2* (Blentic *et al.*, 2003). Another region of expression was the hindgut mesenchyme and associated coelomic epithelium (Fig 2I arrow) and a further region was the roof plate (Fig. 2K arrow). Emerging limb buds do not express *Stra6*, but by stage 23 a domain of expression appeared in the mid-dorsal region adjacent to where the limb bud joined the body (Fig. 2J).

Regulation of Stra6 expression by retinoic acid

In order to determine whether *Stra6* is regulated by RA we performed two experiments. Firstly, to detect any change in expression induced by excess RA, beads soaked in 4mg/ml all-*trans*-RA were placed adjacent to the posterior hindbrain of normal stage 9 chick embryos and the effect examined by *in situ* hybridisation after 18 hours further incubation. Secondly, to detect any effect of the absence of RA we examined the expression of *Stra6* in quail embryos which are devoid of vitamin A and retinoic acid (Dersch and Zile 1993; Dong and Zile 1995).

In the presence of excess RA no effect on *Stra6* could be seen even though there was the potential for an expanded or a decreased domain of gene expression within the hindbrain mesenchyme (data not shown).

To examine *Stra6* expression in the absence of RA we firstly confirmed that the expression of *Stra6* was the same in normal quail embryos as in chick embryos and then revealed two expression domains where *Stra6* was more extensively expressed in the

absence of RA in the vitamin A-deficient (VAD) quail embryos. One of these domains was in the notochord at the posterior end of the embryo which extended anteriorly from the node to the middle of the somites (red arrow in Fig. 2M). In contrast such a domain was not seen in the normal quail embryo at stage 10 (Fig. 2L), nor in the normal chick embryo (Fig. 1J). A second domain which had more extensive expression in the VAD embryos was in the eye and nasal pit (Fig. 2O vs 2N).

Rdh10 expression

Rdh10 expression began at stage 4 (Fig. 3A) in a domain which has the same shape as that of *Raldh2* (Blentic *et al*. 2003), with a sloping anterior edge and spreading all the way to the posterior end of the embryo. Sections revealed that, like *Raldh2*, *Rdh10* was expressed in the ingressing mesodermal cells (Fig. 3B), but was not expressed in the streak or node itself. Almost immediately the expression of *Rdh10* shrank down to a band of mesoderm (Fig. 3C) which was narrower than that of *Raldh2* (Blentic *et al.*, 2003) and subsequently *Rdh10* was expressed in the newly formed somites (Fig. 3D & E). By stage 9/10 (Fig. 3F) *Rdh10* was still expressed in all the somites although expression narrowed down from its presence throughout the whole somite in the first three newly formed somites to just the medial edge in the more mature somites (Fig. 3G). This expression pattern was identical to that of *Stra6* (Fig. 1I) and in sections *Rdh10* expression in mature somites was in the medial lip of the dermamyotome (Fig. 4C). Somitic expression became weaker through subsequent stages and by stage 20 it was restricted to the posterior half of the medial edge, just as *Stra6* was (Fig. 2F). Several other RA metabolism enzymes are expressed in the somite, namely *Cyp1B1* and *Raldh2* although in both of these cases expression is throughout the somite and not just restricted to the medial edge (summary in Fig. 5C).

At stage 9 two new domains of *Rdh10* expression appeared, one in the forming endocardial tube (Fig. 3F, black arrow) and another in the newly differentiating intermediate mesoderm (Fig. 3F, red arrow). Although these are the same domains as *Raldh2*, the latter is more intensely and more widely expressed at this stage. By stage 14 the intermediate mesoderm expression had become stronger (Fig. 3H arrow) and was the major site of expression in the embryo. At this stage (Fig. 3H) the expression of *Rdh10* was clearly absent in the head and began at the level of the first somite, remarkably similar to that of *Raldh2,* although as noted above *Raldh2* is more widespread in the intermediate mesoderm and lateral plate (Blentic *et al.* 2003; Swindell *et al*. 1999).

By stage 16 a domain of expression of *Rdh10* had appeared anterior to the first somite in the eye, but the rest of the body showed the same pattern (Fig. 4A) except that the somite expression had declined in intensity and two streams of expression appeared dorsal to the heart which were presumably neural crest cells in the inflow and outflow tracts of the heart (Fig. 4A arrows). The eye expression was both dorsal and ventral (Fig. 4B) and became more intense by stage 19 (Fig. 4D) but the domains did not expand. This expression in the eye co-localises with *Raldh1* dorsally and *Raldh3* ventrally, but it is very noticeable that these *Rdh10* domains were smaller than those of either *Raldh1* or *Raldh3* (summary in Fig. 5D).

By stage 19 the mesonephros had become the major site of expression of *Rdh10* (Fig. 4C arrow) and this remained so for the continuing period of observation (Fig. 4E &I arrows). The coelomic epithelium lateral to the mesonephros also expressed *Rdh10* strongly (Fig. 4C) and as the limb buds emerged expression spread laterally to encompass the epithelium ventral to the limb bud (Fig. 4E & G). This co-localises with the expression of *Raldh2* in the lateral extraembryonic membrane (Berggren *et al*. 1999). There was no expression of *Rdh10* in the early limb bud itself, but from stage 23 there was a strong dorsal domain in the proximal part of the limb bud (Fig. 4G) which co-localised with *Stra6* (Fig.2J) and may be related to the expression of *Raldh2* in the region of the limb bud where axons wait before entering (Wang and Scott 2008).

The expression at stage 20 revealed the clear posterior bias with only the eye expression and the two stripes dorsal to the heart present in the anterior end of the embryo (Fig. 4F). The somite

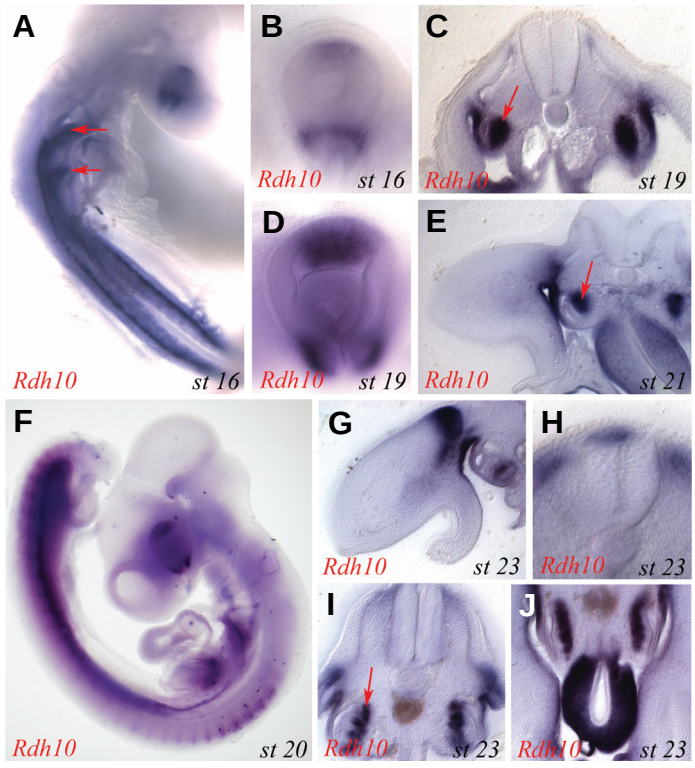

Fig. 4. Expression of *Rdh10* in the stage 16 – 23 chick embryo. (A) *Stage 16 embryo showing similar expression to stage 14 (Fig. 3H) except that two streams of expression have appeared in neural crest cells (arrows) and expression has begun in the dorsal and ventral eye.* **(B)** *Close-up of the stage 16 eye showing dorsal and ventral domains of expression.* **(C)** *Section through a stage 19 embryo showing intense expression in the mesonephros (arrow) and adjacent coelomic epithelium.* **(D)** *Close-up of a stage 19 eye showing the dorsal and ventral domains.* **(E)** *Section through a stage 21 embryo showing expression in the mesonephros (arrow), hindgut mesenchyme, coelomic epithelium medial to the limb bud and within the proximal part of the limb bud.* **(F)** *Stage 20 embryo.* **(G)** *Section through a stage 23 limb bud with intense expression in a proximodorsal region.* **(H)** *Section through the neural tube of a stage 23 embryo showing expression in the roof plate.* **(I)** *Section through a stage 23 embryo showing expression in the mesonephros (arrow).* **(J)** *Section through a stage 23 embryo showing intense expression in the mesenchyme and coelomic epithelium of the hindgut.*

expression of *Rdh10* had decreased in intensity and been reduced to the posterior halves. The strong signal in the posterior of the embryo was due to expression in the mesonephros (Fig. 4C, E, I) and in the mesenchyme of the developing hindgut (Fig. 4J). The latter co-localised with *Stra6* (Fig. 2I). One final domain of *Rdh10* which appeared at these later stages was the roof plate which appeared at stage 23 (Fig 4H) and co-localised with *Stra6* (Fig. 2K).

Regulation of Rdh10 expression by RA

We performed the same experiments on *Rdh10* expression as *Stra6*, namely implanting RA soaked beads adjacent to the posterior hindbrain and examining VAD quail embryos. Unlike *Stra6*, in neither case could any effect of *Rdh10* expression be detected (data not shown) from which we conclude that, as in the case of the *Raldhs* these enzymes are not responsive to RA.

Discussion

According to current concepts of RA signaling, in a cell which can generate RA three components should co-localise: a cell surface receptor to take up retinol, a *Rdh* to convert retinol to retinal in the cytoplasm and a *Raldh* to convert retinal to RA. RA then acts as a paracrine signaling molecule to affect cells at a distance and these distant cells express one of the *Cyp26s* in order to inhibit the RA signal within that cell or to generate a gradient of RA across the gap. We and others have previously documented in detail the expression patterns of the *Raldhs* and the *Cyps* in the chick embryo (Blentic *et al.* 2003; Reijntjes *et al.* 2003; Reijntjes *et al.* 2004; Swindell *et al.* 1999; Cui *et al.* 2003) and now with the data reported here on *Stra6* and *Rdh10* we can determine whether *Stra6*, *Rdh10* and a *Raldh* are indeed localised in the same tissue type and thus examine the validity of this concept. We find that there are three classes of localisations: the first supports the co-localisation concept described above; but the second and third do not and as a result two further novelties of RA signaling are hypothesized.

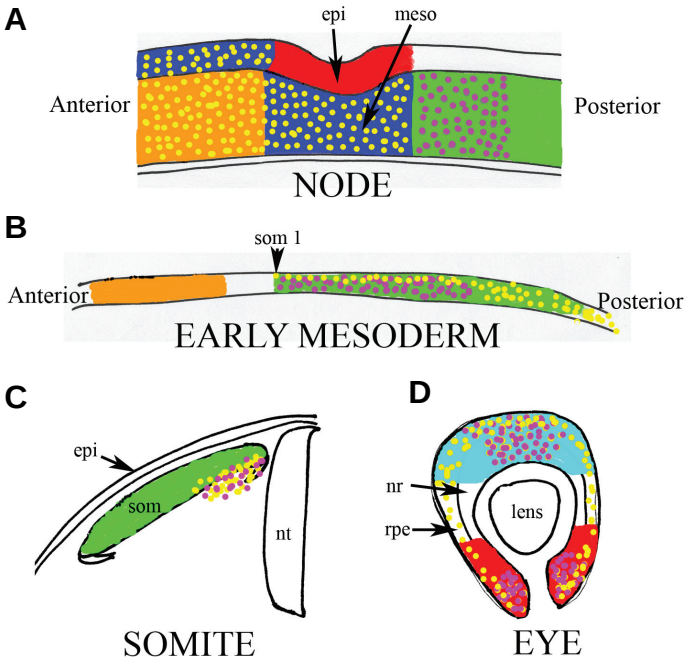

Fig. 5. Summary expression patterns of the retinoic acid metabolic pathway genes in 4 regions of the avian embryo. (A) *Hensen's node in a stage 4 embryo, anterior to the left posterior to the right. Epi = epiblast of the node; meso = mesoderm of the node.* **(B)** *the mesoderm of the stage 5 embryo, anterior to the left posterior to the right. Arrowhead marks the position of the future somite 1 at the join between the head and trunk regions.* **(C)** *the somite. Epi = epidermis; som = somite; nt = neural tube.* **(D)** *the eye. Nr = neural retina; rpe = retinal pigmented epithelium; lens = lens. Colour coding – red =* Raldh3*; green =* Raldh2*; pale blue =* Raldh1*; purple dots =* Rdh10*; yellow dots =* Stra6*; dark blue =* Cyp26A1*; orange =* Cyp26C1.

The first class of localisation is where *Stra6*, *Rdh10* and a *Raldh* occur in the same tissue region. There are two clear examples of this that we have observed: one is the intermediate mesoderm from which develops the mesonephros. This tissue expresses *Stra6*, *Rdh10*, *Raldh1* and *Raldh2*. Another example is the roof plate of the neural tube which expresses *Stra6*, *Rdh10* and *Raldh2*.

The second class of localisation are those regions which superficially show co-expression, but actually reveal an interesting phenomenon recently described in *Xenopus* (Strate *et al.* 2009) where the domain of the *Raldh* is much more extensive than that of *Rdh10*. These regions include the eye, the somites and the early mesoderm and the expression patterns are summarised in Fig. 5B-D. In the eye (Fig. 5D) *Stra6* is expressed throughout, in the retinal pigmented epithelium, *Rdh10* is expressed dorsally in a smaller domain than *Raldh1* and ventrally *Rdh10* is expressed in a smaller domain than *Raldh3*. In the somites (Fig. 5C) *Stra6* and *Rdh10* are expressed medially whereas *Raldh2* is expressed throughout the somite and into the lateral plate (Blentic *et al.* 2003; Swindell *et al.* 1999). As the early mesoderm is generated during gastrulation the ingressing mesenchymal cells begin by expressing *Stra6*, but down-regulate it as they migrate laterally. *Stra6* expression is retained adjacent to the mid-line in these recently ingressed cells as the node moves posteriorly. These mesenchymal cells also express *Rdh10* and *Raldh2* so the full complement of enzymes and cell surface receptor are initially expressed, but it is clear that the domain of *Rdh10* expression is not as extensive in the anteroposterior dimension as that of *Raldh2* as summarised in Fig. 5B. This phenomenon has been seen in *Xenopus* early mesoderm and led to the suggestion that retinal diffuses from the *Rdh10* expressing cells into the *Raldh2* expressing (*Rdh10* negative) posterior cells (Strate *et al.* 2009). This novel concept means that not only does RA diffuse across fields of cells but so does its precursor, retinal and it will be important to conduct tests of this hypothesis.

The third class of localisation is where *Stra6* is expressed, but there is no *Rdh10* or a *Raldh* in the same tissue, only a *Cyp*. One of these regions is the node which is a known site of RA synthesis (Chen *et al.* 1992; Hogan *et al.* 1992) and when RA signaling is inhibited the expression of a range of laterality genes is abolished and cardiac sidedness is randomized (Zile *et al.* 2000). We see the expression of *Stra6* in the mesoderm of the node and anterior to it (yellow dots in Fig. 5A) so retinol is taken up. However there is no *Rdh10* in the node to convert it to retinal, instead *Rdh10* is in the ingressed mesenchyme posterior to the node (purple dots in Fig. 5A), as is *Raldh2.* There is another *Raldh*, *Raldh3,* in the epiblast of the node (solid red in Fig. 5A), but no synthetic enzyme

co-localises with *Stra6*. This suggests that there ought to be another *Rdh* somewhere within the node, preferably co-localised with *Stra6*, in order to generate retinal for use by the cells expressing *Raldh3* in the epiblast of the node.

Other regions where there are no co-localisations is the regressing node (Fig. 1J) and, for a brief period of time, the ventral floor plate of the neural tube (Fig. 1L). The only other enzyme expressed in the regressing node is *Cyp26A1* (Blentic *et al*. 2003) predicting that there should be a *Rdh* and perhaps a *Raldh* to be found localised to this region since the CYP26s cannot synthesise RA from retinol. A further region is the mesenchyme surrounding the posterior hindbrain (Fig. 2B). The hindbrain is the area of the embryo where the role of RA signaling has been studied in the greatest detail as the posterior hindbrain is patterned in the rostrocaudal axis by a graded concentration of RA (Dupe and Lumsden 2001; Gale *et al*. 1999; Kolm *et al*. 1997; White *et al*. 2007). Since no enzymes of the RA synthetic pathway are present within the neuroepithelium the source of the graded RA signal is the somitic mesenchyme posterior to the hindbrain. But dorsoventral patterning in the hindbrain is also responsive to RA and we have previously shown the presence of *Cyp1B1* which can synthesise RA from retinol in the mesenchyme adjacent to the hindbrain (Chambers *et al.* 2007). Over-expression of *Cyp1B1* disrupts the expression of several genes responsible for the generation of ventral progenitor domains and subsequent differentiation of motor neurons in the hindbrain. Now that we have seen *Stra6* there as well (Fig. 2B) we have the potential to generate RA for patterning directly at the posterior hindbrain neuroepithelium.

Finally, we observed that the expression of *Rdh10* is unaffected by the excess RA or the absence of RA and thus behaves like the *Raldhs*. Other factors must therefore control the tissue specific expression and levels of these enzymes rather than their substrate. In contrast to this behaviour, the enzymes at the distal end of the RA pathway, the *Cyp26s*, are highly responsive to RA in excess and in its absence (Reijntjes *et al*. 2005) and are known to possess retinoic acid response elements in their promoter sequences. They respond to excess RA by up-regulation in order to stabilize RA levels. It is interesting therefore that the most proximal gene in the RA pathway also responds to altered levels of RA as we noted the expansion of *Stra6* expression in the absence of RA in certain domains. This suggests that RA may be part of a feedback loop with the cell surface receptor for retinol to increase retinol uptake when RA levels are in decline in an attempt to re-establish appropriate levels.

In conclusion, the expression patterns described here on two new components of the RA synthetic pathway have added considerably to our understanding of how this molecule is generated in the embryo and which regions of the embryo require RA signaling for their proper development. We also suggest that there may be more enzymes yet to be discovered.

Materials & Methods

Embryos

Fertilised hens eggs (mixed flock, Henry Stewart and Co. Ltd., Louth, Lincolnshire) were incubated in a humidified atmosphere at 37°C. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton 1951).

The generation of vitamin A-deficient (VAD) quail embryos and the criteria for normal and VAD quail development have been described before (Dersch and Zile 1993; Dong and Zile 1995).

For RA treatment of embryos, all-*trans* -RA (Sigma) was dissolved in 250 µl dimethylsulphoxide to a concentration of 4mg/ml to which was added anion exchange resin beads AG1-X2, formate form (Bio-RAD). To visualize the beads 1ml of Dulbecco's MOD Medium (Gibco) was added after the excess RA solution was removed. In the embryos at stage 9, part of the vitelline membrane was carefully removed and 1 bead placed by the head/trunk junction. Controls were treated identically except that only dimethylsulphoxide was added to the beads. Embryos were incubated overnight at 37ºC and before fixation the position of the beads noted if they had not become embedded within the embryonic tissue.

Isolation of cDNA clones

Total RNA was purified according to Chomczynski and Sacchi (1987). A cDNA pool was prepared using cloned AMV reverse transcriptase (Invitrogen). Oligo$(dT)_{20}$ as a primer and 2 µg of RNA as a template. PCR was carried out using Advantage cDNA PCR Kit (BD Biosciences) using primers designed from the BBSRC Chicken EST Project (Boardman *et. al*., 2002: http://www.chick.umist.ac.uk).
ChEST421a24 for RDH10:
forward 5'-GGGTGCAGGATCAGAAAAGA-3' and
reverse 5'-ACACAGGTTGAGGACCCAAG-3'
to generate a 403bp product and
ChEST109e12 for Stra6:
forward 5'-CGAGCTCTCTACATCGTCACC-3' and
reverse 5'TTGTTGAGGAGGGTGTAGGC3' to generate a 398bp product. PCR amplification conditions were *Rdh10*: 35 cycles, annealing at 55°C for 30 s, extension at 72°C for 60 s; *Stra6*: 35 cycles, annealing at 58°C for 30 s, extension at 72°C for 60 s. The PCR products were purified by agarose gel electrophoresis, cloned into pGEM-T Easy vector (Promega, USA) and sequenced.

Whole-mount in situ *hybridisation and sectioning*

For both *Rdh10* and *Stra6*, labelling of antisense and sense RNA probes with digoxigenin (DIG) was performed using T7 and SP6 RNA polymerases respectively, according to the protocol supplied by Roche (Germany). Chick whole-mount *in situ* hybridisation was carried out using standard procedures (Acloque *et. al*., 2008). Briefly, prehybridisation and hybridisation were carried out at out at 70°C with a probe concentration of 1 µg/ml and visualised with digoxigenin antibodies conjugated to alkaline phosphatase (Roche, Germany) reacted with NBT/BCIP purple (Roche). After *in situ* hybridisation a proportion of embryos were sectioned. For this embryos were embedded in 3.6ml of vibratome embedding mix consisting of gelatin type A, egg albumin (Sigma) and sucrose (BDH), incubated for 2h at room temperature and then 400 µl gluteraldehyde (Sigma) was added. Sections were cut at 80 µm on a vibratome (Leica VT 1000S), placed on glass microscope slides and mounted in glycerol.

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