

The distribution of endogenous retinoic acid in the chick embryo: implications for developmental mechanisms

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SUMMARY

The aim of these experiments was to determine the endogenous distribution of retinoic acid (RA) across a wide range of embryonic stages in the chick embryo. By high pressure liquid chromatography, it was revealed that dihydroRA is the most prevalent retinoic acid in the chick embryo and that the tissues of the stage 24 embryo differed widely in their total RA content (dihydroRA + all-*trans*-RA). Some tissues such as the heart had very little RA and some such as the neural tube had very high levels, the total variation between these two being 29-fold. We showed that these tissues also synthesised RA and released it into the medium, thus validating the use of the F9 reporter cell system for further analyses of younger staged embryos. With these F9 cells, we showed that, at stage 4, the posterior end of the embryo had barely detectably higher levels of RA than the anterior end, but that a significant level of RA generation was detected as soon as somitogenesis began. Then a sharp on/off boundary of RA was present at the level of the first somite. We could find

no evidence for a posterior-to-anterior gradient of RA. Throughout further development, various consistent observations were made: the developing brain did not generate RA, but the spinal part of the neural tube generated it at very high levels so there must be a sharp on/off boundary in the region of the hindbrain/spinal cord junction; the mesenchyme surrounding the hindbrain generated RA whereas the hindbrain itself did not; there was a variation in RA levels from the midline outwards with the highest levels of RA in the spinal neural tube followed by lower levels in the somites followed by lower levels in the lateral plate; the posterior half of the limb bud generated higher levels than the anterior half. With these observations, we were able to draw maps of endogenous RA throughout these early stages of chick embryogenesis and the developmental implications of these results are discussed.

Key words: Vitamin A, Retinoid, Retinoic acid, Chick

INTRODUCTION

Many different vertebrate embryos at many different stages of development have been used to examine the effects of exogenous retinoids. Gastrulation, neural patterning, neural crest migration, the heart, the eye, blood formation, craniofacial development, limbs, fins and tails, vertebral identity and skin patterning have been studied in fish, amphibian, birds and mammals with the presupposition that the resultant disturbances in the patterning events can tell us about the process of normal development (reviews, Maden and Pizzey, 1997; Maden, 1998a,b; Morriss-Kay, 1997). The validity of this presupposition depends upon whether or not retinoids are endogenous components of the patterning processes of the vertebrate embryo.

The currently available data on the endogenous distribution of retinoids, or retinoic acid (RA) in particular, are very sporadic. Embryos of several different species have been examined, but only at one or two particular stages and there has been no complete spatial analysis throughout the development of any one species. Furthermore, different

techniques have been employed for these analyses and often contradictory data have been generated.

For example, in zebrafish, high pressure liquid chromatography (HPLC) has been used to characterise the different retinoids present throughout development (Costaridis et al., 1996). All-*trans*-RA, but not 9-*cis*-RA or 4-*oxo*-RA was detected at all stages from fertilisation and the unusual feature of these embryos was the very high levels of retinal present in the yolk, as is the case in *Xenopus* (Azuma et al., 1990). No information on spatial distribution was generated, however, in these experiments because of the nature of the methodology. This has been obtained in zebrafish embryos by injecting a reporter construct containing a retinoic acid response element (RARE) linked to a minimal promoter and the *lacZ* gene (Marsh-Armstrong et al., 1995). In the presence of RA, this construct is activated within cells and, after histochemical processing for β -galactosidase, these cells can be identified in the embryo by their blue colour. Using this methodology, the first appearance of blue cells was at the 18- to 21-somite stage when transgene activity was concentrated in the anterior trunk in a variety of cell types including spinal cord, floor plate,

notochord, muscle and epidermis. A similar result was obtained by assaying for RA release using a reporter cell line (Wagner et al., 1992). This is a mouse embryonal carcinoma cell line that has been stably transfected with a RARE-tk- β gal construct. Pieces of embryonic tissue are placed on these RA-responsive cells, which are then processed for β -gal and the number of blue cells reflects the amount of RA released from the tissue. So here is an example of a conflicting data – HPLC tells us that RA is present from fertilisation, yet transgene analysis suggests it is not active until surprisingly late stages, 15 somites stage onwards.

In *Xenopus*, like zebrafish, HPLC studies have revealed the presence of various retinoids, including all-*trans*-RA, 4-*oxo*-RA and 9-*cis*-RA, from fertilisation onwards (Crech-Kraft et al., 1994a,b; Durston et al., 1989; Pijnappel et al., 1993). Spatial distribution studies have shown that, when the anteroposterior (AP) axis becomes apparent at stage 13, the concentration of all-*trans*-RA is two-fold higher at the anterior end than the posterior end and 9-*cis*-RA is absent in the middle but present at both ends (Crech-Kraft et al., 1994a). However, using the F9 reporter cell system, the opposite result has been obtained with a 12-fold higher level of RA activity at the posterior end of the stage 12 embryo compared to the anterior end (Chen et al., 1994).

In the chick embryo, a few selective areas of the embryo have been assayed for RA by various methods. Using the F9 reporter cell system, Hensen's node has been found to release more RA than the surrounding area pellucida (Chen et al., 1992; Wagner et al., 1992); the dorsal neural tube and the ventral floor plate both release RA as does the posterior limb bud mesoderm, but the limb bud ectoderm does not (Wagner et al., 1992). HPLC studies have revealed that the limb bud contains all-*trans*-RA at a higher concentration on the posterior side than the anterior side (Scott et al., 1994; Thaller and Eichele, 1987) and that the major retinoic acid isoform in the chick is not all-*trans*, but didehydroretinoic acid (Scott et al., 1994; Stratford et al., 1996; Thaller and Eichele, 1990). During feather development, the skin of the chick also synthesises RA (Upton et al., 1998). Thus in the chick embryo, although there are no major contradictions, the data on the spatial distribution of RA is extremely sparse.

In the 3- to 4-somite-stage mouse embryo, the node synthesises RA at a higher rate than anterior neural tissue or posterior tissue (Hogan et al., 1992). However, no such area was highlighted using a RARE-*hsp-lacZ* transgenic mouse. This system revealed that activation of the transgene first occurred in all three germ layers in the posterior half of the headfold stage embryo with an anterior border at the node and no obvious AP gradient of staining (Rossant et al., 1991). As development proceeded, a sharp anterior boundary appeared at the level of the first somite, posterior to the hindbrain and otic vesicle, and posteriorly there was a decline in staining behind the last-formed somite (Colbert et al., 1993). A similar anterior border and then posterior decline was seen when whole mouse embryos were laid on F9 reporter cells (Ang et al., 1996). The developing eye was positive in the RARE-*hsp-lacZ* transgenic mouse, which accords with studies on the synthesis of RA in the retina (McCaffery et al., 1992, 1993), but the limb bud was negative, which contrasts with the identification of RA in the mouse limb bud by HPLC (Horton and Maden, 1995; Satre and Kochhar, 1989; Scott et al., 1994) and by the F9 reporter cell

system (Ang et al., 1996). However, an HPLC analysis confirmed the lack of RA in the anterior regions of the embryos, particularly the developing hindbrain, midbrain and forebrain (Horton and Maden, 1995). From day 12.5 onwards in the mouse spinal cord, 'hot spots' of RA synthesis have been identified that correspond to the origins of the limb innervation (Colbert et al., 1993; McCaffery and Drager, 1994).

Thus it is clear that available data on the distribution of endogenous RA in the embryo is patchy and often contradictory. To address this issue, we undertook an analysis using the chick embryo from gastrulation stages onwards, covering the major events of embryogenesis. We used a newly generated F9 reporter cell system, having first established the validity of this system by comparing it to extensive endogenous HPLC and RA synthesis measurements on the same stage of embryo. We revealed that didehydroRA is the major form of retinoic acid in the chick embryo, rather than all-*trans*-RA, and that RA is present in most parts of the embryo except the developing forebrain, midbrain, hindbrain and notochord. However RA is present in widely differing amounts in the remaining tissues and each tissue has a different 'retinoid profile'. Along the anteroposterior axis of the early embryo, the presence of a sharp anterior boundary where RA suddenly appears was detected rather than a posterior-to-anterior gradient. With regard to the absence of RA in the neuroepithelium of the developing brain, the mesenchyme surrounding the developing brain generates RA and so we suggest that this may instruct the neuroepithelium, particularly the hindbrain, in its development or that the developing spinal cord, which also contains high levels of RA, may perform this function.

MATERIALS AND METHODS

Embryos and cells

Fertile eggs were obtained from Needle Farms, Enfield, UK and incubated at 38°C to the required stages (Hamburger and Hamilton, 1951). Embryos were dissected in a balanced salt solution prior to collecting tissues for HPLC or for incubation with radiolabelled retinol or for placing onto F9 cells.

HPLC

HPLC was performed using a Beckman System Gold Hardware with a UV detector (351 nm) in series with a solid scintillant radioisotope detector. For endogenous retinoid measurements, the extraction and HPLC method of Thaller and Eichele (1987) was used. Individual tissues from at least 40 embryos were collected in 100 μ l of ice-cold stabilising solution (5 mg/ml ascorbic acid, Na₃EDTA in PBS, pH adjusted to 7.3 with NaOH) and then sonicated. A 10 μ l sample was taken for protein estimations (Pierce BCA protein assay reagent) and the remaining suspension extracted twice with 2 volumes of 1:8 methyl acetate/ethyl acetate (with butylated hydroxytoluene as an anti-oxidant). The extract solvent was dried down over nitrogen, resuspended in 100 μ l methanol, centrifuged at high speed to remove any particulate matter and placed into autosampler vials for HPLC analysis.

For reverse phase chromatography a 5 μ m C₁₈ LiChrocart column (Merck) with an equivalent precolumn was used and the mobile phases consisted of 1% acetic acid in milliQ water (A) and acetonitrile:methanol 3:1 (B). Retinoids were eluted under the following gradient conditions – 60% B and 40% A, rising linearly to 100% B over 25 minutes at a flow rate of 1 ml/minute. For RA synthesis measurements, the normal phase chromatography method of Kawamura et al. (1993)

was used, with slight modifications. The extract was injected onto a 5 μm LiChrospher 100 NH_2 column (Merck) with an equivalent precolumn and eluted at 1 ml/minute, for 5 minutes initially, with 100% mobile phase C (chloroform:methanol, 9:1) changing over 1 minute to 100% mobile phase D (chloroform:methanol:acetic acid, 9.0:0.9:0.1) for a further 20 minutes. The eluant was monitored both with a uv detector at 351 nm and for radioactivity.

RA synthesis and HPLC

The relevant pieces of tissue from at least 40 embryos were dissected out and incubated overnight at 37°C, 5% CO_2 in 1 ml of medium consisting of buffered DMEM (Gibco) with various additives (penicillin/streptomycin, glutamine, BSA, transferrin, putrescine, sodium selenate, T3, insulin, progesterone, corticosterone) to which 500 nM [^3H]all-*trans*-retinol was added. After incubation, the tissue and medium was separated by brief low-speed centrifugation and the retinoids were extracted from each by sonication, extraction, drying down and resuspension, as described above. For the tissue culture medium, extraction solvent was added directly to the medium.

Reporter cell assay

The generation and characterisation of the F9 reporter cells will be described elsewhere (E. S., unpublished data). Briefly, the cells were stably transfected with 1.8 kb promoter sequence of the mouse retinoic acid $\beta 2$ coupled to the *lacZ* gene. They respond with equal EC_{50} values to all the acidic retinoids in the range of 1-7 nM. For explant assay, the cells were grown on gelatin-coated wells of a 24-well culture dish (Nuclon) to confluence. Just prior to introducing the explants to the wells, the growth medium was aspirated from the F9 cells and replaced with 0.5 ml serum-free medium. The explants were allowed to settle and attach to the monolayer. After overnight incubation, the cultures were fixed and the β -galactosidase expression was visualised. They were gently washed twice with room temperature PBS, so that the explants were not disturbed or the F9 cells were not stripped off, then fixed for 5 minutes at 4°C with 2% paraformaldehyde, 0.2% glutaraldehyde in PBS. They were rinsed gently twice more then covered with 1 ml of X-gal stain per dish and incubated at 37°C overnight. The X-gal stain was replaced with PBS and the blue-stained cells counted on an Olympus inverted microscope fitted with an eyepiece graticule.

RESULTS

Endogenous retinoids in different parts of the stage 24 embryo

The first series of experiments was conducted on stage 24 embryos, which were large enough to be dissected into

component parts and so that enough tissue could be collected for HPLC analysis. In this way, we could ask such questions as do different parts of the embryo contain different relative amounts of retinoids or do different parts of the embryo contain different retinoids? The embryos were dissected into eight parts – neural tube (spinal part only, excluding the brain), somites, eyes, frontonasal mass (including forebrain and nasal placodes), branchial arches (including the floor of the pharynx), limb buds, heart and tail bud – and about 40 embryos used for each sample. The samples were extracted and run on reverse-phase HPLC.

It was clear that there were four major peaks consistently present in these samples (Fig. 1A-D) that corresponded to four known retinoids: all-*trans*-retinol, all-*trans*-retinoic acid, and the two corresponding didehydro derivatives, didehydroretinol and didehydroretinoic acid. Identification was made on the basis of elution times, co-elution with radioactive standards and increases in peak heights when cold spikes were added to the samples. Four other peaks were present, all at much lower levels. Only one of these, 4-*oxo*-retinoic acid could be identified with any certainty and this retinoid was present only in neural tube, somites, limb buds and arches, and not present in the remainder. Because of this, 4-*oxo*-RA was not featured in the analysis of retinoid levels. All-*trans*-retinaldehyde, the intermediate metabolite between retinol and RA, was conspicuously absent, as in the case of the mouse embryo (Horton and Maden, 1995). We have not been able to identify the other 4-*oxo* compounds, 4-*oxo*-retinal or 4-*oxo*-retinol, and so cannot make any conclusions about their endogenous distribution in chick embryos.

Thus considering only the four major retinoids, each peak was quantified in terms of the amount of retinoid per mg protein and the experiments were repeated 3-6 times to examine the consistency of the retinoid measurements. Some tissue measurements were very repeatable (such as limb buds) and the data in Table 1 show low standard deviations for these tissues whereas some tissues were rather variable in absolute amounts (such as tails buds or frontonasal mass). However, what was uniformly consistent was the relative levels of each retinoid (ddRol:ddRA:tRol:tRA) in repeat samples of the same tissue. Table 1 records the data and three examples of chromatograms from different tissues are shown in Fig. 1 to demonstrate the clear differences in retinoid profiles between the tissues. Fig. 1B is a chromatogram from the neural tube

Table 1. Levels of 4 endogenous retinoids in 8 different tissues and measurement of the synthesis of RA in the stage 24 chick embryo

Tissue	Retinoid (ng/mg protein)				Total RAs	ddRA:tRA	Synthesis of RA (fmol/mg)
	ddRol	ddRA	tRol	tRA			
Neural tube	234+29	679+127	42+6	34+9	713	20:1	ND
Somites	553+129	301+28	151+44	23+2	324	13:1	0.72
Eye	421+126	280+98	276+87	20+6	300	14:1	0.88
Tail bud	242+104	214+110	124+50	20+8	234	11:1	0.43
Fnm	884+182	191+45	702+173	41+18	232	5:1	0.16
Arches	495+162	146+83	274+90	21+9	167	7:1	0.69
Limb buds	516+52	104+22	202+3	23+7	127	5:1	0.36
Heart	257+18	0	313+60	25+13	25	–	0.19

Each endogenous HPLC measurement represents the average of three repeats and standard deviations are recorded. Each synthesis measurement represents the average of 3-6 repeats. ddRol, didehydroretinol; ddRA, didehydroretinoic acid; tRol, all-*trans*-retinol; tRA, all-*trans*-retinoic acid; total RAs, sum of the ddRA + tRA columns; ddRA:tRA, ratio between ddRA and tRA; ng/mg, ng of retinoid per mg of protein in the tissue sample; fmol/mg, femtomoles of [^3H] RA generated per mg of protein in the tissue sample; ND, not determined.

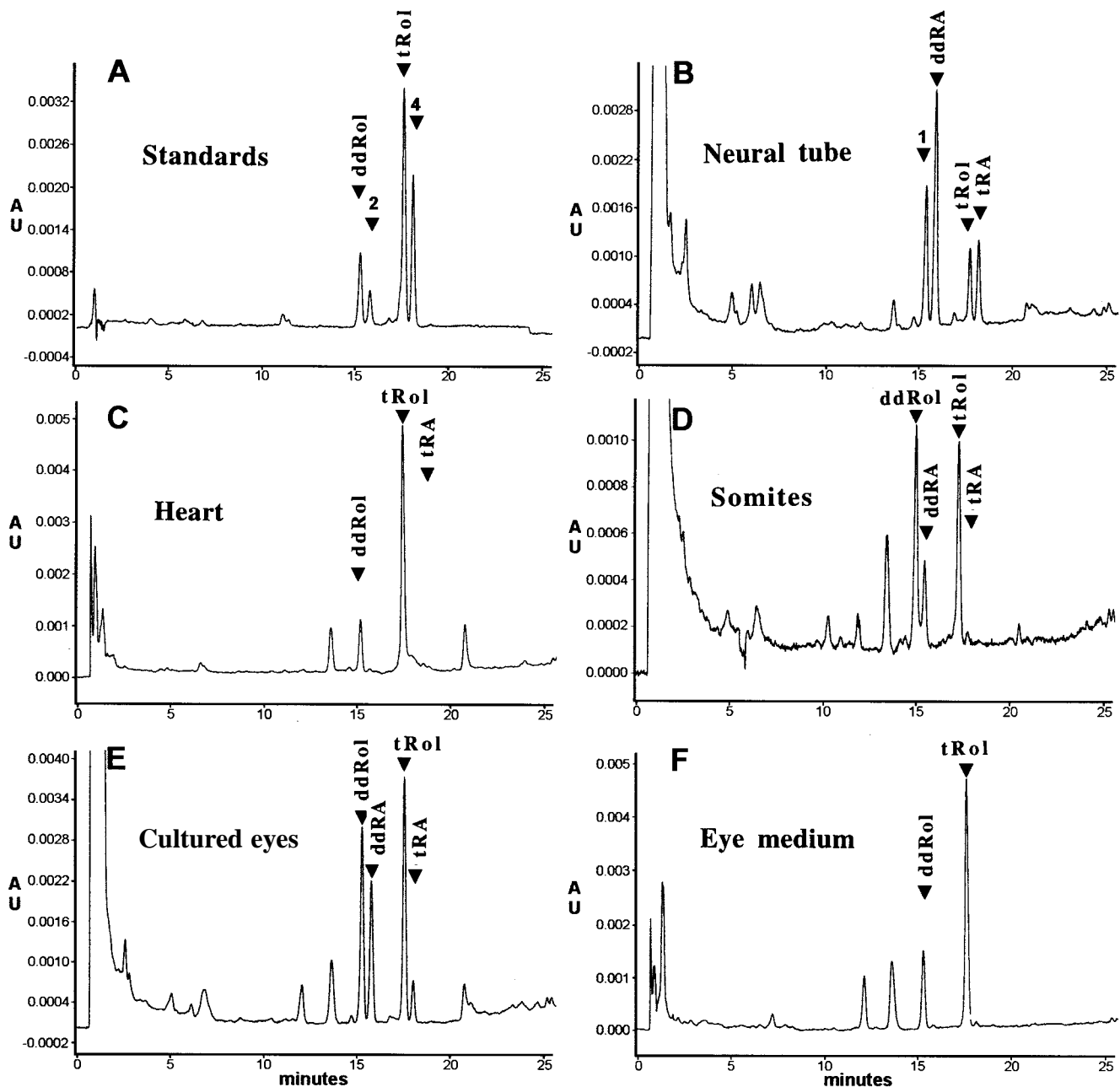


Fig. 1. Chromatograms showing endogenous retinoids in stage 24 chick embryos identified by reverse phase HPLC. (A) Standards eluting at characteristic times. ddRol, didehydroretinol; ddRA, didehydroretinoic acid; tRol, all-*trans*-retinol; tRA, all-*trans*-retinoic acid. (B) Extract of the neural tube. The major peaks present are the 4 retinoid standards from A. (C) Extract of the heart. ddretinol and tRol are present but, in contrast to B, there is no ddRA and only a very small amount of tRA. (D) Extract of the somites. High levels of ddretinol, ddRA and tRol are present but low levels of tRA. (E) Extract of eyes that had been cultured for 6 hours in minimal medium. High levels of the 4 retinoids are still present in the tissues. (F) Extract of the medium from cultured eyes in E. Only the retinols are clearly present, the retinoic acids are barely detectable despite their high levels in the tissue itself as seen in E.

showing high levels of all four retinoids particularly ddRA. Fig. 1C is from the heart showing high tRol, low ddRol, very low tRA and ddRA absent. Fig. 1D is from somites showing high levels of ddRol and tRol, a reasonable level of ddRA and low tRA.

The main conclusions from these results are as follows. Firstly, the major retinoic acid present in all the tissues of the chick embryo except the heart is ddRA, tRA is a minor

component (Table 1). This phenomenon has previously been reported for the chick limb bud (Scott et al., 1994; Thaller and Eichele, 1990), but not for the other tissues of the embryo.

Secondly, most of the tissues contain each of the 4 major retinoids in varying levels (Table 1) rather than a range of different retinoids (Fig. 1B-D). The exception to this is the heart (Fig. 1C), which does not contain ddRA.

Thirdly, each tissue has differing absolute levels of the 4

major retinoids. For example, the frontonasal mass is particularly rich in ddRol whereas the neural tube has a low level of ddRol; the neural tube has an exceptionally high level of ddRA, whereas the limb buds have a low level.

Fourthly, each tissue has a characteristic relative levels of each of these 4 retinoids. For example, the neural tube has a low level of the precursor ddRol but a 3-fold higher level of its metabolite ddRA, presumably reflecting the high rate of usage of ddRA. In contrast, the tail bud has similar levels of ddRol and ddRA and the remaining tissues have a considerable excess (up to 5-fold) of the precursor ddRol over ddRA. In this sense, each tissue could be said to have a unique retinoid profile. A further reflection of this variation is the ddRA:tRA ratio (Table 1, column 7) which is different for different tissues and ranges from 20:1 in the neural tube to 5:1 in the limb buds. This value of 5:1 in the limb bud is a very similar figure to that of 6:1 obtained in a previous analysis of this tissue (Thaller and Eichele, 1990).

Fifthly, by adding the ddRA and tRA values to obtain a total RA content (Table 1, column 6), it can be seen that the tissues have widely differing amounts of total RA. By far the highest level is found in the neural tube, the next highest level is in the eye and somites with approximately half as much, then the tail bud and frontonasal mass, followed by the arches and limb buds with half as much again and finally the heart with very low levels of RA and no ddRA. Thus in this sense too, each tissue of the chick embryo has a different retinoid profile and the amount of variation in RA levels across these eight tissues was 29-fold.

Do these tissues release RA?

It became clear from the above experiments that we could not perform these HPLC analyses on early stages of chick embryogenesis because of the relatively large amount of tissue required. Therefore, we used a F9 reporter cell system that relies upon the presumption that retinoids are released from embryonic tissues and activate adjacent F9 reporter cells that have been transfected with a retinoic acid response element linked to the *lacZ* gene. However, we needed first to demonstrate that retinoids are indeed released from embryonic tissues.

Culturing the tissues represented in Table 1 (from about 50 embryos) for 3-6 hours in DMEM (containing no serum or retinoids) and then quantifying the retinoids in both the tissues and the medium, clearly showed that 3 of the 4 major retinoids were indeed released into the medium. ddRol was readily detectable in the medium from all 8 tissues and, on average, 36% of the total was found in the medium (spread of values = 14-61%). tRol was more readily detected as, on average, 63% of the total was found there (spread of values = 32-82%). tRA was also detected in the medium, but only 19% of the total (spread of values = 5-34%). Surprisingly, ddRA could only be detected in the medium from eyes and not from any other tissue, despite the fact that the neural tube, for example, has a higher endogenous content of ddRA than the eyes. Fig. 1E and F show, respectively, chromatograms of the retinoids remaining in the eyes and those present in the corresponding medium after this culture. Despite the presence of high levels of all 4 retinoids in the tissues, only ddRol and tRol are conspicuously present and there are only very small peaks of ddRA and tRA.

Experiments showed that the rapid disappearance of tRA

and ddRA from the medium is due to the breakdown by the tissue itself rather than the inherent instability of the molecules. When a known amount (100 ng) of each of the 4 retinoids was added to medium and cultured for 6 hours, the presence of tissue caused the retinoids to disappear rapidly. For example, after 6 hours in medium without any tissue 83% of the added tRA was recovered and 55% of the ddRA. When limb bud tissue was present these figures dropped to 30% recovery for tRA and 18% for ddRA. We therefore suggest that tRA is stable in medium whereas ddRA is significantly less stable, but both retinoids are rapidly metabolised (and therefore taken up) by any surrounding embryonic cells that are present. This would also include F9 reporter cells.

Because directly measuring the rates of release of endogenous tRA and ddRA from different embryonic tissues was difficult due to their rapid disappearance from the medium, we subsequently measured the synthesis and release of RA using radioactive retinol.

Synthesis and release of RA from embryonic tissues

We have developed an HPLC method for the study of rates of retinoic acid synthesis that involves taking the relevant tissue from 40-50 embryos and culturing these pieces overnight in 1 ml of defined medium to which 500 nM [³H] all-*trans*-retinol has been added (Stratford et al., 1996). The tissue and medium are then separated, the retinoids extracted from each and passed down a normal-phase HPLC column, which retains the acid metabolites. The radioactive retinoic acid peak is then quantified and different tissues compared. This method therefore provides a useful comparison with endogenous measurements and if [³H]RA is detected in the medium then we know it must have been released by the tissues.

In the absence of any tissue in the medium, there was no metabolic breakdown of the [³H]retinol and no other peak on the chromatograms except for the unmetabolised [³H]retinol which appears in the void volume (Fig. 2A). When only [³H]RA is run on this column, it elutes at 8 minutes (data not shown). When embryonic tissue was present in the culture medium, a peak of [³H]RA was generated in each experiment (e.g. Fig. 2B). After repeating these experiments 4-6 times, it was clear that the amount of [³H]RA generated varied depending on the part of the stage 24 embryo used. Seven different parts of the embryo were examined and the rates of synthesis varied from 0.16 fmol/mg protein in the frontonasal mass to 0.88 fmol/mg protein in the eye (Table 1, column 8). Fig. 2B is a representative chromatogram from the frontonasal mass to show the appearance of a peak of [³H]RA at 8 minutes.

From column 8, it can be seen that the highest rates of synthesis are found in the eye, somites and arches, followed by tail bud and limb bud with half the rate, followed by frontonasal mass and heart with half the rate again. In comparison with endogenous measurements, the high values for eye and somites accord precisely with endogenous measurements, but the value for synthesis in the arches is higher than expected. The tail bud and limb bud agree well with endogenous data, but the frontonasal mass value is lower than expected. The heart gives a low synthesis value, in accord with its very low endogenous level.

Thus these experiments showed that, in all cases, [³H]RA was released into the medium from the tissues that generate it at a rate comparable to their endogenous synthesis; if the

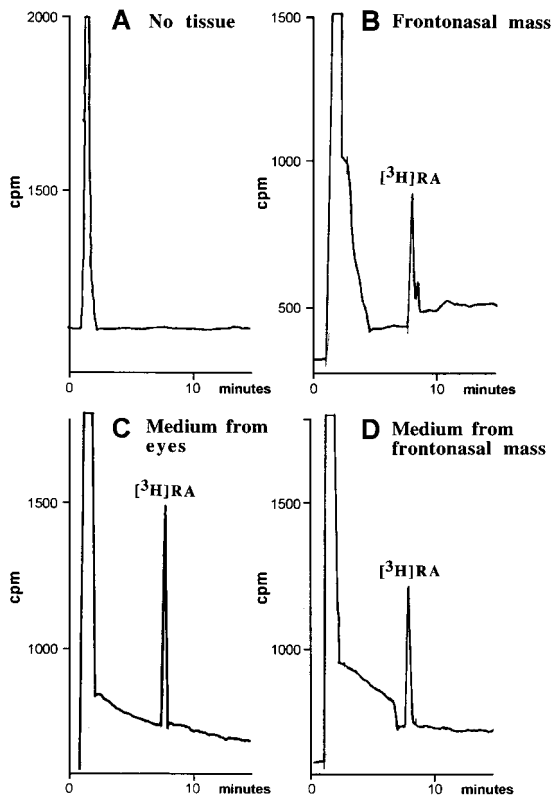


Fig. 2. Examples of measurements of the synthesis of [^3H]RA from [^3H] all-*trans*-retinol by stage 24 chick tissues as measured by normal phase chromatography. (A) Medium with no tissue present only gives a peak of radioactivity in the void volume as [^3H] retinol is not retained on the column. (B) Synthesis by cultured frontonasal mass gives a peak of radioactivity at 8 minutes as well as unmetabolised [^3H] retinol in the void volume. Since [^3H]RA elutes at 8 minutes this demonstrates that a retinoic acid has been generated by this tissue. (C) Radioactivity present in the medium from cultured eyes shows a high peak of [^3H]RA at 8 minutes. (D) Radioactivity present in the medium from cultured frontonasal mass (using the same amount of tissue as in C) shows a smaller peak of radioactive RA than that from the eyes (C).

tissues made a lot of RA, then a lot was released into the medium and if the tissues made little RA, then little was released into the medium (Fig. 2C, eye medium versus Fig. 2D, frontonasal mass medium).

F9 reporter cells

The HPLC system described above is suitable for reasonable amounts of tissue, but not for tiny pieces of tissue such as Hensen's node or single somites, or for the small amount of epithelium that could be dissected from mesenchyme. To solve this problem, several reporter cell lines have been developed (Wagner et al., 1992; Colbert et al., 1993) onto which embryonic tissues are placed. Subsequently, the reporter cells turn blue after β -galactosidase histochemistry. We have generated such a cell line containing 1.8 kb of the mouse RAR β_2 gene, which we have fully characterised (E. S., unpublished data) and find that it is 10 \times more sensitive than the cell line developed by Wagner et al. (1992).

Since the major retinoic acid in the chick embryo is ddRA

and not tRA (Table 1), we first compared the response of this reporter cell line to ddRA versus tRA. The standard curve of response to tRA is shown graphically in Fig. 4A and the response to ddRA was identical. This is shown pictorially in Fig. 3 with tRA on the top row (Fig. 3B-F) and ddRA on the lower row (Fig. 3G-K). Both visually and by cell counts, overall, the response to these two retinoic acids could not be distinguished and concentrations as low as 10^{-10} M can be detected. Therefore this cell line is appropriate for use with the chick embryo and will measure the sum of the retinoic acids.

We first used these reporter cells to ensure that the same results could be obtained with stage 24 chick embryos as were obtained above with endogenous measurement by HPLC. If so, then we could confidently use these cells for an analysis of earlier staged embryos and smaller components of the embryo.

A total of 18 different tissues from stage 24 chick embryos were used and the experiments repeated at least 3 times. A greater variety of tissues were used in these experiments than in the HPLC because less tissue was required (Fig. 4B). Examples of various concentrations and tissues are shown in Fig. 3. The amounts of RA generated by these tissues could be divided into three categories: negligible or low (<10% positive cells), intermediate and high. Fig. 3R-T show examples of the negligible or low category, Fig. 3U-W show examples of the intermediate category and Fig. 3L,M,O,P show examples of the high category.

Since control wells with no tissue in them had about 5-8% β -gal-positive cells present, we conclude that the notochord (Fig. 3T), which consistently scored almost zero in these assays, does not contain any RA and that the forebrain neuroepithelium, midbrain neuroepithelium, hindbrain neuroepithelium and anterior limb bud contain negligible levels of RA. The arches were slightly above these negligible levels and so we assume that they have a low level of RA present. The tissues in the intermediate category were frontonasal mass mesoderm, posterior half limb bud, heart, tail bud and thoracic somites and by comparison to the control curve in Fig. 4A the concentration of RA generated corresponds to 10^{-9} - 10^{-10} M. The tissues in the high category were hindbrain mesoderm, eye, cervical neural tube, cervical somites, brachial neural tube, brachial somites and thoracic neural tube, and comparisons with the control curve (Fig. 4A) suggested that this concentration corresponded to approximately 10^{-8} - 10^{-9} M.

There was excellent agreement between the HPLC data in Table 1 and this reporter cell data (Fig. 4B); the neural tube always scored highest, followed by the somites and eye, then the tail bud and frontonasal mass (mesoderm), and finally arches and limb bud (average of anterior and posterior halves). The only discrepancy was the heart, which scored higher on the reporter cells than would be expected from the endogenous measurements.

We may draw the following conclusions from these reporter cell data. (1) Very little if any, RA can be detected in the notochord, forebrain, midbrain and hindbrain. (2) The value for frontonasal mass in the HPLC study was due to the mesenchyme because when the mesenchyme and forebrain neuroepithelium were separated in the reporter cell assay the neuroepithelium gave a negligible score and the mesenchyme an intermediate score. (3) RA is readily detectable in the mesenchyme surrounding the non-synthesising neuroepithelium of the

forebrain, midbrain and hindbrain. The mesenchyme surrounding the hindbrain produced RA at a particularly high rate. (4) About twice as much RA can be detected in the posterior halves of limb buds as in the anterior halves. (5) Cervical and brachial somites produce more RA than the thoracic somites. (6) The cervical and brachial neural tube produce more RA than the thoracic neural tube. These last two observations are presumably related to the 'hot spots' of RA that have been shown to be present in the brachial and lumbar regions of the mouse spinal cord (McCaffery and Drager, 1994); this has not been shown before for the somites.

Thus having established the validity of the reporter cell system as one that adequately reflects the endogenous measurements of total retinoic acids in the individual tissues of the chick embryo, we could now investigate earlier stages of chick development (stages 20, 15, 10, 8 and 4/5) to see whether the levels of RA changed over time, when RA could first be detected and the distribution in some tissues in more detail.

Changes in the neural tube and somites over time

In this experiment, stage 10, 15, 20 and 24 neural tube and adjacent somites from the cervical and brachial regions were compared (Fig. 3). One complete neural tube was used and equal numbers of somites were used at each stage. Over the first three stages, there was no significant change in either the neural tube or somite RA levels with the neural tube always scoring higher than the somites. Of course, the neural tube was getting larger as were the somites so on a per cell basis there was probably a decline in RA levels. However by stage 24 the amount of RA detected visibly began to decrease. This experiment suggested that early embryos have more endogenous RA than older embryos.

Lack of synthesis in the hindbrain of stage 20 embryos

One consistent feature of these experiments was that the hindbrain neuroepithelium showed no detectable levels of RA. To investigate this in more detail and to look at other features, stage 20 embryos were examined in detail. The hindbrain and the mesenchyme that surrounded the neuroepithelium were each dissected into 5 parts (slightly larger than individual rhombomeres). Along with these tissues, the notochord, somites and spinal neural tube were assayed. There were no detectable levels of RA in the individual components of

the hindbrain neuroepithelium or the notochord (Fig. 4C). In contrast, it is the mesenchyme surrounding the hindbrain neuroepithelium in which the levels of RA are readily detectable. The average value of % β -gal-positive cells for the hindbrain was 6% (control wells with no tissue in them gave values of between 5 and 8%) and the corresponding value for the hindbrain mesenchyme was 16%. The contrast between the cranial neuroepithelium and the surrounding mesenchyme is shown in Fig. 3R,U,S,V, although for tissues from younger

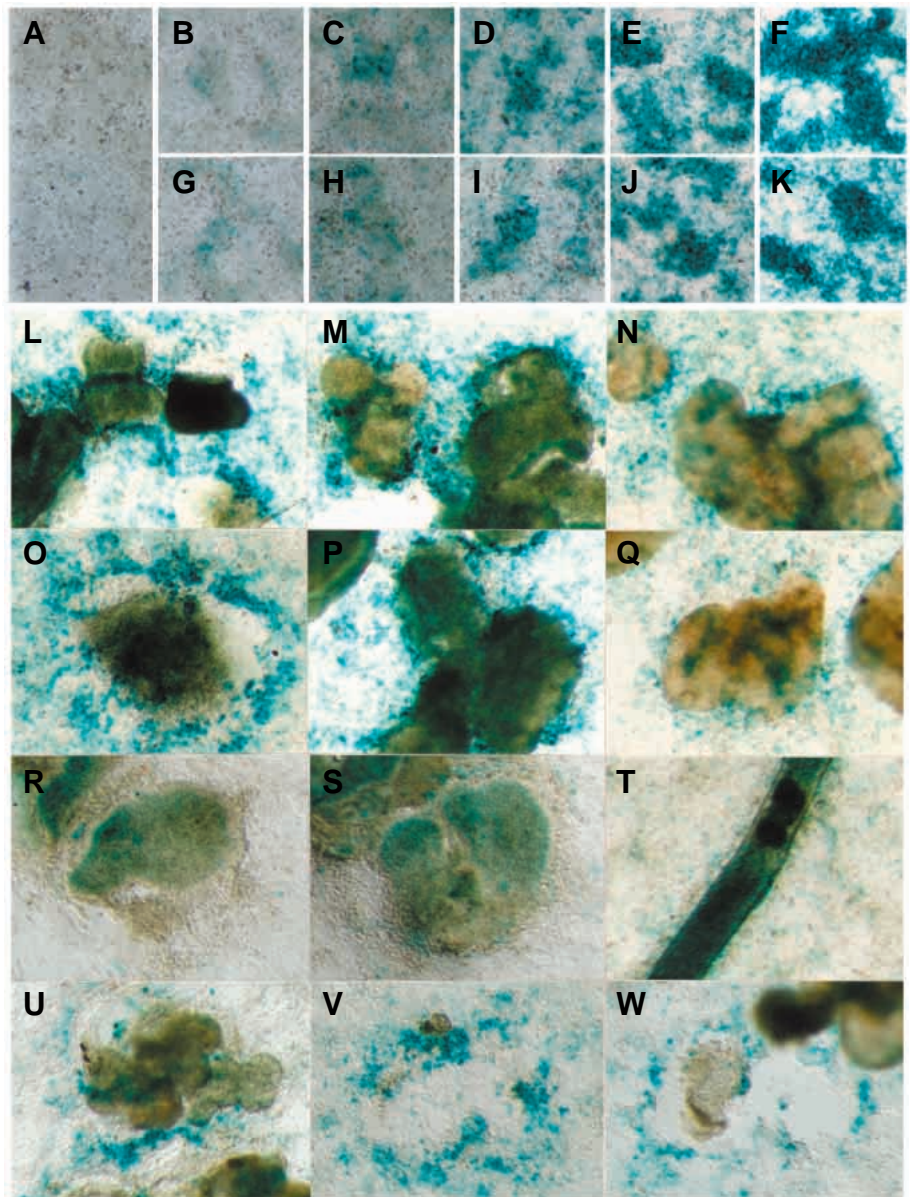


Fig. 3. F9 reporter cells showing the presence of varying numbers of blue cells which have responded to the presence of RA. (B-F) Standard series of tRA concentrations. (G-K) Standard series of ddRA concentrations. (L-W) Various tissues cultured on the reporter cells. (A) No RA; (B) 10^{-10} M tRA; (C) 10^{-9} M tRA; (D) 10^{-8} M tRA; (E) 10^{-7} M tRA; (F) 10^{-6} M tRA; (G) 10^{-10} M ddRA; (H) 10^{-9} M ddRA; (I) 10^{-8} M ddRA; (J) 10^{-7} M ddRA; (K) 10^{-6} M ddRA; (L) stage 10 neural tube; (M) stage 15 neural tube; (N) stage 24 neural tube; (O) stage 10 somites; (P) stage 15 somites; (Q) stage 24 somites; (R) stage 10 hindbrain neuroepithelium; (S) stage 10 midbrain neuroepithelium; (T) stage 24 notochord; (U) stage 10 mesenchyme surrounding the hindbrain; (V) stage 10 mesenchyme surrounding the midbrain and forebrain; (W) stage 10 heart.

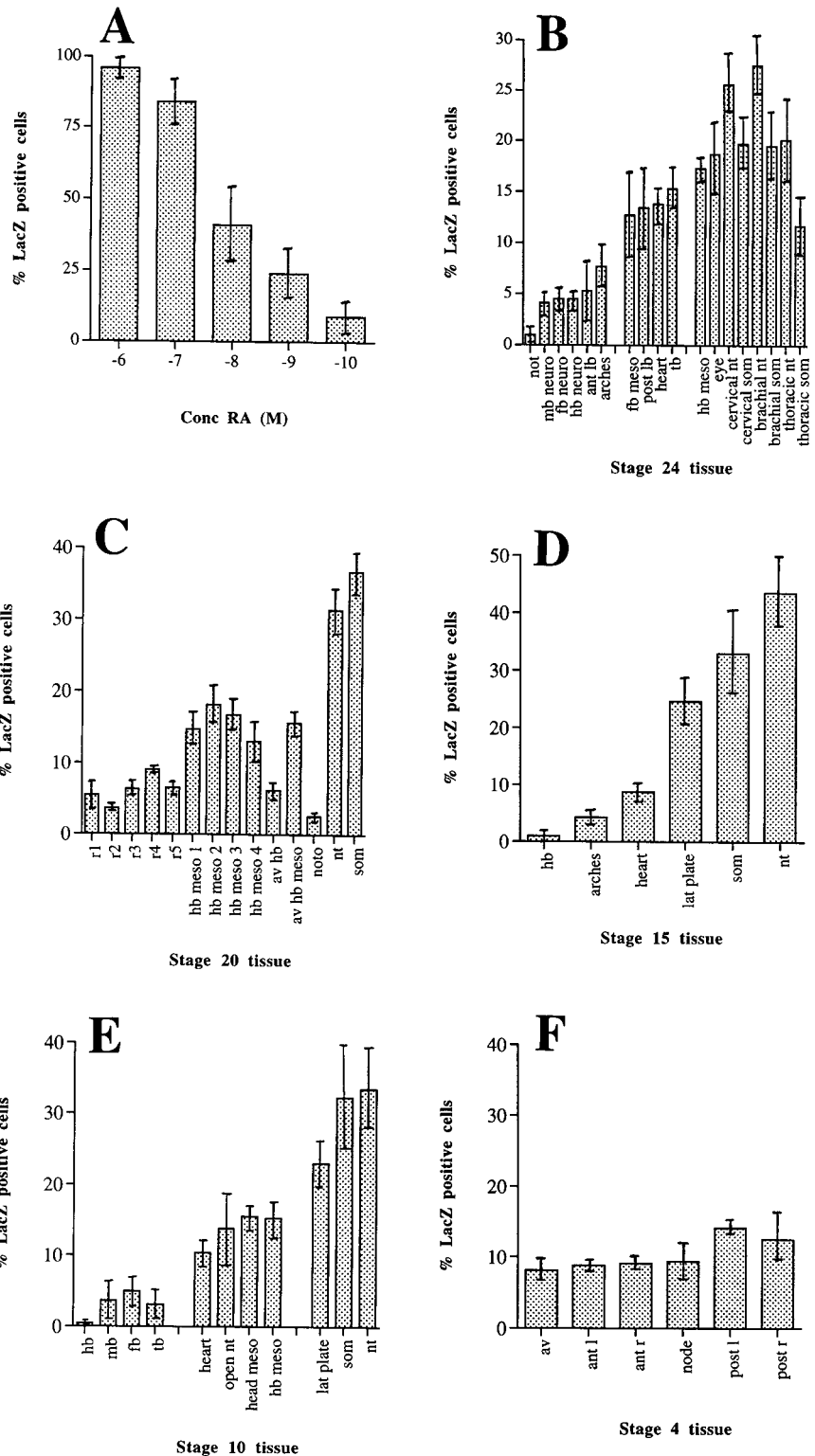


Fig. 4. F9 reporter cell data for a standard concentration range of tRA and for various tissues from various staged chick embryos. Each count of % β -gal-positive cells in a standard eyepiece graticule area was repeated three times and each experiment was repeated at least three times. Standard deviations are marked on all graphs. (A) Standard curve generated for the F9 reporter cell line using tRA. As the concentration of tRA added to the medium increased so the percentage of blue cells increased. (B) Relative amount of RA generated by various tissues from a stage 24 embryo. not, notochord; mb neuro, midbrain neuroepithelium; fb neuro, forebrain neuroepithelium; hb neuro, hindbrain neuroepithelium; ant lb, anterior half limb bud; fb meso, frontonasal mass mesenchyme; post lb, posterior half limb bud; tb, tail bud; hb meso, mesenchyme surrounding the hindbrain; nt, neural tube from various levels; som, somites from various levels. (C) Various tissues from the stage 20 embryo. r1-5, hindbrain neuroepithelium divided into 5 segments; hb meso 1-4, the mesenchyme surrounding the hindbrain divided into 4 segments; av hb, average value for the hindbrain neuroepithelium; av hb meso, average value for the mesenchyme surrounding the hindbrain; noto, notochord; nt, neural tube; som, somites. (D) Various tissues from the stage 15 embryo. lat plate, lateral plate; other abbreviations as before. (E) Various tissues from the stage 10 embryo. hb, hindbrain neuroepithelium; mb, midbrain neuroepithelium; fb, forebrain neuroepithelium; open nt, open neural tube at posterior of embryo; head meso, mesenchyme surrounding the midbrain and forebrain; other abbreviations as before. (F) Various areas of the stage 4 embryo. av, area vasculosa; ant l, anterior left quadrant; ant r, anterior right quadrant; node, equal sized piece including the node; post l, posterior left quadrant; post r, posterior right quadrant.

embryos. The spinal cord and somites of stage 20 embryos generated high levels of RA, as expected.

RA in stage 15 embryos

Six tissues were examined in this stage of embryo, the hindbrain, the branchial arches, the heart, the lateral plate,

somites and neural tube. As at later stages the contrast between the hindbrain and the adjacent spinal neural tube was clear (Fig. 4D). The hindbrain did not generate detectable levels of RA whereas the spinal neural tube produced very high levels. The branchial arches showed negligible or low levels as at stage 24 (Fig. 4B) and the heart also had low levels.

Interestingly in the lateral plate less RA was detected than the somites which in turn had less than the neural tube. There was thus a variation in RA levels from a high level at the midline outwards to a lower level in the lateral plate, an observation that was also made at stage 10 (see below).

RA in stage 10 embryos

Since this is a stage when many patterning events are occurring in the chick embryo (neurulation, somitogenesis etc.), the tissues that could be individually dissected were tested in stage 10 embryos – the component parts of the closed neuroepithelium, the open neuroepithelium, somites, lateral plate, heart and head mesenchyme.

The results shown in Fig. 4E and Fig. 3L,O,R,S,U-W share certain features with later stage tissues. (1) The spinal neural tube and somites released high levels of RA and the lateral plate released lower levels revealing decreasing levels of RA spreading out from the mid-line. (2) The forebrain neuroepithelium, midbrain neuroepithelium (Fig. 3S) and hindbrain neuroepithelium (Fig. 3R) did not produce detectable levels whereas the surrounding mesenchyme (Fig. 3U,V) clearly did. (3) The heart produced intermediate levels of RA (Fig. 3W). (4) The open neural tube at the posterior end of the embryo produced far less RA than the closed neural tube and the tail bud did not generate detectable levels. Thus, in the trunk, the level of RA tended to decrease towards the posterior end of the embryo.

The results of the stage 10 embryo experiments are summarised in Fig. 5C with colour coding representing levels of RA, which also apply for the later stages until stage 24 as there is little change in RA levels between stages. It is apparent, therefore, from Fig. 4 that the brain neuroepithelium is surrounded by mesenchyme that generates RA. RA production in the neuroepithelium begins suddenly in the spinal neural tube at very high levels and decreases posteriorly in the open neural tube. The somites alongside the spinal neural tube also produce RA, but at lower levels and this level continues to decrease laterally in the lateral plate.

We also determined whether there was any detectable variation in RA generation between the first 8 somites in stage 10-12 embryos. The reason for this experiment was the observation that when somites are grafted adjacent to the hindbrain neuroepithelium, they induce a change in the *Hox* gene expression pattern of the neuroepithelium, but the anterior somites (numbers 1-4) behave differently from the more posterior somites (Grapin-Botton et al., 1997; Itasaki et al., 1996). The conclusion of several experiments to test this idea was that there was no detectable difference between adjacent pairs of somites from the first to the eighth. It is only much later in development that any detectable differences between somites appear (Fig. 4B) when the 'hot spots' of RA present in the brachial and lumbar neural tube is also present in brachial and lumbar somites.

RA in stage 4/5 embryos

We next examined embryos when Hensen's node was functioning at stage 4/5 and divided embryos into six pieces attempting to make the pieces of equal sizes – anterior to the node (left and right), node, posterior to the node (left and right) and area vasculosa. The only tissues to give responses that were detectably above control levels were from those from the

posterior end of the embryo (Fig. 4F), but even these values were very low (posterior left, 14.1%; posterior right, 12.5%) compared to later values for tissues such as lateral plate, somites and neural tube (20-35%; Fig. 4E). The remaining scores, including that for the node, were within the range of control values (5-8%). Thus we could not detect any increased levels of RA present in the node.

Early somite stages

Since there was a dramatic increase in RA generation from very low levels at stage 4/5 to very high levels at stage 10, we lastly investigated when this increase comes about. The beginnings of somitogenesis were the stages when high levels of RA could be detected for the first time and a clear boundary of RA was apparent. 2-somite, 4-somite, 5-somite and 8-somite embryos were examined and, in all cases, tissue anterior to the first somite gave control level responses, but tissue posterior to that border gave a strong response (up to 20% β -gal-positive cells) characteristic of the typical response levels of somites. We therefore conclude that these are the stages when RA synthesis is strongly upregulated and an anterior/posterior boundary between high and low levels of RA is established at the axial level of the first somite (Fig. 5B). This on/off boundary remains in place throughout development up to stage 24 in the neuroepithelium but, in the mesoderm, it is diluted by the generation of RA by the cephalic mesoderm, albeit at lower levels than the somites.

Summary drawings of the results from stages 4/5, 8 and 10+ are shown in Fig. 5 with colour codings representing the relative levels of RA production.

DISCUSSION

We began these studies by using HPLC to measure the endogenous levels of various retinoids in the stage 24 chick embryo for two reasons. Firstly, to determine whether different tissues of the embryo have different retinoid profiles and secondly to establish the validity of using the F9 reporter cell system.

The major retinoids present are all-*trans*-retinol (tRol), all-*trans*-RA (tRA), didehydroretinol (ddRol) and didehydroretinoic acid (ddRA). Also present in some tissues was 4-*oxo*-RA and 3 other unidentified peaks, but notable by its absence was all-*trans*-retinaldehyde, as is the case in the mouse embryo (Horton and Maden, 1995). It is clear that different tissues of the embryo at this stage do indeed have different retinoid profiles. ddRA was in excess of tRA by from 5:1 (limb buds) to 20:1 (neural tube) and the total amount of RAs varied by 29-fold across different tissues with the lowest in the heart and the highest in the neural tube. Some tissues had particularly high levels of the RAs such as the neural tube whereas some had particularly high levels of the retinols such as the frontonasal mass. Presumably this variation is significant for the development of the different regions of the embryo and reflects a differential distribution of the enzymes that are involved in the synthesis of the various forms of RA.

In metabolism studies using [3 H] retinol, we demonstrated that, in culture, RA is released from tissues, apparently at a rate proportional to its rate of synthesis and that it is rapidly metabolised by any available cells, presumably including F9

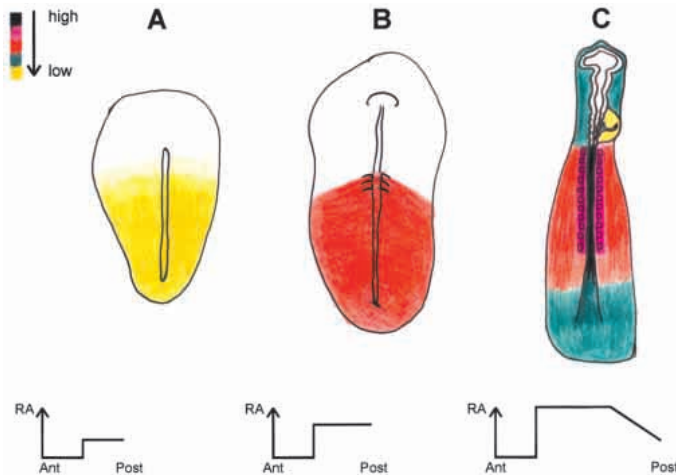


Fig. 5. Summary diagrams of the RA levels in stage 4/5 (A), stage 8 (B) and stage 10+ (C) embryos. Colour codings represent RA levels ranging from absent (white) through low (yellow), intermediate (green, red) to high (purple and black) as shown in the scale, top left. Below each drawing is a representation of the RA levels of a sagittal section from the head (ant) to tail (post) to show the on/off boundary point and its approximate height.

cells. The reason for performing these experiments was to establish the fact that RAs are released from embryonic tissues as the validity of the F9 reporter cell system depends upon this cellular phenomenon. In addition, we directly compared the same stage 24 tissues using HPLC and the F9 reporter cells. Virtually identical results were obtained, namely, the neural tube has the highest levels of RAs followed by the somites and eye, then the tail bud and frontonasal mass, then the arches and limb bud. However, there were a few inconsistencies. Firstly, the heart gave a higher reading on the F9 cells than would have been expected from its very low endogenous RA content and low synthesis rate. Perhaps the heart contains a novel retinoid that is detected on the F9 cells (activates the RAR β promoter), but was not recognised on the HPLC chromatograms. Secondly, the arches gave a low endogenous measurement and F9 cell reading, but a synthesis rate which was higher than expected. Perhaps the rate of breakdown of RA to inactive polar metabolites is especially rapid in this tissue. Thirdly, the frontonasal mass gave a lower synthesis rate than would be expected from reasonable endogenous levels and F9 levels. Perhaps frontonasal mass cells behave differently by holding RA within the cell, which causes a depression of the rate of synthesis.

Nevertheless, the high degree of concordance between the endogenous measurements and the F9 results suggests that our F9 reporter cells are a valid system for detecting endogenous RAs in the embryo. It is worth pointing out that the RAR β RARE can be activated by retinoids other than the retinoic acids e.g. retinaldehydes and retinols, although at a 100-fold and 1000-fold higher concentrations, respectively (E. S., unpublished data). All-*trans*-retinaldehyde could not be detected in chick embryos and the retinols were certainly not in 1000-fold excess (Table 1) so this is unlikely to be a source of confusion in the F9 measurements. Thus we then used the F9 reporter cells to assay earlier embryonic stages to ask questions about where RA is located and when it first becomes detectable in the embryo. Several developmentally

interesting conclusions were drawn from these subsequent results.

RA generation begins at gastrulation stages

The first detectable, but very low, levels of RA were found at stage 4/5 in the posterior half of embryo (Fig. 5A). Neither the area vasculosa, nor the anterior half of the embryo nor Hensen's node gave readings above control levels. We could therefore not provide any support for the concept that the node is a special source of RA synthesis (Hogan et al., 1992). The experiments of Hogan et al. (1992) were performed on the mouse embryo, but it is unlikely that species variation could account for this difference because the rest of our results described here are full in accord with those obtained in transgenic mouse embryos (see below). These mouse embryo experiments were performed at the 3- to 4-somite stage when we would expect the presence of a sharp on/off boundary of RA just anterior to the first somite, as in Fig. 5B, with no marked differences between areas posterior to the boundary. The very low level of synthesis that they obtained for tissue at the anterior end of the embryo would be expected, but a difference between the node piece (posterior to the first somite) and tissue posterior to the node is not what we would have expected from the results present here.

An anteroposterior boundary is established across the early embryo

RA generation increases from stage 4/5 (Fig. 5A) and, by early somite stages, there is a high level of RA from the 1st somite backwards and little RA anterior to this boundary (Fig. 5B). This sharp boundary remains in place in the neuroepithelium at the level of the hindbrain/spinal cord junction throughout subsequent development. In the mesenchyme, it is diluted somewhat by the commencement of RA synthesis by the cephalic mesoderm, although levels in the somites from somite 1 backwards remain higher (Fig. 5C).

The early brain neuroepithelium does not contain RA

No significant activation of the F9 cells occurred when forebrain, midbrain or hindbrain neuroepithelium was assayed at any stage. Yet the spinal part of the neural tube gives the highest activation level of all tissues. From this, we conclude that there is a sharp boundary in RA generation somewhere in the region of the hindbrain/spinal cord junction. We also observed this striking phenomenon in our HPLC studies of endogenous RA in mouse embryos (Horton and Maden, 1995).

These observations support the suggestion that the posterior neural tube produces a morphogen that diffuses anteriorly in the plane of the neuroepithelium into the presumptive hindbrain and is responsible for setting up the pattern of *Hox* gene expression in the hindbrain (Grapin-Botton et al., 1995, 1997). This hypothesis was arrived at following a series of neuroepithelium-grafting experiments: posterior transpositions of hindbrain tissue induce a change in *Hox* gene expression whereas anterior transpositions do not. Our data revealing a sharp boundary of RA synthesis at the level of somite 1 (equivalent to rhombomere 7) with very high levels of RA generated in the spinal cord and the absence of RA generation in the hindbrain neuroepithelium anterior to this boundary fits well with the concept that RA is this morphogen.

Subsequent work (Itasaki et al., 1996; Grapin-Botton et al., 1997) revealed that the same reprogramming of posterior segments of the hindbrain can be performed by grafting somites adjacent to the hindbrain neuroepithelium instead of transplanting the neuroepithelium itself. This also fits well with the data produced here since the somites are also a source of high levels of RA (Fig. 5C).

Patterning in the hindbrain neuroepithelium

Several sets of data from previously published experiments, including those described above, and the results reported here, can be brought together to suggest how the pattern of gene expression in the hindbrain may be set up. Firstly, somite grafting anteriorly (Itasaki et al., 1996) (Grapin-Botton et al., 1997) and rhombomere grafting posteriorly (Grapin-Botton et al., 1995) induce ectopic *Hox* gene expression in the hindbrain neuroepithelium; secondly, the somites and the spinal part of the neural tube contain very high levels of RA; thirdly administration of excess RA changes the *Hox* expression patterns in the hindbrain (Conlon and Rossant, 1992; Marshall et al., 1992; Gale et al., 1996); fourthly, the hindbrain neuroepithelium does not contain RA, but the mesoderm surrounding it contains intermediate levels. These observations together suggest that endogenous RA provided by the spinal part of the neural tube and diffusing into the hindbrain in a planar fashion or provided by the hindbrain mesoderm itself may be responsible for establishing the pattern of *Hox* gene expression in the hindbrain. In support of the former idea, that RA is provided by the spinal cord, our previous experiments on the development of the CNS in the absence of all retinoids have shown that the posterior hindbrain rhombomeres, numbers 4-7/8, do not develop at all and the anterior rhombomeres attach directly onto the spinal cord (Maden et al., 1996; E. G., unpublished data). In support of the latter idea, that the neuroepithelium is patterned by the surrounding mesenchyme, the work of La Mantia et al. (1993) on the development of the olfactory system in the mouse embryo provides a precedent. They have shown that the prosencephalic neuroepithelium does not contain any RA, whereas the mesoderm of the olfactory pit does. This mesenchyme acts as a source of RA to induce the differentiation of the olfactory neurons and to define a forebrain subdivision.

Other areas of the embryo that do not generate RA

Apart from the brain neuroepithelium and the area of the embryo anterior to the AP boundary established in the stage 4-8 embryos (Fig. 5A,B), only two other tissues in the early embryo do not contain any detectable RA and these are the notochord and the epidermis. The earlier observation that notochord grafts and ventral floor plate grafts could, when grafted to the anterior margin of the chick limb bud, induce limb duplications led to the suggestion that this behaviour was due to the presence of RA. Now we know that it is the presence of *shh* (Riddle et al., 1993) which is responsible, a conclusion reinforced by the observations here that the notochord does not contain RA.

Similarity to other embryos: boundaries and not gradients

Finally, it is worth considering how the results described here relate to the data from other embryos. In zebrafish, although

the transgenic studies suggest that RA does not appear until the 18- to 21-somite stage, which is surprisingly late (Marsh-Armstrong et al., 1995), the boundary of expression in the anterior trunk region bears considerable resemblance to the boundary identified in the chick embryo at the level of somite 1.

In *Xenopus*, gradients of retinoids have been the key feature with either a high point at the anterior end (Creech-Kraft et al., 1994a) or a high point at the posterior end (Chen et al., 1994). Our results here, which emphasise sharp on/off boundaries rather than gradients, bear little resemblance to these *Xenopus* studies nor can contribute to a resolution of the discrepancies in the *Xenopus* data. Instead they suggest a completely different concept for the organisation of the anteroposterior axis.

Indeed, our chick results are very similar to those obtained with RAR β lacZ transgenic mouse embryos. In the mouse, activation of the transgene first occurred in all three germ layers in the posterior half of the headfold-stage embryo with an anterior boundary at the node and no obvious AP gradient of staining (Rossant et al., 1991). This is precisely what we obtained here (Fig. 5A). As development proceeded, a sharp anterior boundary appeared at the level of the first somite, posterior to the hindbrain and otic vesicle, and posteriorly there was a decline in staining behind the last-formed somite (Colbert et al., 1993), again, the same picture as in the chick (Fig. 5B,C). A similar anterior boundary and then posterior decline was seen when whole mouse embryos were laid on F9 reporter cells (Ang et al., 1996). From day 10.5 of mouse development onwards the spinal cord displays 'hot spots' of RA synthesis that correspond to the origins of the limb innervation (Colbert et al., 1993; McCaffery and Drager, 1994). Again, we see the same phenomenon in the chick embryo, not only in the neural tube, but also in the somites.

Thus the above data demonstrate that the chick and the mouse embryo behave similarly with regard to the distribution of endogenous RA during embryogenesis. Our task is now to identify and localise the enzymes that are responsible for its generation. Certain alcohol and aldehyde dehydrogenases are surely involved, as ADH-IV, ALDH-I and RALDH-2 begin to be expressed during gastrulation in the mouse embryo from the level of the node posteriorly (Ang and Dueter, 1997; Niederreither et al., 1997). Also involved will be the enzymes that metabolise RA such as CYP26. It will be of great interest to examine the expression of these and other enzymes in the chick embryo to relate their expression patterns to the endogenous variations in RA levels as described here.

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