

# Positional apoptosis during vertebrate CNS development in the absence of endogenous retinoids

Malcolm Maden<sup>1,\*</sup>, Anthony Graham<sup>2</sup>, Emily Gale<sup>1</sup>, Chris Rollinson<sup>3</sup> and Maija Zile<sup>4</sup>

<sup>1</sup>Developmental Biology Research Centre, King's College London, 26-29 Drury Lane, London WC2B 5RL, UK

<sup>2</sup>Department of Experimental Pathology, UMDS, Guy's Hospital, London Bridge, London SE1 9RT, UK

<sup>3</sup>Anatomy and Human Biology Group, King's College London, Strand, London WC2R 2LS, UK

<sup>4</sup>Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Michigan 48824, USA

\*Author for correspondence (e-mail: m.maden@kcl.ac.uk)

## SUMMARY

We have previously shown that quail embryos that develop in the absence of vitamin A have severe defects in their central nervous system. One defect is a completely missing posterior hindbrain. Here we have studied how this comes about by examining cell death using a wholemount technique. In these A- embryos we observe two narrow bands of ectopic apoptosis. One is in the mesenchyme in the region of the first somite and occurs at the 4-6 somite stage, before neural tube closure. The second band follows immediately afterwards and occurs in the neuroepithelium of the presumptive posterior hindbrain at the 6-8 somite stage. Electron microscopy shows that the dying neuroepithelial cells exhibit the characteristics of apoptosis. Rescuing the embryos by injecting retinol before gastrulation completely

prevents these apoptotic events. In an effort to identify some of the genes that may be involved in the apoptotic pathway we show that *Msx-2* is upregulated in the apoptotic neuroepithelium and thus may be involved, whereas *Bmp-4* is not altered and thus presumably not involved. Since these apoptotic events take place at the time of specification of axial identity and segmentation in the mesenchyme and neuroepithelium we conclude that these cells die because they are wrongly specified in terms of their rostrocaudal position, a novel phenomenon which we refer to as positional apoptosis.

Key words: apoptosis, retinoic acid, retinoids, nervous system development, hindbrain, *Msx-2*, *Bmp-4*, quail

## INTRODUCTION

Studies of apoptosis in vertebrates, both in the organism as a whole and in the developing nervous system have primarily concentrated on its function in the control of cell number. Thus the neurotrophic factors are target-derived survival factors which regulate the balance between cell proliferation and cell death in the nervous system (Raff et al., 1993). This process ensures that the number of neurons is precisely matched to the number of target cells they innervate and that neurons which project to inappropriate targets are eliminated because they fail to receive the relevant neurotrophic factor required for survival. Increasing or decreasing the amount of nerve growth factor available to neurons leads to a concomitant increase or decrease in cell death. Similarly in the adult, apoptosis is used to remove inactivated or dangerous cells such as self-reactive lymphocytes, cells infected with viruses or tumour cells and disturbances in this control of cell number leads to disease (Thompson, 1995). The realisation of the continuing balancing act which goes on in the adult between cell proliferation and apoptosis has led to the suggestion that all embryonic cells, not only those in the nervous system, require a supply of survival factors from their neighbours or from the extracellular matrix (Raff et al., 1993). The lack of such 'social controls' may explain why isolated cells in culture die.

We present here an example of what we consider to be a novel form of apoptosis which we have termed positional apoptosis. It occurs in the very early embryo, firstly in the mesoderm and immediately afterwards in the overlying neural tube in a narrow stripe of cells destined to form structures in the posterior hindbrain region of the developing head. The time at which this apoptosis occurs is precisely when the specification of axial identity commences and for this reason we suggest it is caused by the incorrect specification of cells in this region of the embryo at, or after gastrulation. These specification errors are apparently not corrected by regulation, but by the induction of apoptosis, thereby revealing an interaction between the genetic pathway specifying position and that specifying apoptosis.

This positional apoptosis is induced because the embryos concerned have developed in the absence of endogenous retinoids (vitamin A derivatives). Removal of vitamin A from the diet of adults which are subsequently mated leads to the development of embryos under conditions of hypovitaminosis A and this is most readily accomplished in avians (Thompson et al., 1969). It has previously been shown that chick and quail embryos which develop from adults fed on such a diet have a single distended heart tube, situs inversus and the omphalomesenteric veins fail to connect the embryonic heart to the extraembryonic blood islands (Thompson et al., 1969; Heine et al., 1985; Dersch and Zile,

1993). As a result, these embryos, herein referred to as A-embryos, die at about stage 17/18 (after approximately 3 days of development). Most importantly for the work described here, the posterior part of the hindbrain known as the myelencephalon, also fails to develop (Maden et al., 1996).

The normal chick and quail hindbrain is composed of a series of seven segments known as rhombomeres (Vaage, 1969; Lumsden, 1990) which are lineage restricted units resembling insect compartments. Instead of the full complement of rhombomeres, the A-embryo only has 3 rhombomeres. We have identified the missing rhombomeres as those posterior to r3 on the basis of the expression domains of various segmentally expressed genes (Maden et al., 1996). This is clearly a valuable system with which to identify the role of retinoids in CNS development and the genes involved in patterning the CNS, but in order to accomplish this we first needed to know precisely how this hindbrain phenotype came about. We thus undertook a careful analysis of cell death in A-embryos using the TUNEL technique. Normal and A-embryos were examined for apoptotic cells in wholemounts to obtain spatial and temporal information on cell death. In addition, two genes thought to be in the pathway specifying cell death in the chick nervous system, *Msx-2* and bone morphogenetic protein 4 (*Bmp-4*) (Graham et al., 1994), were examined for their involvement in this positional apoptosis.

## MATERIALS AND METHODS

Eggs from Japanese quail (*Coturnix coturnix japonica*) were obtained from the Poultry Research Farm at Michigan State University. Birds were fed a normal or a vitamin A-deficient diet as described previously (Dersch and Zile, 1993). Eggs were collected daily, incubated until the desired stage and fixed in 4% paraformaldehyde. In situ hybridisation was performed according to established protocols. Wholemounts were cleared in 80% glycerol for photography and then sectioned on a vibratome at a thickness of 80  $\mu$ m.

The terminal transferase mediated dUTP-biotin nick end labelling (TUNEL) technique was a modification of a method developed in *Drosophila* (White et al., 1994). Embryos were fixed in 4% paraformaldehyde, washed in PBS/1% Triton X-100 (PBX) and the endogenous peroxidase inactivated by incubation in 0.1% hydrogen peroxide overnight at 4°C. The embryos were again washed in PBX followed by a wash in 1 $\times$  terminal transferase buffer/2.5 mM CoCl<sub>2</sub> in PBX. They were incubated at 37°C in the same buffer but including 0.5 units of terminal transferase per  $\mu$ l and 10  $\mu$ M dUTP (2:1 dUTP:dUTP-biotin) for 3 hours. The embryos were washed in PBX and incubated overnight with rocking with streptavidin-HRP diluted 1:300 in PBX. Finally they were washed in PBX and then stained with diaminobenzidine.

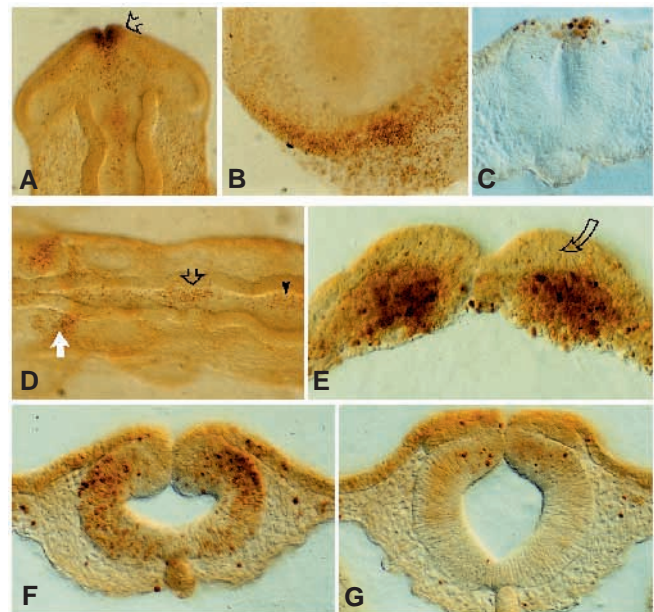
To perform rescues on A-embryos, retinol was dissolved in ethanol at its maximum solubility and then mixed with a solution of Tyrode's solution : A-egg extract 20:1. Each A-egg was injected prior to gastrulation with a volume of this mixture into the albumen so that 2  $\mu$ g retinol was delivered.

For electron microscopy, embryos were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer with 5% sucrose. After two rinses in this buffer the embryos were fixed for 1 hour in 1% osmium tetroxide, rinsed in distilled water three times and stained en bloc with 2% aqueous uranyl acetate. They were dehydrated in an ascending series of ethanols ending with propylene oxide and embedded in Agar 100 (Epon 812 substitute). Semithin sections were cut and stained with toluidine blue. Ultrathin sections were then cut from the selected area, mounted on grids and double stained with aqueous uranyl acetate (5 minutes) and Reynold's lead citrate (3 minutes). The tissue was examined using a Joel 200CX transmission electron microscope.

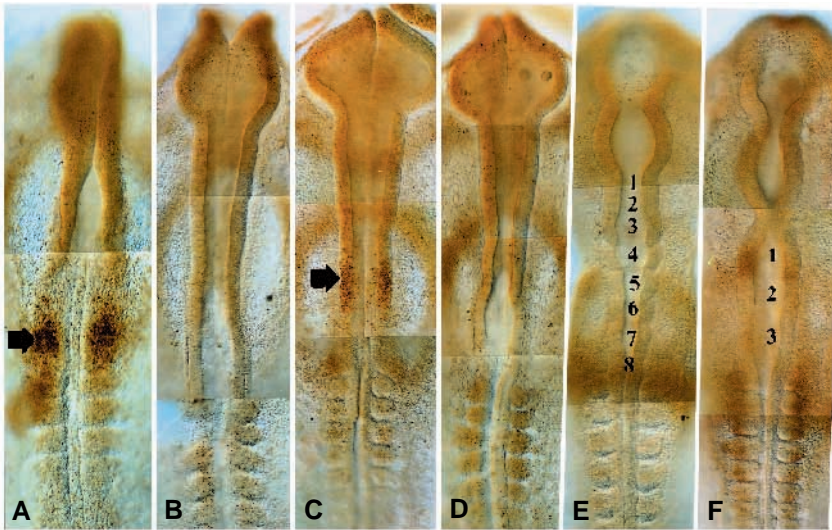
## RESULTS

### Apoptosis in normal quail embryonic development

Normal quail embryos were examined for the presence of apoptotic cells in wholemounts using the TUNEL technique from neural plate stages to stage 14. This technique is invaluable for providing spatial and temporal information. Prior to the 3-4 somite stage apoptosis was only observed in an occasional random cell, but between the 3-4 somite and 10 somite stages apoptosis appeared in three discrete regions of the embryo. The first region was the anterior neuropore (Fig 1A), the second region was the cells of the neural tube which had just completed closure (Fig. 1C) and were located as a group at a constant distance from the last formed somite, the third region was a crescent at the very posterior end of the embryo (Fig. 1B). Sections through the embryo demonstrated that there was very little cell death in the developing neuroepithelium apart from that group of cells which had just completed neurulation (Fig. 1C). After the 10 somite stage, from 11-15 somites, then a new area of apoptosis appeared in the neural crest. This area was centred



**Fig. 1.** TUNEL wholemounts of normal and A- quail embryos. Brown cells are apoptotic cells. (A) normal stage 10 embryo showing extensive cell death in the anterior neuropore (arrow). (B) The posterior end of the embryo in A showing extensive cell death in a crescent. (C) Section through the mid-trunk region of the embryo in A showing a few apoptotic cells in the dorsal midline region which appear soon after neural tube closure. The rest of the neural tube is composed of healthy cells. (D) Stage 12 embryo showing new regions of cell death in the neural crest over the midbrain (arrowhead), the neural crest over rhombomere 3 (open arrow) and in the neural crest from rhombomeres 7 and 8 (white arrow). (E) Section through the band of mesodermal cell death in a 4 somite A- embryo shown in Fig. 2A. The apoptosis is extensive and exclusive to the mesoderm, being absent from the overlying neuroepithelium (arrow). (F) Section through the band of neuroepithelial cell death in a 7 somite A- embryo shown in Fig. 2C. In contrast to E, the apoptosis is now exclusive to the neuroepithelium which by now has completed closure. (G) Same embryo as in F, but two sections (160  $\mu$ m) further rostral showing that here there is no abnormal level of cell death.



**Fig. 2.** TUNEL wholemounts of normal and A- quail embryos. Brown cells are apoptotic cells. (A) 4 somite A- embryo showing a band of apoptosis in the mesoderm (arrow) (see Fig. 1e for section). (B) 7 somite control embryo showing very little apoptosis. (C) 7 somite A- embryo showing a band of apoptosis in the neuroepithelium (arrow) in the region of the future hindbrain (see Fig. 1F for section). (D) 7 somite A- embryo which had been rescued by injecting retinol into the egg prior to incubation. The neuroepithelial cell death is prevented. (E) Normal stage 11 embryo showing the characteristic series of rhombomeres in the hindbrain (numbers 1-8) and very little apoptosis. (F), A- stage 11 embryo showing that the apoptosis has subsided, but now the morphology of the hindbrain is visibly changed as only 3 rhombomeres are visible (numbers 1-3).

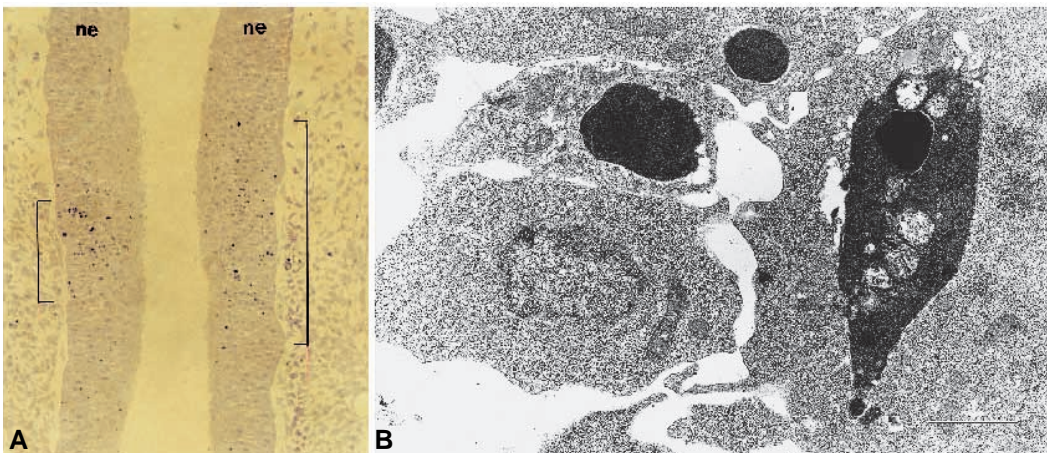
over rhombomere 3, but could also be seen over the midbrain, over rhombomere 5 and in the stream of crest from rhombomeres 7 and 8 (Fig. 1D). Apoptosis of neural crest in the chick hindbrain has been previously documented using vital dyes (Jeffs et al., 1992) and is, in part, concerned with the sculpting of individual streams of neural crest from rhombomeres 2, 4 and 6 by the induction of apoptosis in neural crest from rhombomeres 3 and 5 (Graham et al., 1993). Finally, in later stage embryos apoptosis is seen at a low level in developing organs such as the eye, otic vesicle and heart (data not shown) and Homma et al. (1994) have described apoptosis in the dorsal and ventral regions of the chick neural tube which peak at stage 17 and 18.

### Apoptosis in A- quail embryos

A- quail embryos showed an identical distribution of apoptotic cells up to stage 11 except for two temporally brief and spatially discrete regions. The first wave of apoptosis took place before the closure of the neural tube between the 4 and 6 somite stage (a period of less than 2 hours) and was localised to a band of cells in the region of the first somite (Fig. 2A). Sections through this band demonstrated that the apoptotic cells were present throughout the thickness of the mesoderm, but absent from the overlying open neuroepithelium (Fig. 1E). The width of this band of cells in the rostrocaudal dimension

was approximately 100  $\mu\text{m}$ . The second wave of apoptosis followed immediately after the first wave, occurring between the 6 and 8 somite stage, again a period of less than 2 hours. This band of cell death was in the same position in the rostrocaudal axis as the first band, but now occurred in the neuroepithelium and not in the mesoderm (Fig. 2C). This strip of neuroepithelium was in the region of the future hindbrain (Fig. 2C) where in normal embryos no such death occurred (Fig. 2B). Sections of these 6 somite A- embryos confirmed that the apoptosis occurred within the neuroepithelium and not the mesoderm (Fig. 1F) and that it was a very discrete stripe because a section 160  $\mu\text{m}$  rostral or caudal to the stripe gave the appearance of a normal, healthy neuroepithelium (Fig. 1G). The width of this band of cells in the rostrocaudal dimension was approximately 150-200  $\mu\text{m}$ .

In stage 9/10 (9/10 somites) A- embryos, apoptosis had returned to normal, leaving no trace of these bands of cell death except that by now the morphology of the hindbrain region had begun to change. The result was clearly apparent at stage 11. In normal embryos of this stage the characteristic bulges of the rhombomeres are readily visible (Fig. 2E) whereas in A- embryos only 3 rhombomeres have developed (Fig. 2F) and the first somite is further rostral relative to the otic vesicle, as we have previously described (Maden et al., 1996).



**Fig. 3.** (A) Horizontal semithin section through an A- embryo stained with toluidine blue and examined under the light microscope. Within the neuroepithelium (ne) there is a region of apoptotic cells both on the left and the right which are bracketed. (B) Electron micrograph of cells in the apoptotic region of the neuroepithelium showing heterochromatin condensation and cells which contain large fragments of nuclear and cytoplasmic debris.

### Histology of the region of apoptosis

To confirm that the cells labelled by the TUNEL technique bore the classical histological hallmarks of apoptotic cells an electron microscopic study was performed on 6-8 somite embryos. Semithin sections stained with toluidine blue clearly revealed a band of cell death in the neuroepithelium which was surrounded by healthy cells rostrally, caudally and laterally in the mesenchyme (Fig. 3A). Electron microscopic observations (Fig. 3B) revealed many of the characteristic changes associated with apoptotic cell death including heterochromatin condensation and progressive degeneration of residual nuclear and cytoplasmic structures. Cells adjacent to apoptotic cells often contained nuclear and cytoplasmic debris (apoptotic fragments) which they ingest by phagocytosis for destruction.

### Prevention of apoptosis in rescued embryos

It was crucial to demonstrate that these highly localised areas of apoptosis were directly caused by the absence of retinoids. It is certainly true that in the A- embryos no retinoids are detectable by a variety of techniques including high pressure liquid chromatography (Dong and Zile, 1995; Chen et al., 1995; Twal et al., 1995), but it is conceivable that such a deficit could have consequences which only secondarily affect apoptosis in the mesoderm and CNS. To test this we examined the effect of rescuing A- embryos by injecting retinol (2 µg) into the eggs prior to gastrulation. This single procedure resulted in the complete rescue of A- embryos so that they became indistinguishable from normal embryos when examined for apoptosis at the relevant stages (Fig. 2D). These embryos go on to produce a normal hindbrain and are also fully rescued in other respects such as cardiovascular development, thus a single dose of retinol can restore these deficient embryos to normality and correct the anteroposterior pattern defect in the neuroepithelium. This is a crucial demonstration of the precision of the defect in these A- embryos.

### Is *Msx-2* involved?

In an effort to understand which genes may be involved in this apoptotic pathway we firstly examined the expression of *Msx-2*, as this gene has previously been shown to be involved in cell death both in mesenchymal cells in the interdigital region of the limb (Ganan et al., 1996) and in neural crest cells (Graham et al., 1994). *Msx-2* expression in normal quail embryos up to stage 10 was observed in exactly the same locations as apoptotic cells (Fig. 1A-D), namely the anterior neuropore (Fig. 4A – compare with 1A), in a crescent at the very posterior end of the embryo (Fig. 4B – compare with 1B), in cells which had recently completed neurulation and in the neural crest over rhombomere 3 (Fig. 4C). Thus there is an absolute correlation between morphogenetic cell death and *Msx-2* expression in normal embryos. Sections of stage 10 embryos confirmed that *Msx-2* was expressed in the region where neural crest would be expected and not in the neuroepithelium of r3 (Fig. 4D). After stage 10, as the area of apoptosis in the hindbrain expanded (Fig. 1D), so did the area of *Msx-2* expression (Fig. 4E).

In A- embryos, *Msx-2* expression was strongly up-regulated, but only for a short period of time which coincided with the second period of apoptosis, that in the neuroepithelium. In 6-8 somite A- embryos *Msx-2* up-regulation occurred in the hindbrain neural crest, the neuroepithelium of a discrete segment of the presumptive hindbrain and the ectoderm of the

head (Fig. 4F). It was not expressed in the mesenchyme. The coincidence of *Msx-2* up-regulation in the neuroepithelium which is undergoing apoptosis (cf. Figs 1G and 4F) suggests a role for this gene in neural apoptosis. However, the correlation was certainly not perfect because *Msx-2* was also up-regulated in the cranial ectoderm where no abnormal apoptosis occurred. In contrast, *Msx-2* expression was not induced in the paraxial mesoderm prior to the 6 somite stage, during the period of mesodermal apoptosis. This suggests that a different gene pathway is involved in paraxial mesoderm apoptosis.

To demonstrate that the alteration in *Msx-2* expression was a specific effect of retinoid deprivation, A- embryos which had been rescued with a single injection of retinol prior to gastrulation were examined. These embryos showed normal *Msx-2* expression at the 6-8 somite stage (Fig. 4G), consistent with the suggestion that *Msx-2* is involved in the apoptotic pathway in the developing nervous system.

### Is *Bmp-4* involved?

A second gene thought to be involved both in mesenchymal (Ganan et al., 1996; Yokouchi et al., 1996) and in neural apoptosis is *Bmp-4* (Graham et al., 1994). This is a secreted factor which acts on neighbouring cells to induce apoptosis and we asked whether this gene was up-regulated during these waves of apoptosis in A- embryos. In normal quail embryos up to stage 10 the sites of *Bmp-4* expression are in the open neural tube (Fig. 4H), in a plume streaming from the tail bud (but not in a crescent as *Msx-2* is in Fig. 4B) and at stages 9 and 10 in the neural crest over the anterior hindbrain (not shown, but see Graham et al., 1994). In A- embryos the expression of *Bmp-4* was identical to normal embryos (Fig. 4I) and thus in contrast to *Msx-2*, this gene appears not to be in the pathway of apoptosis triggered by insufficient retinoids.

## DISCUSSION

We have shown here that a form of apoptosis which we call positional apoptosis occurs under certain circumstances as a way of ridding the embryo of cells which we presume have been incorrectly specified in the rostrocaudal axis. The circumstance which induces this phenomenon is the absence of vitamin A (retinoids). When quail embryos develop in the absence of vitamin A we have shown that there are two stripes of apoptosis measuring 100-200 µm wide which occur very early in embryonic development. One is within the mesoderm before neural tube closure at the 4-6 somite stage, and a second one immediately afterwards in the overlying neuroepithelium. This results in the loss of a segment of tissue which in the mesoderm results in the rostral shift of the somites and in the neuroepithelium results in the loss of the posterior rhombomeres, the tissue fated to become the myelencephalon. The administration of retinol in the form of a single dose prior to gastrulation, results in the complete rescue of this A- phenotype and these bands of apoptosis do not appear. The resulting embryos develop normally. Electron microscopic observations confirmed that these dying cells in the neuroepithelium exhibited many of the hallmarks of apoptosis – heterochromatin condensation, degeneration of cytoplasmic structures, heterophagocytosis (Clarke, 1990).

### A novel form of cell death?

Cell death in the developing embryo has long been recognised

as a prominent force in sculpting the limb bud and as a way of ridding the embryo of cells which have fulfilled their function in morphogenesis (Saunders, 1966). In the nervous system too, cell death is a widespread phenomenon being found in the motor columns of the spinal cord, the retina and in a variety of cranial ganglia and brain nuclei (Cowan et al., 1984). This phenomenon is caused, in the nervous system, by a limited supply of trophic factors. Its function is to adjust the magnitude of each neuronal population to the size or functional needs of its projection field as well as eliminating nerves whose axons have grown to the wrong target area. Similarly, in the optic nerve the supply of oligodendrocytes is regulated by apoptosis so that there is a number of oligodendrocytes appropriate to the number and length of axons that need to be myelinated (Raff et al., 1993). More recently, widespread cell death in proliferating cells of the developing mouse cerebral cortex has been described (Blaschke et al., 1996; Voyvodic, 1996) and suggested to be a means of selecting cells which have the desired phenotype, as occurs in the developing thymus.

In all these cases, however, the cell death which occurs is randomly distributed throughout the neuronal population and shows no localisation within the field concerned. This is what one would expect under conditions of a limited supply of a trophic factor and the same randomly located cell death would occur in a dish of cultured cells under the same conditions. In contrast to this situation is the apoptosis we have described here which occurs in a distinct band of cells and eliminates a precisely localised population of mesenchymal and then neuronal cells, resulting in the absence of a segment of the hindbrain, the myelencephalon. We suggest that only the cells within this segment are sensitive to the absence of retinoids because of their position, not because of competition for a limited trophic factor. For this reason we refer to this as positional apoptosis.

Of the genes likely to be involved in these apoptotic events, we have only tentatively identified one, *Msx-2*, and this only in the neuroepithelial apoptosis. There is a complete overlap between areas of apoptosis in the normal embryo and *Msx-2* expression, reinforcing the idea that this gene plays a critical role in the execution of the morphogenetic cell death programme (Graham et al., 1994; Ganan et al., 1996) and *Msx-2* was up-regulated in the apoptotic cells of the presumptive hindbrain neuroepithelium of the A- embryo. However, *Msx-2* was also up-regulated in the ectoderm of the head (Fig. 3F) where no abnormal apoptosis was observed so some further tissue-specific factor must be responsible for selecting the apoptotic pathway in *Msx-2*-expressing cells in the neuroepithelium rather than the ectoderm. We also showed that *Msx-2* was not up-regulated in the mesoderm and so we know nothing about the pathway involved in mesodermal apoptosis.

*Bmp-4* is another gene thought to be involved in the cell death pathway in embryos (Graham et al., 1994; Ganan et al., 1996; Yokouchi et al., 1996), but we could detect no alteration in *Bmp-4* expression in A- embryos. We conclude that this gene is not involved in A- induced apoptosis.

### The sequence of mesodermal and neural specification

The sequence in which these apoptotic events take place coincides precisely with the sequence of specification events thought to occur in the embryo. Classical embryological studies, particularly in amphibians, have shown that mesodermal speci-

fication takes place before neural specification and more recent molecular studies have confirmed this by showing that the anterior borders of *Hox* gene expression are established in the mesoderm before they are established in the neuroepithelium (e.g. Sundin et al., 1990; Murphy and Hill, 1991; Sundin and Eichele, 1992). The mesodermal apoptosis described here occurred about 2 hours before the neuroepithelial apoptosis which is in accord with embryological expectations.

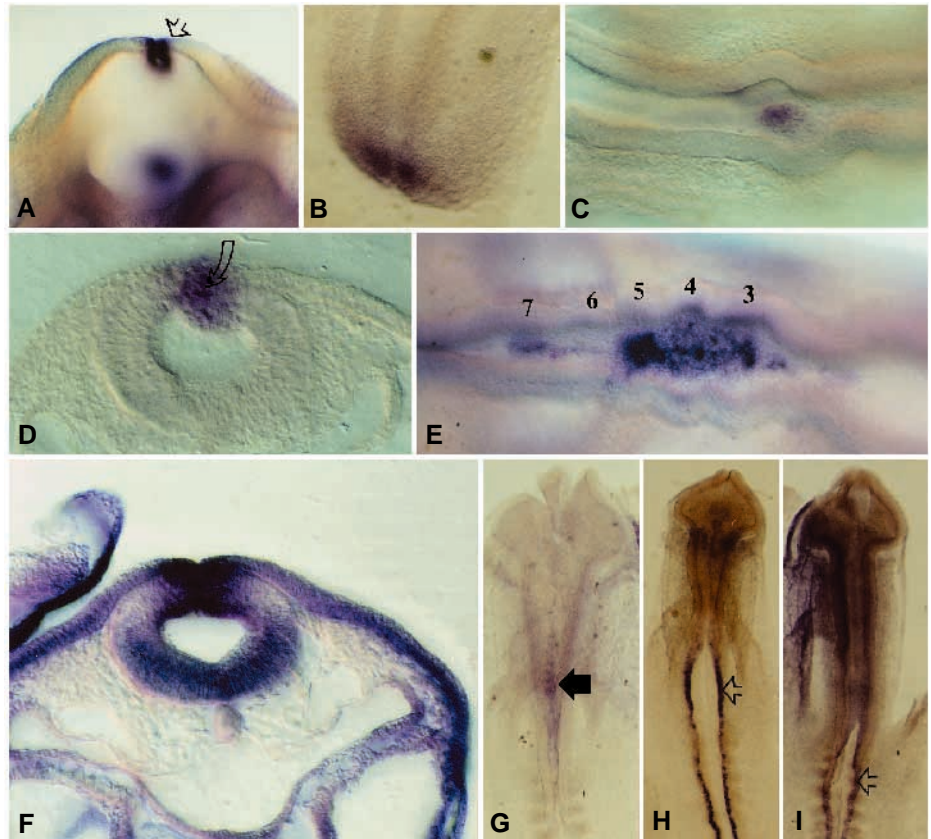
### Primary segmentation

The time at which these apoptotic events occurred coincides with the postulated time of primary segment specification. The band of neural apoptosis takes place exactly at the time of formation of the first visible segment in the hindbrain when the specification of axial identity is determined, namely, the 7 somite stage. This first morphologically visible border within the hindbrain appears at the rhombomere 3/4 border at the 7 somite stage (Vaage, 1969; Lumsden, 1990) and this establishes what one might define as the metencephalon/myelencephalon boundary.

Conlon (1995) has proposed that this primary boundary is established by RA. He suggests that there is a high concentration of RA from the posterior end of the embryo up to the region of this primary boundary. Conversely, anterior to the boundary there is no RA in the normal embryo. This situation is generated by RA synthesis from the node during gastrulation. The presence of RA posterior to the primary boundary establishes the anterior boundary of the expression of the class 1 *Hox* genes and prevents the expression of the *Otx* genes. Conversely, the absence of RA anterior to this boundary means there is no *Hox* gene expression, but allows the expression of the *Otx* genes which establish the forebrain and midbrain pattern. In support of this idea *Hoxb-1*, for example, is expressed up to precisely this primary boundary (the r3/4 border) (Sundin and Eichele, 1990) and this gene contains a retinoic acid response element which is required for appropriate expression (Marshall et al., 1994), and the same applies to the *Hoxa-1* gene (Dupe et al., 1997).

The effects of administration of excess RA also supports this idea. Excess RA leads to defects appearing anterior to the primary boundary, that is, an anterior shift of *Hox* expression domains (Morriss-Kay et al., 1991; Conlon and Rossant, 1992) and a repression of *Otx* and other forebrain genes (Simeone et al., 1995; Gammill and Sive, 1997), leading to either a deletion (Morriss, 1972; Durston et al., 1989; Simeone et al., 1995) or a respecification of anterior hindbrain tissue (Marshall et al., 1992; Gale et al., 1996). Conversely, the results described here where RA is absent should result in the opposite effect, namely a posterior shift in *Otx* and *Hox* expression patterns and defects posterior to the primary boundary. Of the *Hox* genes we have so far examined this is certainly true (Maden et al., 1996) and it will be interesting to look at the *Otx* genes in the future. The tissue defect, rather surprisingly, is not a disrupted posterior hindbrain, but a complete absence caused by precisely localised apoptosis. This is presumably equivalent to the absence of forebrain caused by excess RA levels (Durston et al., 1989; Simeone et al., 1995) and is reminiscent of the deletion of posterior rhombomeres seen in various loss-of-function studies such as those involving the *Hoxa-1* (Mark et al., 1993; Carpenter et al., 1993) and the *kreisler* (McKay et al., 1994) genes. Indeed, localised cell death is clearly apparent in the hindbrain of the *kreisler* mouse and we suggest that the patterns of apoptosis

**Fig. 4.** mRNA expression patterns of *Msx-2* (A-G) and *Bmp-4* genes (H,I) in quail embryos. (A) *Msx-2* expression in a 10 somite normal embryo showing intense localisation at the anterior neuropore (arrow). Compare with the same region in Fig. 1A showing extensive cell death. (B) *Msx-2* expression at the posterior end of the normal embryo in A. Compare with cell death in Fig. 1B. (C) *Msx-2* expression in the hindbrain of a 10 somite normal embryo showing expression only in the neural crest over rhombomere 3. (D) Section through the embryo in C showing that only the neural crest (arrow) and not the neuroepithelium expresses *Msx-2*. (E) More extensive expression of *Msx-2* in the neural crest over rhombomeres 3, 4, 5 and 7 in a stage 12 normal embryo. This expression domain has expanded from stage 10 (see C) as the region of cell death expands (see Fig. 1D). (F) Section through the posterior hindbrain level (see arrow in Fig. 2C) of a 7 somite A- embryo showing the up-regulation of *Msx-2* within a band of neuroepithelium where cells are undergoing apoptosis (see Fig. 2C). (G) *Msx-2* expression in a rescued A- 7 somite embryo showing that the excessively high levels of expression of this gene in the A- embryo (shown in F) have been returned to the normal levels (shown in C) such that the only expression domain is in the neural crest over the anterior hindbrain (arrow). (H) *Bmp-4* expression in a normal 7 somite embryo showing expression in the open neuroepithelium (arrow). The other site of expression at the posterior end of the embryo is not shown. Later embryos also show *Bmp-4* expression in the neural crest over the anterior hindbrain. (I) *Bmp-4* expression in a 7 somite A- embryo showing no change in the expression pattern as the only site of expression is in the open neural tube (arrow).



should be studied in all these cases involving targeted disruptions of the *Hox* genes. Thus we feel that the results presented here concerning the defects caused by the absence of RA fit very well with the primary boundary model of Conlon (1995).

### The size of the segmental fields

Since the size of the region of apoptosis in the neuroepithelium is 150-200µm and the result is the loss of the myelencephalon, this suggests that this may be the size of the myelencephalic field when it is established as a segment of the hindbrain. This seems a highly appropriate size both theoretically and compared to others which typically extend less than 50 cells (Wolpert, 1969).

### Vertical vs planar signalling in the neuroepithelium

It is interesting to consider whether these two apoptotic events in the mesoderm and then in the neuroepithelium are causally connected. Does the mesodermal cell death directly cause the neuroepithelial death? One scenario could be the following. The primary boundary (Conlon, 1995) is established in the mesoderm following gastrulation and in the absence of RA the incorrect specification leads to the instigation of the cell death programme in those cells at the primary boundary. If there is a very precise vertical signalling of pattern from the mesoderm to the overlying neuroepithelium, then the loss of a 'myelencephalon-inducing mesodermal' field would result in the absence of a specification

signal for the neuroepithelial myelencephalic field. The neuroepithelial cells which fail to receive a signal also instigate the cell death programme. This hypothesis lays the primary defect caused by the absence of vitamin A at the feet of mesoderm and assumes that the neuroepithelium is specified by vertical signalling from the mesoderm.

An alternative scenario in which planar signalling is involved in the specification of the neuroepithelium rather than vertical signalling implies that endogenous retinoids are required both during gastrulation in the mesoderm and during planar signalling within the neuroepithelium. In this case the mesodermal apoptosis would be caused by the failure of specification of the mesodermal primary boundary as above. The subsequent neuroepithelial apoptosis would be caused by a similar requirement for retinoids in planar signalling within the neuroepithelium and their absence results in the failure of specification of the corresponding neuroepithelial primary boundary. We clearly do not have enough embryological data to decide between these alternatives, but since excess retinoids can affect both mesodermal specification and neuroepithelial specification separately, at least in *Xenopus* (Ruiz i Altaba and Jessell, 1991a,b), perhaps the latter hypothesis is more appropriate.

In conclusion, this work demonstrates the value of this retinoid-deficient model as a unique system for identifying

which genes are involved in specification of the mesoderm and the neuroepithelium at the stage when these events are taking place in the embryo. We do not need to rely on deductions made from alterations in the anatomy of later embryos. By taking embryos just prior to the 4-6 and 6-8 somite stages we can ask which genes are mis-expressed and have incorrect anterior borders. *Hox* genes are obvious candidates for such a role, but by performing the appropriate cloning exercises we anticipate that the A- embryo will be an good model system to discover new genes involved in embryonic pattern specification.

This work was supported by the Wellcome Trust (M. M. and E. G.), the USDA (M. Z.) and the Michigan Agricultural Experiment Station at MSU.

## REFERENCES

- Blaschke, A. J., Staley, K. and Chun, J. (1996). Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. *Development* **122**, 1165-1174.
- Carpenter, E. M., Goddard, J. M., Chisaka, O., Manley, N. R. and Capecchi, M. R. (1993). Loss of Hox-A1 (Hox-1.6) function results in the reorganisation of the murine hindbrain. *Development* **118**, 1063-1075.
- Chen, Y.-P., Dong, D., Kostetskii, I. and Zile, M. H. (1995). Hensen's node from vitamin A-deficient quail embryo induces chick limb bud duplication and retains its normal asymmetric expression of *sonic hedgehog* (Shh). *Dev. Biol.* **173**, 256-264.
- Clarke, P. G. H. (1990). Developmental cell death: morphological diversity and multiple mechanisms. *Anat. Embryol.* **181**, 195-213.
- Conlon, R. A. (1995). Retinoic acid and pattern formation in vertebrates. *Trends Genet.* **11**, 314-319.
- Conlon, R. A. and Rossant, J. (1992). Exogenous retinoic acid rapidly induces anterior ectopic expression of murine Hox-2 genes in vivo. *Development* **116**, 357-368.
- Cowan, W. M., Fawcett, J. W., O'Leary, D. D. M. and Stanfield, B. B. (1984). Regressive events in neurogenesis. *Science* **225**, 1258-1265.
- Dersch, H. and Zile, M. H. (1993). Induction of normal cardiovascular development in the vitamin A-deprived quail embryo by natural retinoids. *Dev. Biol.* **160**, 424-433.
- Dong, D. and Zile, M. H. (1995). Endogenous retinoids in the early avian embryo. *Biochem. Biophys. Res. Comm.* **217**, 1026-1031.
- Dupe, V., Davenne, M., Brocard, J., Dolle, P., Mark, M., Dierich, A., Chambon, P. and Rijli, F. M. (1997). In vivo functional analysis of the *Hoxa-1* 3' retinoic acid response element (3' RARE). *Development* **124**, 399-410.
- Durston, A. J., Timmermans, J. P. M., Hage, W. J., Hendricks, H. F. J., de Vries, N. J., Heideveld, M. and Nieuwkoop, P. D. (1989). Retinoic acid causes an anteroposterior transformation in the developing nervous system. *Nature* **340**, 140-144.
- Gale, E., Prince, V., Lumsden, A., Clarke, J., Holder, N. and Maden, M. (1996). Late effects of retinoic acid on neural crest and aspects of rhombomere identity. *Development* **122**, 783-793.
- Gammill, L. S. and Sive, H. (1997). Identification of *otx2* target genes and restrictions in ectodermal competence during *Xenopus* cement gland formation. *Development* **124**, 471-481.
- Ganan, Y., Macias, D., Duterte-Coquillaud, M. Ros, M. A. and Hurle, J. M. (1996). Role of TGF $\beta$ s and BMPs as signals controlling the position of the digits and the areas of interdigital cell death in the developing chick limb autopod. *Development* **122**, 2349-2357.
- Graham, A., Heyman, I. and Lumsden, A. (1993). Even-numbered rhombomeres control the apoptotic elimination of neural crest cells from odd-numbered rhombomeres in the chick hindbrain. *Development* **119**, 233-245.
- Graham, A., Francis-West, P., Brickell, P. and Lumsden, A. (1994). The signalling molecule BMP4 mediates apoptosis in the rhombencephalic neural crest. *Nature* **372**, 684-686.
- Heine, U. I., Roberts, A. B., Munoz, N. S., Roche, N. S. and Sporn, M. B. (1985). Effects of retinoid deficiency on the development of the heart and vascular system of the quail embryo. *Virchows Arch. (Cell Pathol.)* **50**, 135-152.
- Homma, S., Yaginuma, H. and Oppenheim, R. W. (1994). Programmed cell death during the earliest stages of spinal cord development in the chick embryo: a possible means of early phenotypic selection. *J. Comp. Neurol.* **345**, 377-395.
- Jeffs, P., Jaques, K. and Osmond, M. (1992). Cell death in cranial neural crest development. *Anat. Embryol.* **185**, 583-588.
- Lumsden, A. (1990). The cellular basis of segmentation in the developing hindbrain. *Trends Neurosci.* **8**, 329-335.
- Maden, M., Gale, E., Kostetskii, I. and Zile, M. (1996). Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. *Curr. Biol.* **6**, 417-426.
- Mark, M., Lufkin, T., Vonesch, J. L., Ruberte, E., Olivo, J. C., Gorry, P., Lumsden, A. and Chambon, P. (1993). Two rhombomeres are altered in Hoxa-1 mutant mice. *Development* **119**, 319-338.
- Marshall, H., Nonchev, S., Sham, M. H., Muchamore, I., Lumsden, A. and Krumlauf, R. (1992). Retinoic acid alters hindbrain Hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity. *Nature* **360**, 737-741.
- Marshall, H., Studer, M., Popperl, H., Aparicio, S., Kuriowa, A., Brenner, S. and Krumlauf, R. (1994). A conserved retinoic acid response element required for early expression of the homeobox gene Hoxb-1. *Nature* **370**, 567-571.
- McKay, I. J., Muchamore, I., Krumlauf, R., Maden, M., Lumsden, A., Lewis, J. (1994). The *kreisler* mouse: a hindbrain segmentation mutant that lacks two rhombomeres. *Development* **120**, 2199-2211.
- Morriss, G. M. (1972). Morphogenesis of the malformations induced in rat embryos by maternal hypervitaminosis A. *J. Anat.* **113**, 241-250.
- Morriss-Kay, G. M., Murphy, P., Hill, R. E. and Davidson, D. R. (1991). Effects of retinoic acid excess on expression of Hox-2. 9 and Krox-20 and on morphological segmentation in the hindbrain of mouse embryos. *EMBO J.* **10**, 2985-2995.
- Murphy, P. and Hill, R. E. (1991). Expression of the mouse labial-like homeobox-containing genes, Hox 2. 9 and Hox 1. 6, during segmentation of the hindbrain. *Development* **111**, 61-74.
- Raff, M. C., Barres, B. A., Burne, J. F., Coles, H. S., Ishizaki, Y. and Jacobson, M. D. (1993). Programmed cell death and the control of cell survival: lessons from the nervous system. *Science* **262**, 695-700.
- Ruiz i Altaba, A. and Jessell, T. (1991a). Retinoic acid modifies mesodermal patterning in early *Xenopus* embryos. *Genes Dev.* **5**, 175-187.
- Ruiz i Altaba, A. and Jessell, T. (1991b). Retinoic acid modifies the pattern of cell differentiation in the central nervous system of neurula stage *Xenopus* embryos. *Development* **116**, 81-93.
- Saunders, J. W. (1966). Death in embryonic systems. *Science* **154**, 604-612.
- Simeone, A., Avantsaggiato, V., Moroni, M., Mavilio, F., Arra, C., Cotelli, F., Nigro, V. and Acampora, D. (1995). Retinoic acid induces stage-specific anteroposterior transformation of rostral central nervous system. *Mech. Dev.* **51**, 83-89.
- Sundin, O. H. and Eichele, G. (1990). A homeo domain protein reveals the metameric nature of the developing chick hindbrain. *Genes Dev.* **4**, 1267-1276.
- Sundin, O. and Eichele, G. (1992). An early marker of axial pattern in the chick embryo and its respecification by retinoic acid. *Development* **114**, 841-852.
- Sundin, O., Busse, H. G., Rogers, M. B., Gudas, L. J. and Eichele, G. (1990). Region-specific expression in early chick and mouse embryos of Ghox-lab and Hox 1. 6, vertebrate homeobox-containing genes related to *Drosophila* labial. *Development* **108**, 47-58.
- Thompson, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science* **267**, 1456-1462.
- Thompson, J. N., Howell, J. McC., Pitt, G. A. J. and McLaughlin, C. I. (1969). The biological activity of retinoic acid in the domestic fowl and the effects of vitamin A deficiency in the chick embryo. *Br. J. Nutr.* **23**, 471-490.
- Twal, W. O., Roze, L. and Zile, M. H. (1995). Anti-retinoic acid monoclonal antibody localizes all-trans-retinoic acid in target cells and blocks normal development in early quail embryo. *Dev. Biol.* **168**, 225-234.
- Vaage, S. (1969). The segmentation of the primitive neural tube in chick embryos (*Gallus domesticus*). *Adv. Anat. Embryol. Cell Biol.* **41**, 1-88.
- Voyvodic, J. T. (1996). Cell death in cortical development: how much? Why? So what? *Neuron* **16**, 693-696.
- White, K., Grether, M. E., Abrams, J. M., Young, L., Farell, K. and Steller, H. (1994). Genetic control of programmed cell death in *Drosophila*. *Science* **264**, 677-683.
- Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. *J. Theoret. Biol.* **25**, 1-47.
- Yokouchi, Y., Sakiyama, J., Kameda, T., Iba, H., Susuki, A., Ueno, N. and Kuriowa, A. (1996). BMP-2/-4 mediate programmed cell death in chicken limb buds. *Development* **122**, 3725-3734.