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Conserved segmental expression of *Krox-20* in the vertebrate hindbrain and its relationship to lineage restriction

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Summary

The zinc-finger gene *Krox-20* is expressed in two alternating segments, rhombomeres (r) 3 and 5, in the developing mouse hindbrain. This expression pattern is established prior to rhombomere formation in the mouse, but it is not known how the timing of expression relates to cellular events of segmentation, such as lineage restriction. We have cloned *Krox-20* sequences from *Xenopus* and the chick and shown that its alternating expression pattern is conserved in these systems, suggesting that its role in hindbrain development is conserved. Analysis of the early stages of *Krox-20* expression in the chick show that both domains of expression precede the restriction of cell lineage to specific rhombomeres, consistent with a role of this gene in early events of hindbrain segmentation. The finding that expression is not coincident with lineage restriction indicates that early expression may not reflect an irreversible commitment of cells to r3 and r5 and/or may be mosaic.

Key words: *Krox-20*, segmentation, hindbrain, rhombomeres.

Introduction

A conserved feature of the development of the vertebrate central nervous system is the transient formation of repeated bulges, termed rhombomeres (r), in the hindbrain. Studies of the chick hindbrain at the cellular level have shown that rhombomeres are a manifestation at the morphological level of a process of segmentation (see Lumsden and Guthrie, this volume). Rhombomeric constrictions appear in a defined sequence in the early neural epithelium (Vaage, 1969), and upon forming, the movement of cells across these boundaries is restricted, thus confining cells and their clonal descendants to specific rhombomeres (Fraser *et al.* 1990). As a consequence, the hindbrain is subdivided into a series of compartmental units prior to the onset of neurogenesis. At later stages of development this partitioning correlates with, and presumably underlies, the segmental organisation of nerves, for example the branchial motor nerves, each of which is generated in an adjacent pair of rhombomeres (Lumsden and Keynes, 1989).

The genetic basis of the formation of rhombomeres and the specification of their phenotype is largely obscure. By analogy with segmentation in *Drosophila* development (Akam, 1987; Ingham, 1988) it is likely that certain of the genes with critical roles in these processes will be expressed in segment-restricted domains. Several such genes encoding putative transcription factors have been found in the mouse, including the *Hox* homeobox-containing genes (see Hunt *et al.*, this volume). *Hox-2.6*, *-2.7* and *-2.8* have anterior boundaries of expression at r6/7, r4/5 and r2/3, respectively, and thus pairs of rhombomeres express particular combinations of these genes (Wilkinson *et al.* 1989*b*). In contrast, *Hox-2.9* expression becomes restricted to a single rhombomere, r4 (Murphy *et al.* 1989; Wilkinson *et al.* 1989*b*; Sundin and Eichele, 1990; Murphy and Hill, 1991). The expression of these genes in rhombomeric patterns suggests that they may have an analogous role to their *Drosophila* counterparts in the specification of segmental identity.

We have fewer clues as to the role of another putative regulatory gene, *Krox-20*, which is expressed in a segmental pattern distinct from that of the *Hox* genes. *Krox-20* encodes a protein with three zinc-finger domains, and was first identified as a gene whose transcription is rapidly up-regulated upon treating quiescent fibroblasts with serum or purified growth factors (Chavrier *et al.* 1988). This is a primary response, as it occurs in the presence of cycloheximide, and presumably involves growth factors acting through signal transduction pathways to activate transcription of the *Krox-20* gene. *Krox-20* protein binds DNA in a sequence-specific manner and several lines of evidence suggest that it may act as a transcription factor (Chavrier *et al.* 1990; Nardelli *et al.* 1991). *Krox-20* is expressed in two alternating rhombomeres, r3 and r5, in the 9.5 day old mouse embryo hindbrain (Wilkinson *et al.* 1989*a*). This expression pattern correlates with several other features of hindbrain development that also exhibit a two-segment periodicity. Reticular and

branchial motor nerves differentiate in r2, r4 and r6 prior to r3 and r5 (Lumsden and Keynes, 1989). In addition, r3 and r5 are unique in not forming migratory neural crest, thus separating the neural crest cell populations derived from r2, r4 and r6 which stream into successive branchial arches (Lumsden *et al.* 1991). Furthermore, grafting experiments indicate that an alternation in cellular properties may underlie the formation of rhombomere boundaries; the juxtaposition of r3 and r5 does not lead to boundary formation, but a boundary is generated when either of these are grafted adjacent to any of the even-numbered rhombomeres (Guthrie and Lumsden, 1991). Finally, each of the branchial motor nerves arises from two adjacent rhombomeres (Lumsden and Keynes, 1989) and pairs of rhombomeres out of phase from these express particular combinations of *Hox-2* genes (Wilkinson *et al.* 1989*b*).

Studies of *Krox-20* expression can provide further lines of circumstantial evidence as to the role of this gene. It would be expected that the conservation of rhombomeres throughout vertebrates will be matched by a conservation of the expression domains of genes with roles in segmental development, and thus it is pertinent to examine *Krox-20* expression in other species. In addition, it is important to document in detail the timing of expression relative to the other events of hindbrain development. Here, we discuss evidence that the pattern of *Krox-20* expression is conserved between mammals, birds and amphibians, and show that expression in the chick hindbrain precedes the establishment of lineage restriction.

Conserved structure and expression of *Krox-20*

Two cDNA clones potentially corresponding to the *Xenopus* homologue of *Krox-20* were obtained by screening a neurula-stage embryo cDNA library at moderate stringency with a probe from the zinc-finger domain of mouse *Krox-20* (L. C. Bradley *et al.*, unpublished data). DNA sequence analysis indicates that these clones encode a protein with three zinc-fingers identical to those of mouse *Krox-20* (Fig. 1) and with 56% amino acid sequence identity in non-finger regions. These clones do not correspond to *Xenopus* homologues of the closely related *Krox-24* gene of the mouse (Lemaire *et al.* 1988), which has 6 amino acid sequence differences in the zinc-fingers and only 39% identity in non-finger regions. These data indicate that we have cloned the *Xenopus* homologue of *Krox-20*.

A similar screening strategy was used to isolate clones cross-hybridising to mouse *Krox-20* from a stage 15 chick cDNA library. However, sequence analysis indicates that none of these correspond to *Krox-20*. Therefore, a different strategy employing the polymerase chain reaction was used. Redundant oligonucleotides were designed that correspond to amino acid sequences in the first and third zinc-fingers of *Krox-20* but that are different in *Krox-24* (see Fig. 1). These were used to amplify sequences from chick genomic

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Krox-20 M   VHERPYPCPAEGCDRRFSRSDELTRHIRIH
        X   ----------------------------
        C       *******-------------T---
Krox-24 M   P-----A--V-S-----------------

Krox-20 M     TGHKPFQCRICMRNFSRSDHLTTHIRTH
        X     ----------------------------
        C     ----------------------------
Krox-24 M     --Q------------------------

Krox-20 M     TGEKPFACDYCGRKFARSDERKRHTKIH
        X     ----------------------------
        C     ----********
Krox-24 M     ---------I------------------
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Fig. 1. Conserved structure of the zinc-fingers of *Krox-20*. The deduced amino acid sequences of the zinc-fingers of mouse (M), *Xenopus* (X) and chick (C) *Krox-20* and mouse *Krox-24* are compared. Dashes indicate amino acid identity with mouse *Krox-20*, and asterisks the regions that correspond to the oligonucleotides used to amplify *Krox-20* from chick genomic DNA. *Xenopus Krox-20* sequences (Bradley *et al.* unpublished data), mouse *Krox-20* sequences from Chavrier *et al.* (1988) and mouse *Krox-24* sequences from Lemaire *et al.* (1988).

DNA which were then subcloned and sequenced. Sequence comparisons indicate that sequences from the chick homologue of *Krox-20* have been obtained by this strategy (Fig. 1). A single conservative amino acid substitution is predicted from the chick sequence, which is unlikely to represent a PCR artifact as it was found in 2 independent clones.

We analysed the expression pattern of *Krox-20* by the *in situ* hybridisation of *Xenopus* and chick embryos with homologous probes (Fig. 2). *Krox-20* is expressed in two domains in the hindbrain of the stage 28 *Xenopus* embryo, but since rhombomeres are not conspicuous until later stages, we cannot at present correlate these domains with hindbrain segments. Two stripes of *Krox-20* expression were also observed in the chick embryo and, as for the mouse, these correspond to r3 and r5. These data indicate that the alternating expression of *Krox-20* in the early hindbrain is conserved between the mouse, chick and *Xenopus*, suggesting that this gene has a conserved role in hindbrain development. But what might this role be? We have sought further clues by analysing the onset of *Krox-20* expression in order to assess whether it might act upstream or downstream of other events of hindbrain segmentation, in particular focusing on the establishment of lineage restriction.

Establishment of *Krox-20* expression in the neural epithelium

The two domains of *Krox-20* expression are established in the early neural plate of the mouse embryo, prior to the morphological appearance of rhombomeres (Wil-

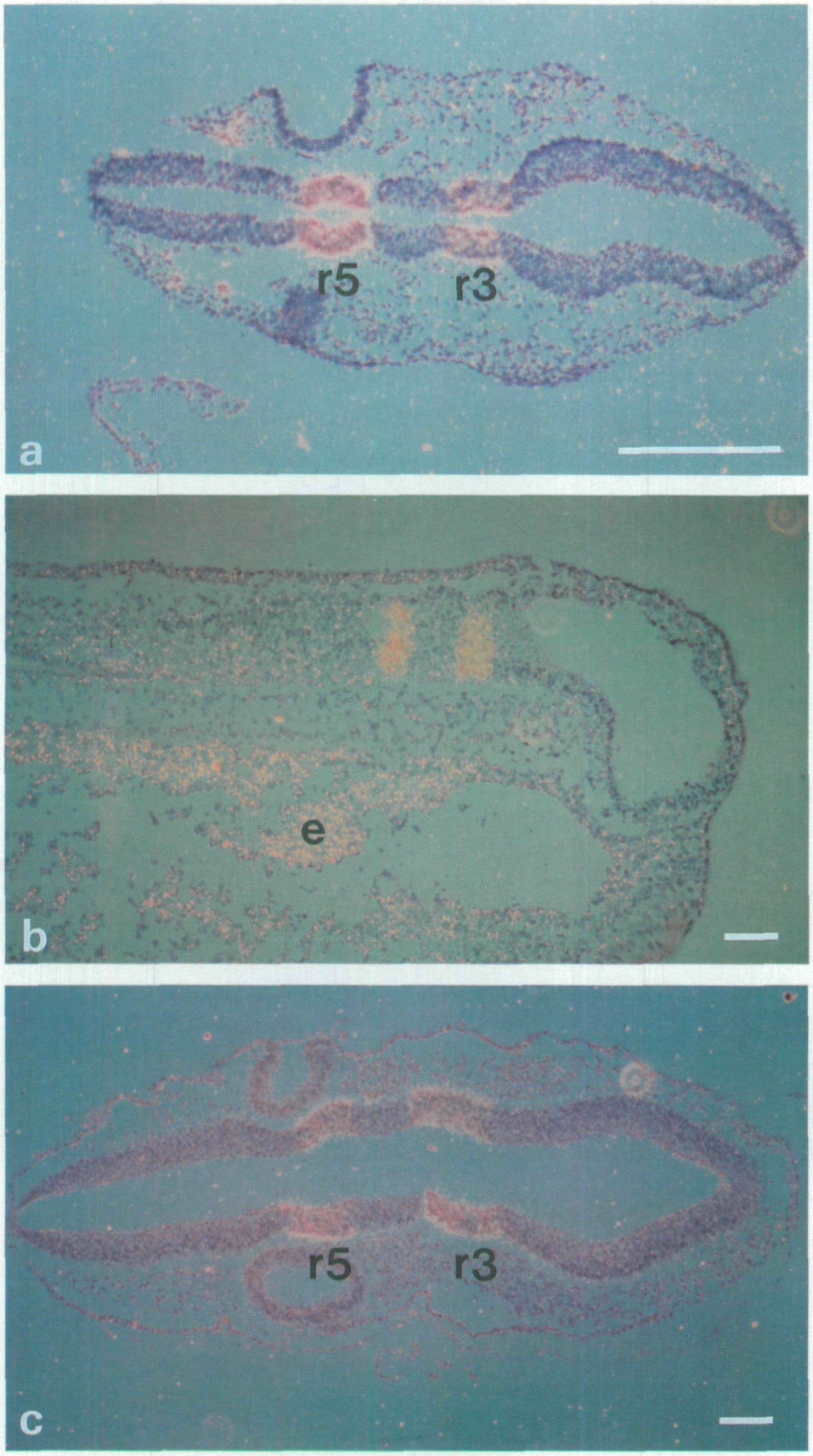

Fig. 2. Conserved patterns of *Krox-20* expression in mouse, *Xenopus* and chick. *In situ* hybridisation was carried out using appropriate homologous *Krox-20* probes as described (Wilkinson and Green, 1990). (a) 9.5 day mouse embryo; (b) stage 28 *Xenopus* embryo; (c) stage 15 chick embryo. r, rhombomere. The apparent signal in the endoderm (e) of the *Xenopus* embryo is due to the refraction of light by yolky cells, not the hybridisation of probe. Anterior is to the right in all photographs. Bar=100 μm.

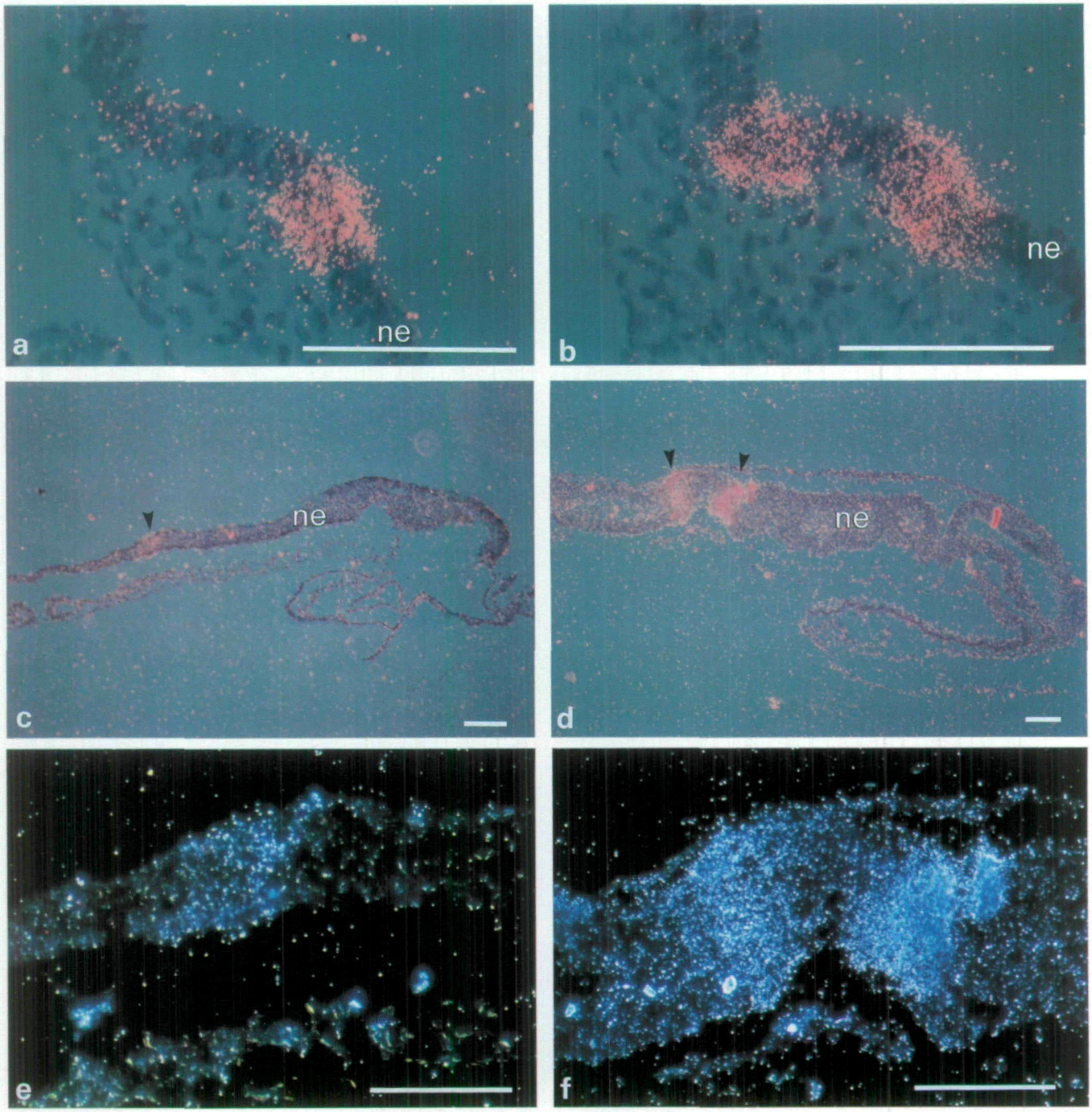

Fig. 3. Onset of *Krox-20* expression in the mouse and chick embryo. *In situ* hybridisation analysis was carried out to examine the early stages of *Krox-20* expression. (a) 8 day mouse embryo; (b) 8.5 day mouse embryo; (c,e) 3 somite chick embryo; (d,f) 7 somite chick embryo. e and f are higher magnification views of the embryos shown in c and d. The arrows indicate sites of *Krox-20* expression. ne, neural epithelium. Anterior is to the right in all photographs. a and b are from Wilkinson *et al.* (1989*b*). Bar=100 μm.

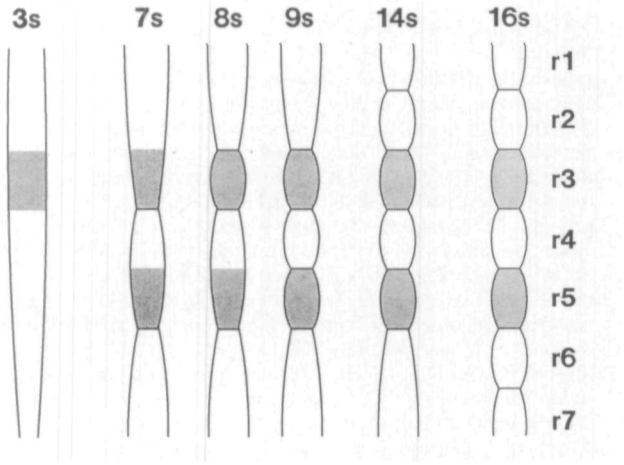

Fig. 4. *Krox-20* expression and rhombomere boundary formation. The diagram indicates the expression of *Krox-20* (shaded) and the time course of rhombomere boundary formation in the developing chick hindbrain (data from Vaage, 1969). s, somite stage; r, rhombomere.

kinson *et al.* 1989*a*,*b*). At 8 days of development (~5 somite-stage), *Krox-20* is expressed in a single domain, and by 8.5 days a second, more posterior, domain has appeared (Fig. 3). The analysis of *Xenopus* embryos has revealed that, in this system too, the two domains of *Krox-20* expression are established at early neurula stages (L. C. Bradley, unpublished data). The onset of expression in the early neural plate is also found in the chick, and we can compare these data with the timing of the cellular events of segmentation which have been relatively well characterised in this system. A single domain of *Krox-20* expression is detected in the hindbrain of the 3 somite chick embryo, and a second, more posterior, stripe of expression is detected in the 7 somite embryo (Fig. 3). The order in which these domains of expression are established correlates with the formation of r3 before r5 (Vaage, 1969). However, *Krox-20* expression is detected prior to the formation of rhombomeres (Fig. 4), at least 8 h in advance for the anterior domain and 3 h in advance for the posterior domain; these are minimal estimates, not only in view of the limited sensitivity of *in situ* hybridisation but, for the latter, also because we have not analysed 5 and 6 somite embryos. Since the spatial restriction of cell lineage occurs coincident with rhombomere boundary formation in the chick (Fraser *et al.* 1990), our data show that *Krox-20* expression is initiated before compartments have formed.

Implications for the function of *Krox-20*

The observation that boundaries do not form when r3 and r5 are juxtaposed, but are generated when either of these is grafted adjacent to an even-numbered rhombomere (Guthrie and Lumsden, 1991), suggests that r3 and r5 share cellular properties, perhaps involving cell adhesion, that underlie lineage restriction. Both the spatial pattern and timing of *Krox-20* expression are consistent with it regulating genes with direct roles in the formation of compartments. However, this idea is certainly over-simplistic, since if does not explain why the boundaries flanking r3, for example, do not form simultaneously, or how the r1/r2 and r6/r7 boundaries are generated.

It must be emphasised that these expression studies of *Krox-20* provide no direct evidence regarding gene function, and are also consistent with roles in other events of hindbrain segmentation. The expression in r3 and r5 also precedes, and correlates with, the emigration of neural crest from alternate rhombomeres (Lumsden *et al.* 1991), the alternation in neuronal differentiation (Lumsden and Keynes, 1989), the expression of a carbohydrate epitope recognised by the HNK-1 antibody (Kuratani, 1991) and the expression of *Hox-2* genes with anterior limits at two segment intervals (Wilkinson *et al.* 1989*b*). In *Drosophila*, the coupling of high-level expression of homeotic genes to segment boundaries occurs in part through their regulation by pair-rule genes (reviewed by Ingham, 1988). Despite the obvious differences between segmentation in *Drosophila* and vertebrates, it is possible that there is an analogous regulation of *Hox* expression by genes, such as *Krox-20*, which encode transcription factors and have segment-restricted expression. According to this, *Krox-20*, in combination with other genes, could regulate the high-level expression of *Hox-2.7* and *Hox-2.8* in r5 and r3–r5, respectively (Wilkinson *et al.* 1989*b*), and the restriction of *Hox-2.9* expression to r4 (Murphy *et al.* 1989; Wilkinson *et al.* 1989*b*; Sundin and Eichele, 1990; Murphy and Hill, 1991). The finding that *Krox-20* protein binding sites are present in the *Hox-1.6* gene (Chavrier *et al.* 1990) is consistent with this idea, and it is pertinent to ascertain whether such sites also exist in other *Hox* genes and to test their significance *in vivo*.

Relationship between *Krox-20* expression and cell commitment

Regardless of its function, *Krox-20* is a marker of r3 and r5, and thus expression could indicate a commitment of cells to these rhombomeres. However, if this commitment is irrevocable, then lineage restriction should be coincident with the onset of expression, and not at later stages as shown here. There are two models that explain these data. The initial expression of *Krox-20* could be mosaic, consisting of a mixture of committed, expressing cells and uncommitted, non-expressing cells; clonal descendants of the former cells would be restricted to r3 and r5, whereas progeny of the latter could be subsequently recruited to either odd- or even-numbered rhombomeres. According to this view, the finding that some clones marked before rhombomere formation are restricted, whereas others are not (Fraser *et al.* 1990), could in part be due to a mosaicism in cellular commitment and not only a consequence of whether clones have spread across prospective boundaries. A second possibility is that *Krox-20* expression is not a reliable marker of cell commitment and that it can

be down-regulated in, for example, cells that have migrated into prospective even-numbered rhombomeres. As a first step towards addressing these possibilities, it will be important to analyse the expression of *Krox-20* at a single cell resolution.

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