small amount of block that did develop recovered slowly (Fig. 3C), like block of wild-type channels, indicating that F1764A did not alter the escape pathway, so the drug was trapped when the activation or inactivation gates closed.

Our results lead to a model of the local anesthetic receptor site in the pore of the Na⁺ channel. Mutations F1764A, Y1771A, and I1760A, which had the strongest effects on use-dependent block, are oriented on the same face of the IVS6 helix (Fig. 4). F1764A and Y1771A reduced open and inactivated channel affinity by one to two orders of magnitude, and F1764A also had a smaller effect on resting channel affinity, suggesting that the native residues at these positions contribute to the free energy of drug binding. F1764 and Y1771 are hydrophobic (23), aromatic residues separated by two turns of the S6 helix (Fig. 4), so they are about 11 Å apart. Effective local anesthetics are approximately 10 to 15 Å in length (24), with positively charged and hydrophobic moieties at either end that could interact with these residues through hydrophobic (25) or π electron (26) interactions. Therefore, we propose that F1764 and Y1771 are determinants of the local anesthetic binding site and that substitution of these residues with alanine destabilizes drug binding by reducing the hydrophobicity and aromaticity at these positions. I1760 is oriented on the same face of the helix as F1764 and Y1771 and is therefore well positioned to modulate extracellular access to the local anesthetic binding site. Replacement of the bulky isoleucine residue at position 1760 with alanine allows QX314 to reach the site from the extracellular medium, perhaps by passing directly through the pore from the outside. Thus, I1760 likely corresponds to a narrow region in the pore, just to the extracellular side of the local anesthetic binding site. The mutations I1761A, V1766A, and N1769A increased resting block without altering inactivated state affinity. Because these amino acids are oriented away from the face containing F1764, Y1771, and I1760 (Fig. 4), they may be oriented away from the channel pore. Mutations to alanine at these positions may increase channel sensitivity to drugs through indirect effects on the local anesthetic site, perhaps by partially inducing the inactivated binding site conformation in functionally resting Na⁺ channels.

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Y, Tyr. Each amino acid is indicated by its one-letter code. The number indicates its position in the chain, and A at the end indicates that alanine has been substituted for the native amino acid. Except where noted by error bars, the data presented in the figures are single examples from three or more experiments that led to the same conclusion.

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Role of a Conserved Retinoic Acid Response Element in Rhombomere Restriction of *Hoxb-1*

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After activation in mesoderm and neuroectoderm, expression of the *Hoxb-1* gene is progressively restricted to rhombomere (r) 4 in the hindbrain. Analysis of the chick and mouse *Hoxb-1* genes identified positive and negative regulatory regions that cooperate to mediate segment-restricted expression during rhombomere formation. An enhancer generates expression extending into r3 and r5, and a repressor limits this domain to r4. The repressor contains a conserved retinoic acid response element, point mutations in which allow expression to spread into adjacent rhombomeres. Retinoids and their nuclear receptors may therefore participate in sharpening segment-restricted expression of *Hoxb-1* during rhombomere boundary formation.

Rhombomeres are segmental units of organization in the vertebrate hindbrain (1). Cell mixing can occur between prospective neighboring segments before rhombomeres become lineage-restricted cellular compartments (2). Later, boundaries form between

odd- and even-numbered units; however, cells can mix when even- or odd-numbered rhombomeres are grafted adjacent to each other, which suggests that the formation and maintenance of boundaries are dependent on signaling between odd and even segments (3). Some of the Hox homeobox genes important for regulating axial patterning have limits of expression that coincide with rhombomere boundaries (4, 5). For example, expression of the Hoxb-1 gene is progressively restricted to r4 in the hindbrain (4, 6–8). These patterns of expression, combined with mutational analysis (9,

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10), have implicated the *Hox* genes in segmentation and in regulation of rhombomere identities. However, little is known about the processes that regulate the segment-restricted expression of *Hox* genes (11) and how or if they relate to the cellular formation and sharpening of rhombomeric boundaries. To approach these questions, we analyzed the *Hoxb-1* gene in transgenic mice.

In the mouse hindbrain, between 9.0 and 9.5 days post coitum (dpc), Hoxb-1 expression maps precisely to r4, with sharp boundaries at the r3/r4 and r4/r5 junctions

(4, 6, 7). There is also expression in neural crest cells that migrate from r4 and populate the second branchial arch (12). These same neural crest and r4-restricted domains of expression are generated by a 7.5-kb fragment containing the Hoxb-1 gene fused with the Escherichia coli β -galactosidase (lacZ) gene [construct number (cn) 1, Fig. 1A] (13). Deletion analysis was done on this fragment to identify regulatory regions involved in r4 restriction (14, 15) (Fig. 1). A 1-kb fragment 5' of Hoxb-1 (cn 3) functioned as an enhancer on a heterologous promoter–lacZ reporter gene and generated



Fig. 1. Mapping and analysis of evolutionarily conserved *Hoxb-1* enhancer and repressor regions in transgenic mice. In (A) and (B), on the left are the *lacZ* reporter constructs (*14*) used to monitor regulatory activity. Arrows indicate the 5' to 3' orientation of the regulatory regions in the vectors. On the right is the transgenic analysis. Abbreviations: Cn, construct number; Tg, total number of expressing lines and F₀ embryos; r4, expression in rhombomere 4; and r3/5, expression in rhombomeres 3 and 5. (**A**) Identification of the r4 enhancer and r3/r5 repressor regions. The Stu I–Hind III fragment (cn 6) corresponds to a minimal r4 enhancer region, and the Apa I–Spe I fragment (cn 8) contains the r3/r5 repressor. (**B**) Mapping of a conserved mouse (cns 12 to 18) and chick (cns 19 and 20) r3/r5 repressor: Construct 18 has point mutations in the DR2 element that block r3/r5 repression. (**C**) Map of functional repressor regions, location, and sequence of a conserved DR2-RARE. Asterisk indicates point mutations in the RARE used for cn 18 and for in vitro binding. M, mouse; c, chick. Restriction sites: RV, Eco RV; A, Apa I; Sc, Sac I; Sp, Spe I; R, Eco RI; St, Stu I; Sm, Sma I; H, Hind III; P, Pst I; X, Xho I; K, Kpn I.

a stripe of expression in the region of r4 and its associated neural crest (Fig. 2A). Successive deletions of this 5' enhancer (cns 4 to 6) (Fig. 2, B and C) mapped the regulatory region responsible for this activity to a 332– base pair (bp) Stu I–Hind III fragment (cn 6). In all embryos expressing the transgene, we observed reproducible staining in r4 and in the neural crest. Frequently (in 36% of embryos), expression was seen in r6 (Fig. 2, B and C), which is consistent with the idea that even-numbered rhombomeres, such as r4 and r6, may share properties that could include common regulatory factors (3).

Despite the fact that the enhancer generates expression in r4 (Fig. 2, A and C), detailed analysis of the patterns reveals that the domain is not strictly limited to r4 and that some patches of expression are found in adjacent rhombomeres. Transgene expression spread into cells in r3 and r5 at a time when the endogenous gene and the 7.5-kb Hoxb-1-lacZ transgene (cn 1) were strictly limited to r4 (Fig. 2, D and E). This suggests the absence of a negative control region that restricts expression in domains other than r4. We localized such a regulatory domain by adding flanking regions back to the 1-kb enhancer construct (cn 3). Addition of 3' flanking sequences (cn 2) or 445 bp of 5' flanking DNA (cn 7) did not alter the expanded r4 pattern (Fig. 1A). However, a 741-bp 5' flanking Apa I-Spe I fragment (cn 8) acted as a functional repressor and restricted transgene expression to r4 with sharp boundaries at the r3/r4 and r4/r5 junctions (Fig. 2, G and H). Single or tandem copies of the repressor alone were unable to stimulate expression in the hindbrain (cn 9; Fig. 1A), which suggests that it works cooperatively with the r4 enhancer to restrict Hoxb-1 expression.

In situ analysis in chicks showed that Hoxb-1 has a similar r4-restricted expression pattern (6). Therefore, the 5' flanking regions of the chick Hoxb-1 gene were cloned and tested in transgenic mice to determine if similar combinations of cisacting regulatory regions are involved in its regulation. A construct containing 1.6 kb of 5' flanking DNA (cn 10) produced expression in a domain spanning r2 to r6, with the greatest amounts in r4 and r6 and patchy staining in r2, r3, and r5 (Fig. 2F). This pattern is similar to that generated by the mouse r4 enhancer lacking the repressor (Fig. 2D). The ectopic expression in r2 and r6 might again be a consequence of common characteristics in even-numbered rhombomeres (3). Addition of more 5' flanking DNA (cn 11) specifically eliminated only the expression in r3 and r5 (Fig. 21). This implies that the chick Hoxb-1 gene also contains a regulatory region that functions as an r3/r5 repressor, and both activation and repression are required for

r4-restricted expression of *Hoxb-1* in chick and mouse embryos.

To determine if the repressor region restricts spreading from r4 by specifically blocking expression in r3 and r5, we used an enhancer region from the Hoxb-2 gene (11). When tested on heterologous promoters, this enhancer stimulated expression in r3 and r5 (8, 11, 16). However, when attached to two Hoxb-1-lacZ transgenes (cns 12 and 13; Fig. 1B), the enhancer was unable to stimulate expression in r3 and r5 (Fig. 3A). This confirms that a region of the Hoxb-1 gene inhibits expression in r3 and r5. Further deletion analysis (cns 14 to 17; Fig. 1B) showed that the repressor region is located in the Apa I-Spe I fragment and that elements in both the Apa I-Sac I and Sac I-Spe I subfragments were necessary but not sufficient to cooperate in blocking r3/r5 expression (Fig. 3, B and C).

We also used this assay to examine the chick Hoxb-1 regulatory regions and to locate potential r3/r5 restriction elements. The Hind III–Kpn I (cn 20) but not the Pst I (cn 19) subfragment inhibited expression in r3 and r5 (Fig. 3, D and E). This indicates functionally equivalent repressor regions in the mouse and chick Hoxb-1 genes, which coincide with regulatory regions (cns 8 and 11) involved in limiting expression of Hoxb-1 to r4.

To identify specific components of the repressor activity, we compared the sequence between the mouse and chick regions that display repressor function in transgenic mice. Despite the low overall degree of sequence identity, alignment of these fragments reveals a motif related to the consensus sequence for a retinoic acid response element (RARE) (17), with a spacing of 2 bp between the direct repeats (DR2) (Fig. 1C). In electrophoretic mobility shift assays (EMSAs) (18), heterodimers between RARa or RARy and retinoid X receptor α (RXR α) receptors, but not homodimers, bound to double-stranded oligonucleotides spanning the mouse DR2 element (Fig. 4A). Three point mutations (19) in each half of the direct repeats (mDR2; Fig. 1C) eliminated the ability of the RAR-RXR heterodimers to bind to this site in vitro (Fig. 4A).

In a transgenic construct (cn 18), containing the version of the mouse DR2 inactivated by mutation, linked to the r3/r5 enhancer, expression was no longer limited to r4 (Fig. 4, B and C) and was similar to that that would be seen if the entire repressor region were deleted. With a construct lacking the r3/r5 enhancer, the mutated mouse RARE allowed transgene expression to spread from r4 into adjacent rhombomeres. Therefore, this *Hoxb-1* 5' RARE is an essential component of the repressor region. Through sequence homology, we have as yet been unable to identify additional components of the repressor.

Ectopic exposure of mouse embryos to retinoic acid (RA) at 7.5 dpc alters Hoxb-1 expression (7, 8, 20, 21) and

Fig. 2. A conserved enhancer and repressor cooperate to regulate r4-restricted Hoxb-1 expression. (A through C) Lateral views of 9.0 to 9.5 dpc embryos expressing progressive deletions that map an r4 enhancer to a Stu I-Hind III fragment. There is reproducible staining in r4 and in neural crest (nc) migrating from r4 into the second branchial arch. In (B) and (C) there is ectopic expression in r6. (D) Dorsal view of a 9.0 dpc mouse embryo, and (E) a coronal section of that embryo showing that expression is not confined to r4 but extends into r3 and r5. (G) Dorsal view of a 9.0 dpc embryo and (H) a coronal section of that embryo, demonstrating that addition of the repressor restricts expression to r4. (F) and (I) show dorsal views of transgenic mouse embryos expressing the lacZ reporter gene under the control of chick Hoxb-1 regulatory regions with (F) and without (I) the r3/r5 retransforms r2 to an r4 identity (8). In association with this transformation, Hoxb-1 expression is induced in r2 and in first-arch neural crest (8, 21). Therefore, we examined the RA response of several chick and mouse transgenes and mapped



pressor region. Arrows denote that the only difference between the presence and absence of the repressor is the lack of expression in r3/r5. Ov, otic vesicle. Constructs tested: (A) 3, (B) 5, (C) 6, (D) and (E) 3, (G) and (H) 8, (F) 11, and (I) 10, according to the map in Fig. 1.



Fig. 3. The repressor region in Hoxb-1 actively blocks expression stimulated by an r3/r5 enhancer. (A) An r3/r5 enhancer is not capable of stimulating r3/r5 expression in the presence of the Apa I-Eco RV repressor. (B) Deletion of the repressor restores the ability of the enhancer to generate expression in r3/r5. (C) The Apa I-Sac I fragment containing the DR2-RARE is not sufficient to repress the r3/r5 expression domain. In the 5' flanking regions of the chick Hoxb-1 gene, the Hind III-Kpn I fragment (D) but not the Pst I fragment (E) is able to repress r3/r5 expression. Ov, otic vesicle; nc, neural crest. Constructs tested: (A) 12, (B) 14, (C) 16, (D) 19, and (E) 20, according to the map in Fig. 1.

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the regions involved in directing this activity. The 5' RARE is not required for RA-stimulated expression in r2, as constructs lacking the DR2 (cns 6 and 10) do respond to induction by RA and are expressed in r2 and first-arch neural crest (Fig. 4, D and E). In mouse and chick embryos, this ectopic RA response maps to the same minimal enhancers (cns 6 and 10), which give expression in the region of r4. Sequencing of these regions in both species did not reveal any additional RAREs. This suggests that RA-induced expression in r2 is probably mediated by an indirect mechanism, working through the enhancer that generates broad r4 expression.

The 5' RARE in the *Hoxb-1* gene appears to directly repress expression in r3 and r5 that spreads from r4, which suggests that retinoids and their nuclear receptors could be involved in regulating the sharpening of *Hox* expression during the formation of

Fig. 4. A conserved RARE is required for r3/r5 repressor activity but not for the ectopic response of Hoxb-1 to RA. (A) EMSAs with wildtype (5' DR2) and mutant (5' mDR2) RAREs show that only the wildtype RARE binds RAR-RXR heterodimers. Sequences of the oligonucleotides are given below their respective lanes. (B and C) Expression in 9.5 dpc embryos with a wildtype RARE (B) and a mutated RARE (C). Arrows indicate that point mutations in the RARE abolish the repressor activity, permitting expression in r3/r5 (C). (D and E) Lateral views of 9.0 to 9.5 dpc RA-treated embryos with mouse (D) and chick (E) Hoxb-1 transgenes that lack the 5' BAREs. Both mouse and chick r4 enhancers mediate an ectopic response to RA (8) by generating an additional stripe of expression in r2 and its neural crest (marked by arrowheads). Ov, otic vesicle; nc, neural crest. Constructs tested: (B) 12, (C) 18, (D) 6, (E) 10, according to the map in Fig. 1.

rhombomere boundaries. Such a function for a RARE is unusual, as RARs and RXRs are usually implicated in positive regulation. Although retinoid receptors are expressed in the hindbrain, some with specific rhombomere boundaries (22), there is no simple distribution that accounts for repression in r3 and r5 but not in r4. However, it is also important to consider factors that can inhibit retinoid action. Cytoplasmic retinoic acid binding proteins (CRABPs) and some chicken ovalbumin upstream promoter (COUP)-related nuclear receptors are expressed at high levels in r4 and in evennumbered rhombomeres (22). CRABP may keep retinoic acid below threshold levels in r4, and the COUP-related receptors competitively interact with the RXRs to prevent heterodimerization with RARs. Therefore, we suggest that it is the combination of RAR-RXR receptors and their antagonizing agents that restricts



repression of Hoxb-1 in r3 and r5.

Another DR2-RARE is located 3' of the mouse Hoxb-1 gene (23). Unlike the negative function of the 5' RARE, this 3' RARE appears to play a positive role in Hoxb-1 regulation because it is necessary to establish the early neural pattern of expression (22). Therefore, multiple RAREs, each with different functions, are involved and are required to regulate specific aspects of Hoxb-1 expression.

Restriction of Hoxb-1 expression to r4 is achieved by a combination of elements that first activate expression in a broad domain and later repress expression in certain subregions. This is analogous to the situation in Drosophila in which segmental expression is mediated by varying combinations of positive and negative regulation (24) and is distinct from the case of Hoxb-2, in which segmental expression in r3 and r5 is directly generated by the spatially restricted expression of the upstream activator Krox-20 in r3 and r5 (10, 11). Negative regulation, such as that of Hoxb-1, could have important implications for maintaining rhombomere identities during boundary formation. Before boundary formation, when clonal descendants of cells can still contribute to more than one segment, some cells in presumptive r3 and r5 will be expressing r4 markers. In later stages of development, expression of these markers is confined to r4, a result achievable by cell death, cell sorting, or respecification (or all three). Our experiments show that the down-regulation of endogenous Hoxb-1 expression in r3 and r5 occurs while those cells are still capable of expressing Hoxb-1. This may serve to separate their identities from that of cells in r4, which would occur as a part of the process of respecification of cells from an r4 to an r3/r5 identity. Therefore, the molecular mechanisms involving the RARE that repress Hoxb-1 expression in r3/r5 may in turn serve to generate the boundaries of rhombomere compartments.

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- 14. A 7.5-kb Eco RV fragment of the Hoxb-1 gene from cosmid 8D (25) was inserted into the pKS+ vector. Constructs 1, 2, and 12 to 20 contain the lacZ gene in frame in the first exon. In cn 12 to 20, a 2.0-kb r3/r5 enhancer (11) from Hoxb-2 was inserted at the 3' end. Constructs 13 to 15 were digested with Apa I, Sac I, and Spe I at their 5' ends, respectively. In cns 16, 17, 19, and 20, fragments were inserted into the Spe I site of cn 15. Construct 18 contains point mutations in the RARE (19). Constructs 10, 11, 19, and 20 have fragments of chick Hoxb-1 inserted into the Spe I site of cn 15. Constructs 3 (sense), 7, 10, and 11 contain regulatory regions in the promoterless vector pZA (26). For cns 3 (antisense), 4 to 6, 8, and 9, fragments were inserted into the Spe I site of pGZ40, which uses the human β-globin promoter (26). In cn 17, a 750-bp fragment from the mouse

anti-Müllerian hormone gene was inserted in cn 15.

- 15. Animal experiments were done according to procedures regulated under the project licenses issued by the United Kingdom Home Office and by the Genetic Manipulation Advisory Group. Generation and analysis of transgenic mice from F₁ hybrids (CBA × C57BL/6) were as described (8, 16, 27, 28). The anaesthetic in embryo transfer was Avertin at a dose of 0.015 to 0.017 ml of a 2.5% solution per gram of body weight, injected intraperitoneally. RA was administered to pregnant females (7.5 dpc) by gavage application of 200 μ l of sesame seed oil containing all *trans*-RA for a final dose of 20 mg per kilogram of body weight, as described (8).
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 The EMSAs were done with purified human RARα
- and RARγ and with human RXRα, as described (29).
 The point mutations in the RARE were generated by
- inverse polymerase chain reaction (PCR). Oligos used were: 5'-ACCCTTACCTGCCTGGACTTGC-CCTAGCTC and 5'-ACCGGGAGGGATCTCCCGTA-AACAGTGGC. The PCR product was purified and selfligated and mutations were confirmed by complete sequencing. The fragment containing the point mutations replaced the Apa I–Sac I fragment of cn 13.
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Control of Thalamocortical Afferent Rearrangement by Postsynaptic Activity in Developing Visual Cortex

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The formation of specific connections in the developing central nervous system is thought to result from mechanisms that increase the strengths of synapses at which pre- and postsynaptic activity are correlated and decrease it otherwise. In the visual cortex, initially widespread inputs normally sort out into eye-specific patches during early life. If only one eye can see during this period, its patches are much larger than normal, and patches from the occluded eye become much smaller. Anatomical experiments here show that closedeye inputs expand within a region of cortex that is silenced, establishing that inhibition of common target cells gives less active inputs a competitive advantage.

Ocular dominance columns (alternate patches of visual cortex that receive input from the two eyes) form in normal development by a selective loss of the initially widespread and overlapping branches of the geniculocortical afferents and the selective growth of new arbor in territory dominated by one eye or the other (1). During this period of segregation, cortical neurons and their input afferents show dramatic plasticity in response to changes in the visual environment (2). Closing the lid of one eye during this period in early postnatal life causes most visual cortical neurons to lose response to the deprived eye and respond instead exclusively to the nondeprived eve (3). Such monocular deprivation also causes an anatomical expansion of the cortical territory into which geniculocortical afferents carrying information from the nondeprived eye terminate, as well as a complementary shrinkage of territory serving the deprived eye (4).

Experiments in which neural activity in visual cortex was suppressed by the sodium channel blocker tetrodotoxin demonstrated that ocular dominance plasticity requires neural activity in the cortex (5). Furthermore, microelectrode experiments indicated that the suppression of postsynaptic visual cortical activity by cortical infusion with the γ -aminobutyric acid type A (GABA_A)-receptor agonist muscimol during the period of monocular deprivation shifted ocular dominance toward the deprived eye (6). This result suggested that postsynaptic activity in the cortex plays a crucial role in ocular dominance plasticity, but it was not clear whether it revealed mechanisms that nor-

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mally operate during development or was, instead, a pharmacological curiosity. Because the formation of cortical columns in normal development ultimately involves anatomical plasticity, the present study aimed to determine whether the pharmacological control of postsynaptic activity also controlled the anatomical rearrangements of presynaptic geniculocortical afferents. For this purpose, we labeled geniculocortical afferent termination in regions of layer IV of the visual cortex in which postsynaptic activity was inhibited during the period of monocular deprivation, and we compared the patterns of labeling with those in the control cortex.

Postsynaptic activity in one hemisphere of the primary visual cortex of 4-week-old kittens was inhibited by infusing the cortex with muscimol solution. Four weeks is midway through the process of ocular dominance column segregation. Two days after starting the infusion, kittens were deprived of vision in the contralateral eye by eyelid suture. Geniculocortical afferent termination in the visual cortex was labeled by transneuronal transport of [³H]proline, which was injected into one eye (7). In normal older animals, the patches of cortex labeled transneuronally were almost precisely complementary, which allows one to infer the distribution of the unlabeled eye's inputs in experimental material (Fig. 1). After 2 or 4 weeks of muscimol infusion, the region inactivated by muscimol was delineated physiologically by mapping the activity of cortical cells with microelectrodes (Fig. 2A). Drug infusion was then stopped, and the ocular dominance of cortical cells was determined after the effects of muscimol had subsided (8). Thereafter, the ani-

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