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Cell Lineage Restrictions in the Chick Embryo Hindbrain

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Cell lineage restrictions in the chick embryo hindbrain

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SUMMARY

During development of the chick embryo, early neuronal differentiation and axonogenesis in the hindbrain follow a segmented pattern in register with the segmented morphology of this region. Cell marking experiments have shown that the segments, or rhombomeres, are lineage-restriction units each constructing a defined piece of the hindbrain. This raises the interesting possibility that, as in the developing fly, metamerism is used to generate level-specific anatomical structures with great and reliable precision. In the hindbrain, as for many invertebrates, lineage ancestry may be important in the determination of cell fate. The segmentation seen in this body region could therefore reflect a similar condition once present in the ancestor common to vertebrates and invertebrates.

INTRODUCTION

To understand how the complex structure of the vertebrate brain develops it is necessary to identify the mechanisms that generate regional variations in the identity, arrangement and connectivity of nerve cells. One of the possible mechanisms involved, early subdivision of the neural epithelium by segmentation, would simplify a number of the developmental problems involved in the formation of regional patterns and the generation of different cells in their correct numbers, and positions. It would permit the construction of the ground plan of the central nervous system (CNS) which only later becomes built up and overridden by integrative aspects of development.

Morphological evidence of transient segmentation at the early stages of neural tube develop has long been recognized. Alternative swellings and constrictions along the neuraxis were first described in the last century by von Baer (1828), and were christened 'neuromeres' by Orr (1887). Despite many descriptive accounts of neuromeres in various species (McClure 1890; Gräper 1913; Neal 1918; Streeter 1933; Bergquist & Källén 1954; Vaage 1969; Kuhlenbeck 1973), however, their status has never been satisfactorily agreed. Some workers hold that neuromeres are either fixation artefacts or the products of mechanical interactions between the neural tube and adjacent mesoderm; others hold that neuromeres (particularly the rhombomeres in the hindbrain region) are evidence in favour of intrinsic neural segmentation, having perhaps arisen in adaptation to the adjacent branchial arch segmentation. Neal (1918) nevertheless argued that there is no consistent relationship between individual rhombomeres and specific cranial nerves, leading him to conclude that 'it would seem impossible to regard the rhombomeres as metameric structures.' The majority of early studies were concerned more with the phylogeny of head segmentation than with the ontogeny of the hindbrain; one reason for the lack of consensus would appear to be that insufficient attention has been paid to cellular and molecular patterns that might underlie the morphology.

A major question remains, therefore, as to whether or not the neuromeres are developmentally significant. If they are true segments, the following would be expected. First, that the overt neuromeric pattern should be produced by intrinsic patterns of cell proliferation and/or changes in cell shape. Second, that the pattern corresponds with an underlying segmental pattern of cellular and/or molecular differentiation. Third, that the cells of adjoining rhombomeres do not mix with each other, creating lineage restrictions and independent spatial domains in the continuous neuroepithelial sheet. Fourth, that regulatory genes should be expressed in patterns which relate in some way to the neuromeric pattern. Recent observations and experiments suggest that these expectations are met by the rhombomeres of the higher vertebrate hindbrain.

PATTERNS OF CELL PROLIFERATION

Immediately after the neural plate closes, the rhombencephalic neural tube of the chick embryo becomes constricted at evenly spaced positions along its length to define eight rhombomeres (figure 1). Each comprises an external (basal, pial) ballooning of the epithelium and is contiguous with its neighbouring rhombomere at an internal (apical, ventricular) transverse boundary ridge. Rhombomeres are first visible at Hamburger and Hamilton stage nine and persist to stage 24 (Vaage 1969).

Cells are already organized in units that correspond to the future segments at the time these constrictions appear. In a study of mitotic patterning in the chick embryo CNS by using colchicine, Källén (1962) showed that mitoses accumulate maximally around the centres of future rhombomeres and that the boundaries

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are presaged by mitotic minima. Although a later study (Tuckett *et al.* 1985) found no such segmental patterns, we have recently confirmed Källén's findings in untreated embryos (Guthrie *et al.*, in preparation); the centres of rhombomeres have a higher mitotic density and shorter cell-cycle time than the boundaries.

The thymidine analogue, bromodeoxyuridine (BrdU, detectable by anti-BrdU antibodies), has been administered to visualize the positions of S-phase nuclei in the neuroepithelium during the process of rhombomere formation (Guthrie *et al.* in preparation). The pattern of BrdU uptake by hindbrain cells indicates that radial interkinetic nuclear migration between ventricular and pial surfaces, which is typical of this epithelium (Sauer 1935), is normal within rhombomeres but reduced or absent at their boundaries. At the time these form, neuroepithelial cell bodies remain close to the ventricular surface throughout the cell cycle.

The periodic constrictions which transform the more or less cylindrical neural tube into a segmented one could involve one or both of two morphogenetic processes. Reduced cell division at the presumptive rhombomere boundaries would exert a choke on the expanding diameter of the tube. The accumulation of cell bodies on the ventricular border at these regions would further accentuate their inflection towards the lumen by the effect of cell wedging.

SEGMENTAL PATTERNS OF NEURONAL DEVELOPMENT

A recent study (Lumsden & Keynes 1989) has revealed that from the time the first neurons differentiate (stages 11 and 12), the boundaries between rhombomeres become colonized by transversely oriented axons which grow through preformed intercellular channels on the pial side of the epithelium, directly beneath the ridges on the ventricular surface. The impaired or impeded interkinetic nuclear migration of boundary cells may contribute to the formation of these wide intercellular spaces in the future marginal zone; as cell bodies accumulate at the ventricular side of the epithelium so the pial side becomes progressively devoid of cell bodies. The tendrilous neuroepithelial cell end feet which extend across the channels to the basal surface are also associated with axon growth-promoting matrix glycoprotein, laminin.

Neurogenesis of both the reticular formation and the branchial motor system conforms to the rhombomeric pattern (Lumsden & Keynes 1989); the consecutive cranial nerves (V, VII and IX) derive from a specific, consecutive pairing of rhombomeres, each pair lying in register with an adjacent branchial arch (figure 1). Thus the fifth nerve derives from rhombomeres 2 and 3 (r2 and 3), opposite the 1st arch; the seventh from r4 and 5, opposite the 2nd arch; and the ninth from r6 and 7, opposite the 3rd arch. The temporal pattern of early neurogenesis thus involves a superimposed two-segment periodicity; the rostral member of each rhombomere pair differentiating before the caudal member, and containing the motor nerve root.

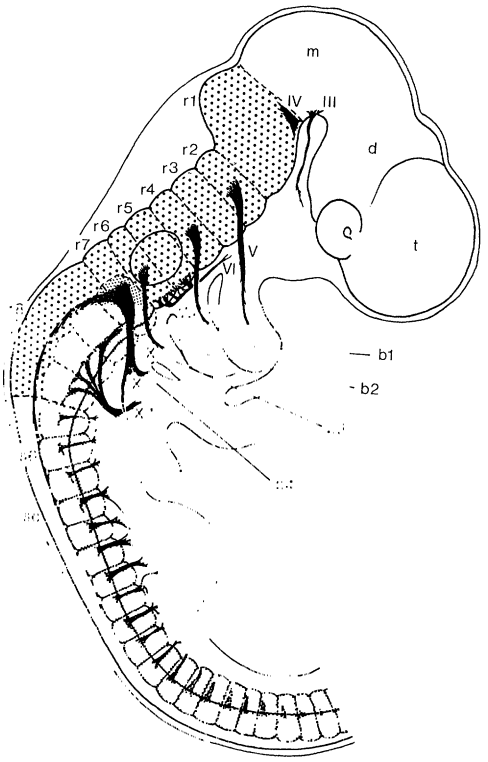

Figure 1. Two segmented systems are shown in a three-day chick embryo: the paraxial mesoderm is subdivided into somites (fine stipple; s6, somite number six) lying alongside the spinal cord (sc), and the rhombencephalon (coarse stipple) is subdivided into rhombomeres (r1–r8). The principal early divisions of the brain (t, telencephalon; d, diencephalon; m, mesencephalon; r, rhombencephalon) may also represent a meristic series. The cranial motor nerves (III–XII) are shown but not the cranial ganglia. The exit points of branchial motor nerves (V, VII, IX) are in alternate rhombomeres, in register with the first three branchial arches (b1–b3).

Whereas the Vth, VIIth and IXth nerve nuclei occupy serially adjacent position along the rostrocaudal axis, later forming a continuous column of branchial and visceral efferent cell bodies, the nuclei of the somatic motor system originate and remain in a discontinuous cell column. The generation of these cells conforms, nevertheless, to a segmental pattern. The neurons of the IVth nerve lie in r1; those of the VIth nerve arise *en bloc* between the r4/5 and the r6/7 boundaries, occupying two rhombomeres which are out of phase with the adjacent branchial motor nuclei (VII and IX). Finally, the XIIth nerve nucleus lies in the region of the medulla adjacent to the occipital somites O2–O4 (r8).

CELL LINEAGE RESTRICTION

The relative stasis of diving cells, together with a local increase in cell–cell adhesion (Lumsden & Keynes 1989), suggests that the boundaries constitute relatively immobile populations which could form barriers to the translocation of cells along the A–P plane, from one rhombomere to the next. The possibility that rhombomeres might be domains of lineage restriction has

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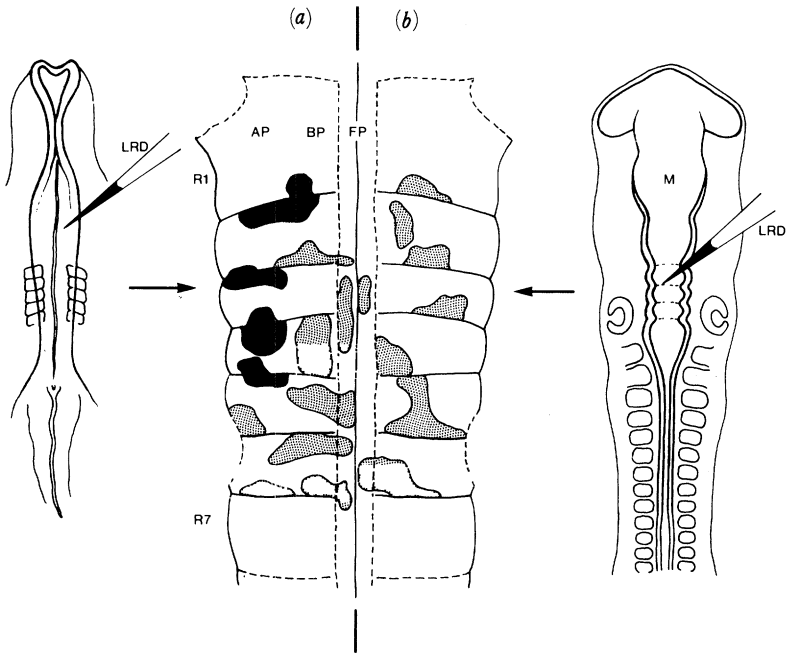

Figure 2. The positions of labelled cell clones in relation to boundaries. Single neuroepithelial cells were filled with lysinated rhodamine dextran (LRD) and the labelled progeny mapped after 2 days further incubation. The single resulting clones are shown superimposed on a line drawing of a stage 19 hindbrain represented as a flat-mount. Early-marked clones ((*a*), left) sometimes spread into two rhombomeres, spanning the boundary between, whereas others were confined at the boundaries. Clones marked after the formation of rhombomere boundaries ((*b*), right) were always confined to one side of a boundary. AP, alar plate; BP, basal plate; FP, floor plate; M, midbrain; R1, R8, rhombomeres 1 and 8. From Fraser *et al.* (1990) with permission of Macmillan Magazines Ltd.

been tested recently by cell marking experiments in the chick hindbrain (Fraser *et al.* 1990). By intracellular marking with fluorescent dextrans *in ovo* we could identify the descendants of single neuroepithelial cells for up to six generations. When a cell was marked early, before the appearance of rhombomere boundaries, its labelled descendants were sometimes dispersed into two rhombomeres, the clone appearing to have crossed the intervening boundary. When a cell was marked later, after boundary appearance, its clone was still dispersed widely within the rhombomere of origin but was now confined within its boundaries. When clones abut boundaries they elongate transversely, acquiring a D-shaped outline (figure 2).

We conclude that once they become defined, rhombomeres are polyclonal lineage restriction units. The cells within each unit are designated to form only that precise part of the hindbrain by the absence of mixing with the cells of adjoining rhombomeres. The time of allocation of the polyclonal founder cells appears to coincide with the formation of visible boundaries between the units. The descendants of cells marked before this event may end up in two rhombomeres, the clone becoming bisected by the formation of a boundary. After the founder cells are allocated, they and their descendants are confined to one side of the boundary or the other. The rhombomeres therefore share characteristics with the compartments of insects (Garcia-Bellido *et al.* 1973; Lawrence 1989).

What cellular mechanism restricts lineages at rhombomere boundaries? Perhaps the simplest way would be a mechanical barrier that prevents cells from moving across it (figure 3). This concept is exemplified by the wing imaginal disc of *Drosophila*, where dorsal and ventral compartments are partitioned by a zone of non-dividing cells (O'Brochta & Bryant 1985). Perhaps the border zone of static cells at rhombomere boundaries could partition the hindbrain in a similar way? A further indication that a mechanical restriction could exist is seen in the zebrafish hindbrain where a zone of cells equivalent in extent to that observed in the chick is sharply defined by a 'glial curtain' (Trevarrow *et al.* 1990). Alternatively, the barrier to cell movement at boundaries may be other than a mechanical one furnished by the border cells. First, we observed that labelled clones consistently spread up to the centre lines of boundaries and were not restricted at the interface between the rhombomere centres and the border cells (Fraser *et al.* 1990); clones in neighbouring rhombomeres would, therefore, meet each other directly and would not be separated by intervening cells. Second, we have extirpated the border cells at rhombomere boundaries and then observed the behaviour of clones descended from cells marked at the edges of the gap; despite rapid replacement of the missing tissue, none of the labelled clones crossed into the adjoining rhombomere (S. E. Fraser, R. Keynes and A. Lumsden, unpublished results). Since the boundary reforms without any cell mixing between neighbouring rhombomeres, the restriction would seem to be independent of a unique set of border cells. It is therefore possible that cells of a single rhombomere share some specific surface property that renders them immiscible with those of adjoining rhombomeres (Figure 3), as is presumed to be the case at the

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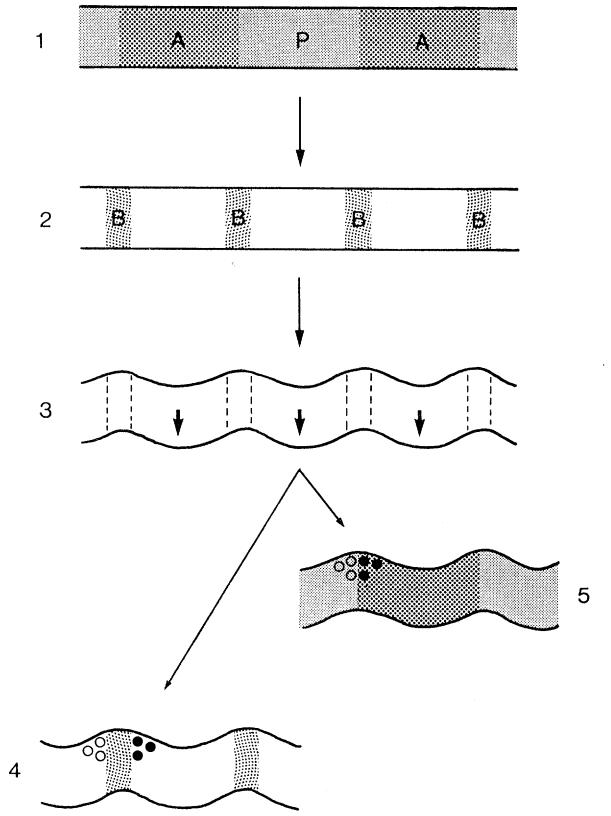

Figure 3. A scheme for two possible alternative bases for cell lineage restriction in the developing hindbrain. A series of diagrams shows the neuroepithelium in longitudinal section, ventricular to the top. (1) At some time just before boundary formation, cells are allocated in groups along the rostrocaudal axis of the neural tube. These could possess alternate cell states, A and P. *Krox-20*, for example, could be involved in specifying one of these states. (2) Cells bordering (B) the interface between A and P become reduced in their capacity for cell division. (3) Morphogenesis of the rhombomeres is related to the differential rates of division, the more rapidly proliferating regions increasing the diameter of the neural tube (short arrows) thereby producing bulges. (4) Border cells become specialized later in development; they could create a restriction to cell movement, simply by presenting a mechanical barrier between neighbouring rhombomeres, in which case A cells (dots) and P cells (circles) would not necessarily differ in their adhesion or miscibility. (5) Alternatively, the border cells are not directly responsible for the impediment to cell movement, the restriction being created by the immiscibility of A and P cells. In this case, the restriction would occur at a line in the middle of the boundary and not, as in (4), at the interfaces between the central regions and the border cells.

boundary between the anterior and posterior compartments of the fly wing (Morata & Lawrence 1975). As yet nothing is known about the relative adhesion of cells from individual rhombomeres, nor have any cell-surface markers been found which distinguish one rhombomere from another. We have, however, observed the alternate segmental expression of the HNK-1 epitope; in r3 and r5, HNK-1 antibody stains the entire radial thickness of the neuroepithelium, whereas in r2 and r4 antibody staining is restricted to young motor neurons (Figure 4. Guthrie *et al.* in preparation). HNK-1 recognizes a sulphated carbohydrate epitope

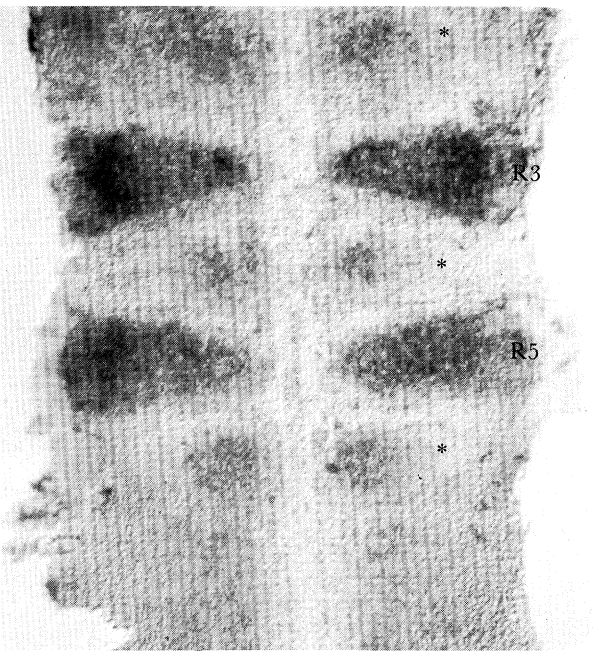

Figure 4. A wholemount hindbrain from a stage 15 chick embryo stained by the indirect immunoperoxidase method with HNK-1 antibody. Rhombomeres 3 and 5 contain HNK-1+ neuroepithelial cells, whereas rhombomeres 2, 4 and 6 contain small groups of HNK-1+ branchial motor neurones, close to the midline (asterisks).

(L2) on various adhesion glycoproteins (including NCAM and L1) which may itself be involved with adhesion (Keilhauer *et al.* 1985).

SEGMENTAL PATTERNS OF GENE EXPRESSION

Compartments in *Drosophila* each contain all the surviving descendants of a small set of founder cells whose development is directed by specific regulatory genes (Garcia-Bellido 1975). In defining a compartment, the realm of action of regulatory or selector genes has become as important as cell lineage restriction (Lawrence 1990). The two-segment repeat pattern of both carbohydrate structure expression and neuronal differentiation suggests the action of classes of genes similar to the 'pair rule' genes described for segmentation in *Drosophila* (Ingham 1988). During cellularization of the blastoderm, genes of the pair-rule class are transiently expressed in an alternate series of seven stripes, domains which define the initial segmentation of the anterio-posterior axis of the embryo. Specific combinations of pair-rule genes also determine the subsequent elaboration of pattern in each of these parasegments. Position-specific regulation of the pair-rule genes depends on *gap* gene domains established by diffusion of transcripts in the syncytial blastoderm.

Regulatory genes with striped expression patterns in the hindbrain, in register with the emerging segmentation, have now been identified in both mouse (Wilkinson *et al.* 1989*a*) and chick (L. Nieto & D. G. Wilkinson, in preparation); the zinc finger-encoding gene *Krox-20* is expressed within the confines of r3 and r5 at the time these rhombomeres become defined, but

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it is not expressed in r2, r4 and r6. This expression pattern suggests a function in setting up the repeat pattern.

Other genes with putative regulatory roles have been found to be expressed in rhombomere boundary-restricted domains in the mouse embryo (Wilkinson *et al.* 1989*b*); rhombomere boundaries coincide with the limits of expression of homeodomain-encoding genes belonging to the *Hox-2* cluster. For example, *Hox-2.1, Hox-2.6, Hox-2.7* and *Hox-2.8* are expressed up to the spinal cord/r8 boundary, the r6/7 boundary, the r4/5 boundary and the r2/3 boundary, respectively. At the same stage, by contrast, *Hox-2.9* is expressed within and is coextensive with one rhombomere, r4, in both mouse (Murphy *et al.* 1989; Wilkinson *et al.* 1989*b*) and chick (Muchamore & Krumlauf, in preparation). The *Hox-2* genes are expressed at high levels after rhombomeres become defined, the restriction to cell movement across rhombomere boundaries perhaps playing a role in establishing and maintaining their sharp anterior limits of expression.

Hox-2 genes (Graham *et al.* 1989) show close sequence similarity with the homeotic regulator *Ant-c* and *Bx-c* genes of *Drosophila* (Akam 1989), which are expressed at high levels in specific parasegments and determine their eventual phenotypic character parasegments and determine their eventual phenotypic character. In view of their function in *Drosophila* and their evolutionary conservation, the vertebrate *Hox* genes are attractive candidates for a role in the specification of rhombomere identity, and thus of segmented nuclei in the hindbrain. Like the insect epidermis, therefore, the developing vertebrate hindbrain may use compartments as units for pattern formation.

DETERMINATION OF NEURONAL FATE

The formation of compartments, with specific patterns of selector gene expression and neuronal differentiation, suggests that in the hindbrain, development may be more determinate than it is in other regions of the vertebrate body. Progenitor cells may be specified early and their partitioning into non-mixing groups allows their determined progeny to maintain, through subsequent cell divisions, the patterns or differentiation appropriate for their axial level.

Some preliminary observations from our cell-marking study (Fraser *et al.* 1990) are consistent with this idea. In the normal chick embryo at the stage we analysed the labelled clones, there are at least seven different phenotypes of hindbrain neuron. Yet clones each contained only one of these neuronal types, and clones in different animals which occupied coextensive or overlapping positions in the hindbrain contained homogeneous populations of different neuronal types. These findings suggest that individual neuronal fates are determined several cell divisions before final differentiation and that cell lineage accordingly plays some part in the patterning and generation of specific types of hindbrain neuron. This is in sharp contrast to the indeterminacy seen in other CNS areas, such as the cortex of rodents (Price & Thurlow 1988; Walsh & Cepko 1988), the mesencephalic tectum of the chick (Gray *et al.* 1988), and the retinae of both rodents (Turner & Cepko 1987) and frogs (Wetts & Fraser 1988), where a single parent cell produces a variety of neuronal phenotypes. Here cell fate is determined, presumably by local interactions, at or after the last cell division.

CONCLUSIONS

Recent studies have confirmed segmentation as a fundamental process in development of the hindbrain. Here, segmentation is based on early lineage restriction and the differential activation of regulatory genes. It remains to be seen whether other domains in the embryonic CNS, such as forebrain and midbrain are segmented even though there is less obvious morphological evidence of the process. Another prospect is to analyse the contribution of hindbrain segments in the construction of the head as a whole. There is, for example, an obvious match between segmentation of the hindbrain and the adjacent branchial arches. The migration of neural crest cells from specific rhombomeres into individual arches may be one way in which the registration is set up. Thus the hindbrain may hold further clues to both the phylogeny and ontogeny of head segmentation.

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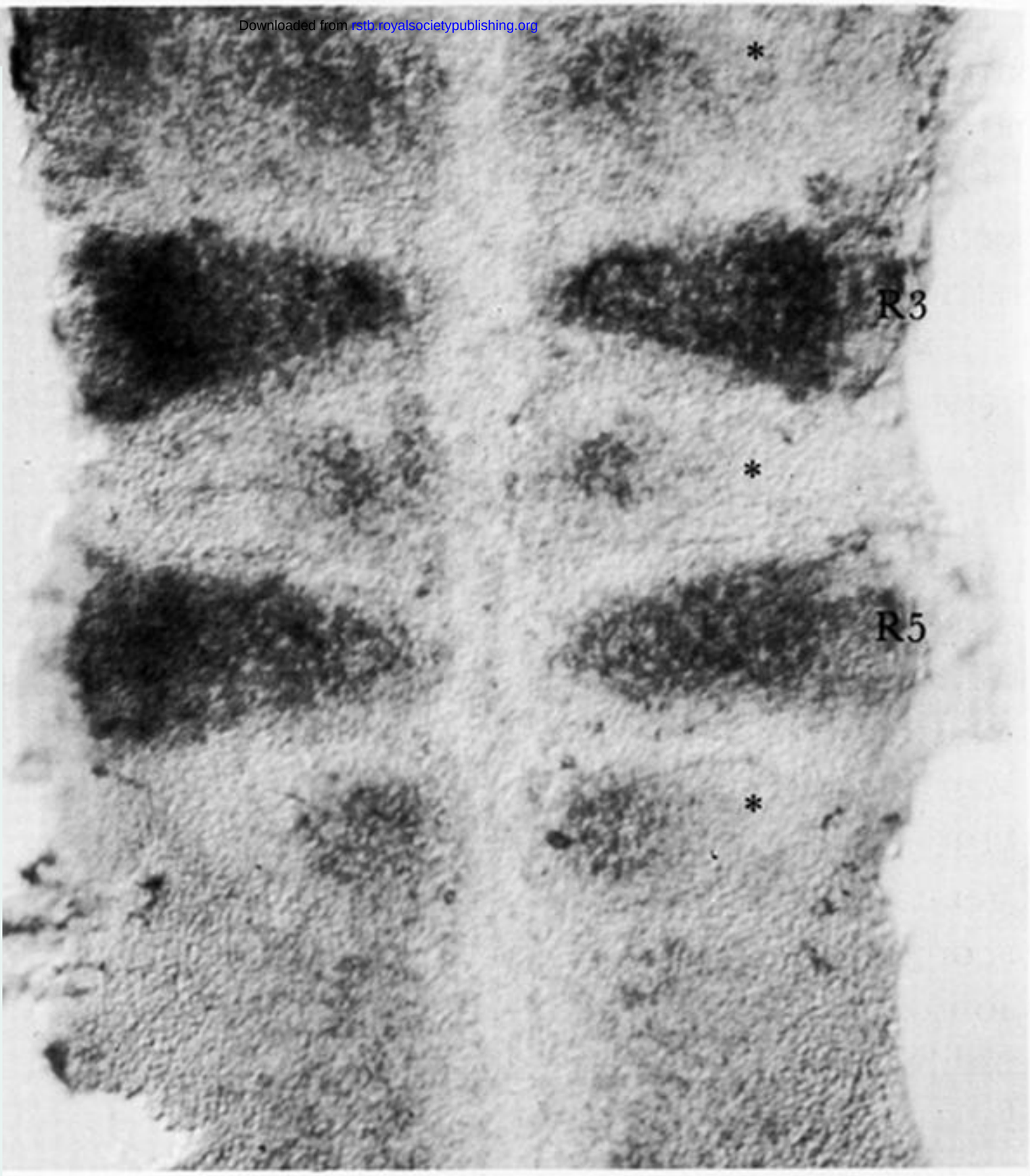

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