

Axial (HNF3 β) and retinoic acid receptors are regulators of the zebrafish *sonic hedgehog* promoter

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The signalling molecule Sonic hedgehog is involved in a multitude of distinct patterning processes during vertebrate embryogenesis. In the nascent body axis of the zebrafish embryo, *sonic hedgehog* is co-expressed with *axial* (HNF3 β in mammals), a transcription regulator of the winged helix family. We show here that misexpression of *axial* leads to ectopic activation of *sonic hedgehog* expression in the zebrafish, suggesting that *axial* is a regulator of *sonic hedgehog* transcription. The *sonic hedgehog* gene was cloned from zebrafish and its promoter was characterized with respect to activation by *axial*. Expression of *axial* or rat HNF3 β in HeLa cells results in activation of co-transfected *sonic hedgehog* promoter–CAT fusion genes. This effect is mediated by two Axial (HNF3 β) recognition sequences. We furthermore identified a retinoic acid response element (RARE) in the *sonic hedgehog* upstream region which can be bound by retinoic acid receptor (RAR) and retinoid X receptor (RXR) heterodimers *in vitro* and confers retinoic acid inducibility to the *sonic hedgehog* promoter in the HeLa cell system. Our results suggest that both Axial (HNF3 β) and retinoic acid receptors are direct regulators of the *sonic hedgehog* gene.

Keywords: *axial* (HNF3 β)/floor plate/notochord/retinoic acid/*sonic hedgehog*

Introduction

Cell–cell signalling plays a crucial role in the differentiation of specific cell types during development. Sonic hedgehog (Shh), one of several vertebrate members of the hedgehog family of signalling molecules, has been implicated in the induction of ventral cell fates in the neural tube, anteroposterior specification of the limb, patterning of the somites as well as establishment of asymmetry across the left–right axis (Echelard *et al.*, 1993; Krauss *et al.*, 1993; Riddle *et al.*, 1993; Fan and Tessier-Lavigne, 1994; Johnson *et al.*, 1994; Laufer *et al.*, 1994; Roelink *et al.*, 1994; Ekker *et al.*, 1995; Ericson *et al.*, 1995; Fan *et al.*, 1995; Levin *et al.*, 1995; Münsterberg *et al.*, 1995; Chiang *et al.*, 1996; Currie and Ingham, 1996).

This multitude of distinct processes controlled by Shh during embryogenesis is reflected in both temporally and

spatially restricted expression of the *shh* gene (Echelard *et al.*, 1993; Krauss *et al.*, 1993; Riddle *et al.*, 1993; Roelink *et al.*, 1994; Ekker *et al.*, 1995). Several lines of evidence show that the spatially restricted expression is essential for orderly inductive patterning of embryonic tissues by Shh. Aberrant expression of *shh* in the neural tube, for example, results in ectopic activation of target genes at dorsal aspects of the neural tube (Echelard *et al.*, 1993; Krauss *et al.*, 1993; Barth and Wilson, 1995; Ekker *et al.*, 1995; MacDonald *et al.*, 1995; Concordet *et al.*, 1996; Hauptmann and Gerster, 1996). Similarly, ectopic expression of *shh* in the anterior wing bud causes mirror image duplications in the wing (Riddle *et al.*, 1993; Laufer *et al.*, 1994). Also, the absolute levels of Shh protein secreted from a localized source may be crucial; high levels of Shh protein induce floor plate, whereas lower levels induce motor neurons in neural plate explants (Marti *et al.*, 1995; Roelink *et al.*, 1995).

Little is known about the mechanisms underlying the spatially restricted expression of *shh*. In the developing body axis of vertebrate embryos, *shh* expression is tightly correlated with expression of HNF3 β , a transcription regulator of the winged-helix class (Ang *et al.*, 1993; Echelard *et al.*, 1993; Krauss *et al.*, 1993; Monahagan *et al.*, 1993; Riddle *et al.*, 1993; Ruiz i Altaba *et al.*, 1993; Sasaki and Hogan, 1993; Strähle *et al.*, 1993; Roelink *et al.*, 1994; Ekker *et al.*, 1995). Targeted mutation of HNF3 β in mouse results in impaired notochord development and loss of *shh* expression (Ang and Rossant, 1994; Weinstein *et al.*, 1994). Overexpression of HNF3 β induces aberrant activation of *shh* in the neural tube of *Xenopus* and mouse embryos (Ruiz i Altaba *et al.*, 1995a; Hynes *et al.*, 1995). In the chicken wing bud and the zebrafish fin bud, administration of retinoic acid leads similarly to activation of *shh*, indicating retinoic acid receptors (RARs) as further potential regulators of *shh* expression (Riddle *et al.*, 1993; Helms *et al.*, 1994; Akimenko and Ekker, 1995; Stratford *et al.*, 1996). It remains unclear, however, whether *shh* expression is regulated directly by HNF3 β and RARs or whether the experimental manipulations of the embryos affect *shh* expression indirectly.

In the zebrafish embryo, as in other vertebrate embryos, *axial* (*axl*), the zebrafish homologue of HNF3 β , and *shh* are expressed in overlapping domains in the embryonic shield (zebrafish organizer), the notochord and the floor plate (Krauss *et al.*, 1993; Strähle *et al.*, 1993, 1996). Onset of *axl* expression in the embryonic shield precedes that of *shh* (Krauss *et al.*, 1993; Strähle *et al.*, 1993), consistent with the notion that *shh* expression might be regulated by *axl*. To test this, we misexpressed *axl* in the zebrafish embryo and show here that this results in ectopic activation of *shh* expression in a wide variety of different cells normally not expressing the gene. We cloned the *shh* gene from zebrafish and characterized its promoter in a

cell system. Evidence is provided that Axl (HNF3 β) and RARs are direct regulators of the *shh* promoter.

Results

Ectopic expression of *axl* activates *shh* transcription

The spatial and temporal relationships between *axl* and *shh* in the developing axis of the zebrafish embryo suggest that *axl* may regulate *shh* transcription. To test this possibility, we constructed an expression plasmid (pCMVax1) in which the *axl* cDNA is placed under the control of the cytomegalovirus (CMV) enhancer/promoter (Boshart *et al.*, 1985). Plasmid DNA injected into 1–4 cell stage embryos is highly mosaically distributed (Westerfield *et al.*, 1992) and, since the CMV control region is active in many different cell types in the zebrafish embryo (J.Wegner, U.Strähle and M.Westerfield, unpublished), such injection generates embryos mosaic for *axl*-expressing cells throughout the body. Injected embryos were fixed after either 12 or 24 h development and hybridized with a *shh* probe. Embryos at 12 h of development show strong ectopic expression of *shh* at lateral and ventral positions of the embryo in both the ectoderm and the mesendoderm (Figure 1A and B). At 24 h of development, ectopic *shh* expression is detectable in pCMVax1-injected embryos ($n = 94$) in many different cell types (Figure 1C) that do not usually express *shh* (Krauss *et al.*, 1993). These ectopic sites of expression include cells of the yolk sac (Figure 1D), the gut (Figure 1E), the dorsal aspects of the neural tube (Figure 1F) and the somites (Figure 1G). Embryos ($n = 53$) injected with CMV β (MacGregor and Caskey, 1989), in which the *axl* coding region has been replaced by *lacZ*, did not show ectopic activation of *shh* expression (data not shown).

To assess the distribution of misexpressed Axl with respect to ectopic activation of *shh* in injected embryos, the *axl* coding region was tagged with six copies of the myc epitope recognized by monoclonal antibody 9E10 (Evan *et al.*, 1985). In 11 h old embryos ($n = 54$) double-labelled with antibody 9E10 and *shh* RNA probe, a large number of cells ectopically expressing *shh* showed co-expression of myc-axial. However, a substantial number of cells expressed 9E10 immunoreactivity but did not show detectable *shh* expression. Similar results were obtained when CMV β was co-injected with pCMVax1, the untagged version of the *axl* expression plasmid (data not shown). This indicates that *shh* cannot be activated in all cells misexpressing *axl*. A small fraction of cells was also found which showed *shh* expression but in which 9E10 immunoreactivity could not be detected. In summary, the ectopic activation of *shh* in a predominantly cell-autonomous fashion suggests that Axl may be a direct regulator of the *shh* gene.

Cloning of the *shh* gene

To investigate the molecular mechanism of the observed activation of *shh* by Axl in the embryo, we screened a zebrafish genomic library (Molven *et al.*, 1991) with a *shh* cDNA fragment (Krauss *et al.*, 1993) to isolate the *shh* promoter. Two clones (λ shh5 and λ shh7) were isolated and shown to overlap by restriction mapping (Figure 2).

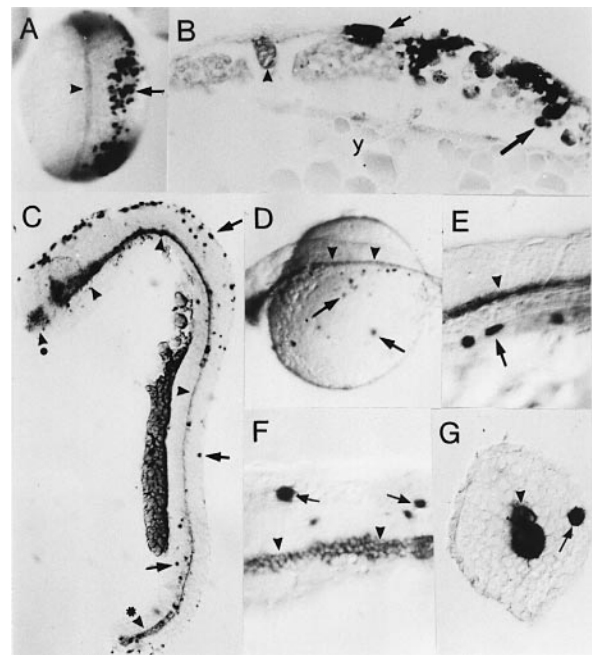


Fig. 1. Ectopic expression of *axl* leads to transcription of *shh* in a wide variety of cell types. Plasmids carrying the *axl* cDNA under control of the CMV enhancer/promoter (pCMVax1) were injected into one of the blastomeres of cleavage stage embryos up to the 4-cell stage. Embryos were fixed either after 12 (A and B) or 24 h development (C–G) and hybridized to the *shh* probe. (A) Five-somite stage embryo injected with pCMVax1. Strong activation of *shh* expression in lateral parts of the embryo (arrow) is evident in addition to normal expression along the dorsal midline (arrowhead). Dorsal view, anterior up. (B) Transverse section through the embryo shown in (A). Cells from ectodermal (thin short arrow) and mesodermal (thick arrow) origin express *shh* in injected embryos. The arrowhead points to *shh* expression in the notochord and neuroectoderm. Dorsal up. The position of the yolk is indicated by y. (C) pCMVax1-injected embryo stained with the *shh* probe at 24 h of development. *shh* is normally expressed in the ventral diencephalon (arrowhead with dot) and in the ventral neural tube from the midbrain to the tail (arrowheads). The nascent notochord in the tail bud also expresses *shh* still at this stage in uninjected embryos (arrowhead with asterisk). In addition to the normal expression, numerous cells express *shh* ectopically (arrows point out some cells) in embryos injected with pCMVax1. The orientation is anterior up, dorsal left. The yolk was removed. (D) Ectopic activation of *shh* in cells of the yolk sac (arrow). (E) Ectopic expression of *shh* in the endoderm (arrow heads). (Lateral view at the level of the anterior trunk, dorsal up; arrowhead, normal *shh* expression in neural tube.) (F) Ectopic activation of *shh* in the anterior dorsal neural tube. (G) Transverse section through the tail of a pCMVax1-injected embryo showing ectopic activation of *shh* in a somitic cell (arrow). The arrowhead indicates normal expression in neural tube and notochord.

After subcloning into plasmid vectors, the exon–intron structure was established by sequence analysis using primers complementary to the cDNA sequence (Figure 2). Both phage inserts span the entire *shh* coding region. The mature *shh* mRNA is encoded by three exons, and the exon–intron junctions are conserved among zebrafish *shh*, mouse *shh* and *Drosophila hedgehog* (Lee *et al.*, 1992; Mohler and Vani, 1992; Chiang *et al.*, 1996). The start sites were mapped by RNase protection analysis and 5' RACE. Two major transcription start sites were found, one 253 bp and the other 339 bp upstream of the translation initiation codon (Figure 3) corresponding roughly to the ends of previously isolated cDNA clones (Krauss *et al.*, 1993; Roelink *et al.*, 1994). The second start site will be

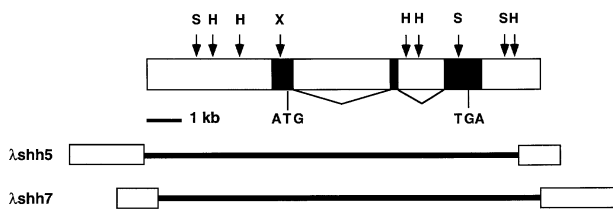


Fig. 2. Genomic structure of the zebrafish *shh* gene. The exons (black boxes) covering the coding region are outlined (ATG, translation initiation codon; TGA, translation termination codon). Exon 1 comprises the 5' non-coding sequence and the first 100 amino acids, exon 2 encodes amino acids 101–187 and exon 3 encodes amino acids 188–418. The two independently isolated phages (λ shh5 and λ shh7) from which the map was deduced are outlined. Restriction sites indicated with vertical arrows are: S, *SalI*; H, *HindIII*; X, *XhoI*. Bar, 1 kb.

referred to as position +1. Both start sites are used equally during development at least up to 48 h (data not shown).

A 376 bp *shh* promoter fragment is sufficient for transactivation by zebrafish *axl* and rat HNF3 β

To test whether *axl* transactivates the *shh* promoter, a 2.5 kb *SalI*–*XhoI* fragment containing ~2.2 kb of upstream sequence was cloned in front of the coding region of chloramphenicol acetyl transferase (CAT), and co-transfection experiments in HeLa cells were carried out. Co-transfection of the –2200shhCAT reporter construct with pCMVaxl but not with the control plasmid CMV β led to a strong increase in CAT activity (Figure 4). Transfection of –563shhCAT containing 563 bp of *shh* promoter sequence elicited the same response, when co-transfected with pCMVaxl. Replacement of the *shh* sequences by the herpes simplex thymidine kinase promoter (–105 to +51; McKnight and Kingsbury, 1982) did not show transactivation by *axl* (data not shown), suggesting that the increase in CAT activity in response to *axl* is mediated by the *shh* sequences inserted upstream of the CAT coding region. To delineate the sequences required for transactivation by Axl more precisely, 5' deletions of the *shh* upstream region were generated and tested by co-transfection with pCMVaxl or CMV β . Plasmid –155shhCAT harbouring 376 bp of *shh* sequence shows the same response to co-expression of *axl* as the plasmids containing larger *shh* inserts (Figure 4). Thus, 376 bp of *shh* sequence comprising 155 bp upstream of the transcription start site is sufficient to mediate transactivation by Axl.

Zebrafish *axl* is closely related to mammalian HNF3 β , sharing 97% sequence homology in the winged-helix DNA-binding domain (Lai *et al.*, 1991; Strähle *et al.*, 1993). It is thus likely that HNF3 β and Axl have similar DNA recognition sequences. To test whether HNF3 β activates the zebrafish *shh* promoter, increasing amounts of CMVHNF3 β coding for rat HNF3 β under control of the CMV promoter/enhancer (Pani *et al.*, 1992) were transfected into HeLa cells together with a constant amount of –155shhCAT. For comparison, increasing amounts of pCMVaxl were transfected with –155shhCAT in parallel. HNF3 β (Figure 5A) increases expression of CAT activity from –155shhCAT similarly to Axl (Figure 5B). Thus, Axl and HNF3 β appear also to be functionally equivalent and are likely to recognize similar or identical DNA-binding sequences.

The sequence of the promoter region (Figure 3) was

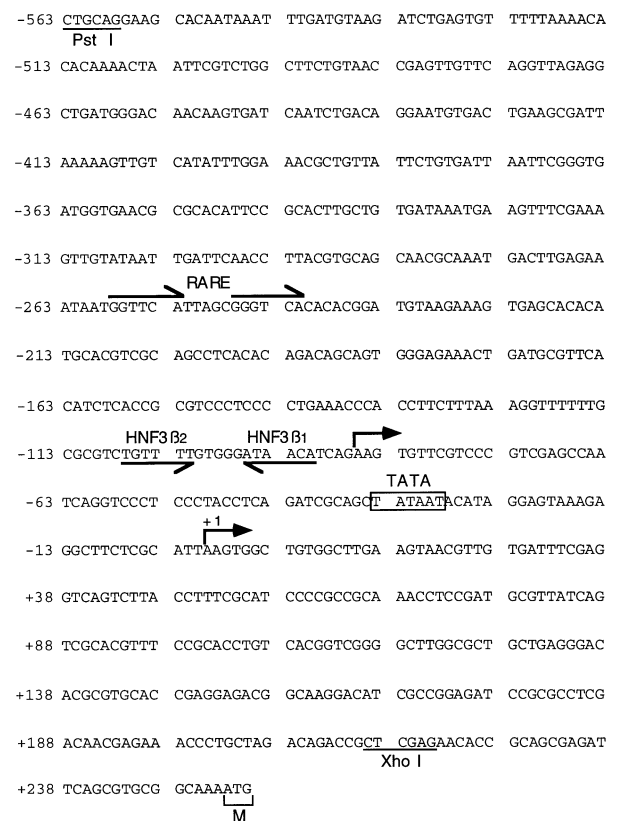


Fig. 3. Sequence of the *shh* promoter. Transcription start sites and translation initiation codon are indicated by horizontal arrows and M, respectively. The HNF3 β -binding sites (HNF3 β 1 and HNF3 β 2) which show strong homology to the HNF3 β recognition sequence are located upstream of the start sites. The DR5 RARE and a putative TATA box are indicated.

searched for homologies to the HNF3 β consensus sequence [5'-(G/A) (T/C) (C/A) A A (C/T) A-3']; (Overdier *et al.*, 1994; Roux *et al.*, 1995; Kaufmann and Knöchel, 1996)]. Two HNF3 β -binding site homologies (HNF3 β 1 and 2) reside within the 155 bp upstream sequence mediating transactivation by Axl and HNF3 β (Figure 3).

Two closely spaced HNF3 β -binding sites are required for activation of the *shh* promoter

To test whether the putative HNF3 β -binding sequences may be relevant to activation of the *shh* promoter, we first investigated whether they bind Axl and HNF3 β protein by gel retardation analysis. An Axl–GST fusion protein encoding full-length Axl and a HNF3 β –GST fusion protein, which represents a truncated version of rat HNF3 β but which shows DNA-binding specificity identical to full-length protein (Overdier *et al.*, 1994), were expressed in *Escherichia coli* and partially purified. Recombinant Axl–GST (Figure 6A, left panel) or HNF3 β –GST (Figure 6B, left panel) were incubated with oligonucleotides covering the binding site homologies HNF3 β 1 and 2 (Figure 6, Oligo 1 and 2, respectively) or with an oligonucleotide (Figure 6, Oligo 3) containing the sequence from –143 to –112 of the *shh* upstream region. Both recombinant proteins specifically retarded oligonucleotides containing elements HNF3 β 1 and HNF3 β 2 (Figure 6A and B, left panels). Oligonucleotide 3 did not bind either fusion protein. Mutation of the HNF3 β consensus

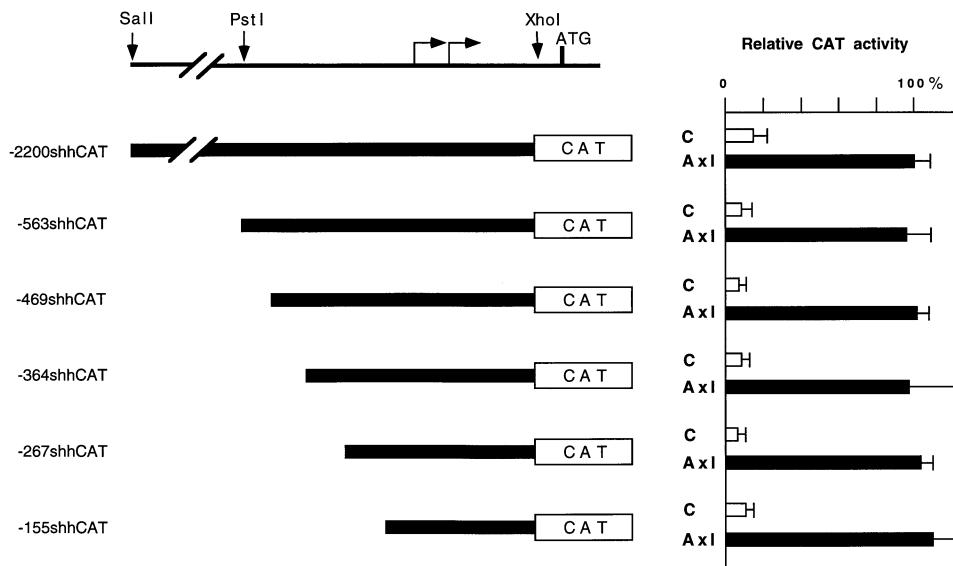


Fig. 4. Deletion analysis of the *shh* promoter. A 155 bp *shh* promoter sequence is sufficient for transactivation by Axl in HeLa cells. HeLa cells were transfected with *shh*-CAT fusion genes together with the *axl* expression plasmid pCMVaxl (Axl) or the control plasmid CMV β (C). In CMV β , the *axl* coding region has been replaced by *lacZ*. Plasmids -2200shhCAT and -563shhCAT contain the 2.5 kb *SalI*-*XhoI* and the 0.8 kb *PstI*-*XhoI* *shh* fragments in front of the coding region of CAT. The extent of *shh* sequence in 5' deletion plasmids -469shhCAT, -364shhCAT, -267shhCAT and -155shhCAT is indicated by black bars in the schematic drawing of the constructs. The transcription start sites (horizontal arrows) and relevant restriction sites (*XhoI*, *PstI*, *SalI*) are indicated. CAT enzymatic activity was determined in extracts from transfected cells after 48 h. The bar diagram shows the average of three independent transfections done in parallel. Standard deviations are indicated. Relative CAT activity was calculated by normalizing the protein concentration and setting the CAT activity obtained from -2200shhCAT as 100%.

sequences (Oligo 1' and 2' respectively; see Materials and methods) by a series of base changes abolishes binding of Axl-GST (Figure 6A, right panel) and HNF3 β -GST (Figure 6B, right panel). Oligonucleotides containing HNF3 β 1 and HNF3 β 2 sites are bound specifically by proteins present in whole cell extract prepared from 24 h old zebrafish embryos (Figure 6C, left panel). Formation of a specific complex was abolished when HNF3 β 1 and HNF3 β 2 sites were mutated (Figure 6C, right panel), suggesting that proteins are present in whole cell extract that interact with the same sequence as Axl and HNF3 β .

Thus, elements HNF3 β 1 and 2 may be important for activation of the *shh* promoter by Axl and HNF3 β . To assess this hypothesis, element HNF3 β 1 or element HNF3 β 2 on their own or in combination were mutated by the same cluster of nucleotide changes in the centre of each element which abolished protein binding *in vitro*. Mutation of either HNF3 β 1 (-563shhCAT-M1) or HNF3 β 2 (-563shhCAT-M2) led to a decrease in CAT activity by ~50%, relative to -563shhCAT when transfected into HeLa cells together with pCMVaxl (Figure 7A). -563shhCAT-DM carrying mutations in both elements shows a 5-fold reduction of CAT activity in comparison with the wild-type construct -563shhCAT (Figure 7A). Identical results were obtained in transfection with CMVHNF3 β ; CAT activity was moderately decreased by each single mutation, while mutation of both elements led to a strong reduction of CAT activity (Figure 7B). The basal level of expression in the absence of Axl or HNF3 β was not affected by the mutations. In summary, these data show that elements HNF3 β 1 and 2 mediate transactivation of the *shh* promoter by Axl and HNF3 β . As a weak response to co-transfection of either pCMVaxl or CMVHNF3 β remains in the double mutant, however, other sequences may marginally contribute to transactivation.

Retinoic acid receptors activate the *shh* promoter

In zebrafish embryos treated with retinoic acid (RA), *shh* expression is ectopically activated in the anterior fin buds and its expression in the neural tube of the tail is expanded (Akimenko and Ekker, 1995). Implantation of beads soaked in RA into the anterior limb bud of chicken embryos leads similarly to ectopic activation of *shh* expression (Riddle *et al.*, 1993), suggesting that *shh* may be regulated by RARs. We noted a DR5-type retinoic acid response element (Figure 3; RARE, GGTTCATTA-GCGGGTCA) between position -242 and -258 in the *shh* upstream region. To test whether this element confers RA inducibility to the *shh* promoter, we first tested whether RARs can bind to the element by gel retardation experiments. RARs bind to DR5-type target sequences as heterodimers between RARs and RXRs (Yu *et al.*, 1991; Berrodin *et al.*, 1992; Bugge *et al.*, 1992; Kliewer *et al.*, 1992; Marks *et al.*, 1992; Zhang *et al.*, 1992). An oligonucleotide harbouring the *shh* DR5 element was specifically retarded when incubated with a mixture of recombinant mouse RAR α and RXR α (Figure 8A, Oligo 2, left panel). Oligonucleotides containing a single repeat of the hexamers AGGTCA or TGGTCA, found at position +36 and -478 in the *shh* promoter upstream region, respectively, did not show retardation when incubated with RAR α /RXR α (Figure 8A, Oligo 1 and 3). Mutation of the RAR-binding site in oligonucleotide 2 prevented complex formation (Figure 8A, left panel, Oligo 2'). Oligonucleotide 2 is specifically bound by proteins present in whole cell extract prepared from 24 h old zebrafish embryos (Figure 8B, left panel) and binding of extract protein is abolished when the DR5 element is mutated (Figure 8B, right panel, Oligo 2').

To test whether the zebrafish *shh* promoter is inducible by RA, the -563shhCAT expression plasmid was co-

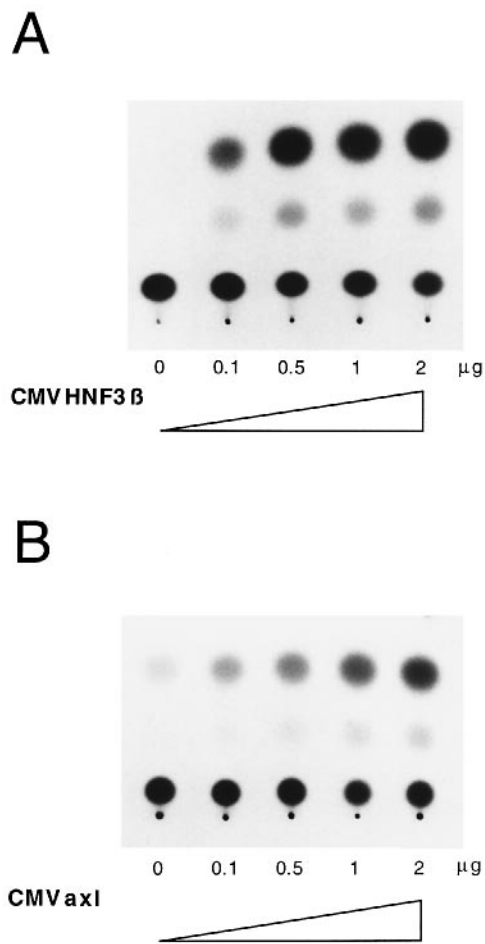


Fig. 5. Rat HNF3 β induces CAT expression from -155shhCAT in a manner similar to zebrafish *Axl*. Increasing amounts of CMVHNF3 β (A) or pCMVaxl (B) were transfected into HeLa cells together with -155shhCAT. CAT enzymatic activity was determined by assaying 400 μ g of extract protein. The autoradiographs were exposed for 24 h.

transfected into HeLa cells with plasmid RAR β encoding human RAR β 2 (Zelent *et al.*, 1989). The HeLa cells employed express RXRs endogenously (Leid *et al.*, 1992). Transfection of plasmid RAR β and administration of all-*trans* retinoic acid (t-RA, 10^{-7} M) resulted reproducibly in a 2.5- to 4-fold increase in CAT activity in comparison with controls exposed to ethanol vehicle alone (Figure 9). This effect is dependent on co-transfection of plasmid RAR β as t-RA did not cause an increase in CAT activity in the absence of co-transfected RAR β . Administration of 9-*cis* RA (10^{-7} M) that activates RXR did not induce an increase in CAT activity, suggesting that endogenous RXRs on their own cannot mediate the effect. Also, co-transfection of plasmid RXR α encoding human RXR α (Elder *et al.*, 1992) did not result in an increase in CAT activity in the presence of 9-*cis* RA (Figure 9).

To test whether the induction of CAT activity by RAR β in the presence of t-RA is mediated by the DR5 RARE located at position -258/-242 of the *shh* upstream sequence, the element was mutated in -563shhCAT by the same series of nucleotide exchanges which abolished protein binding *in vitro*. The resulting plasmid -563shhCAT-DR5-M did not show a response to co-transfection of plasmid RAR β and administration of t-RA

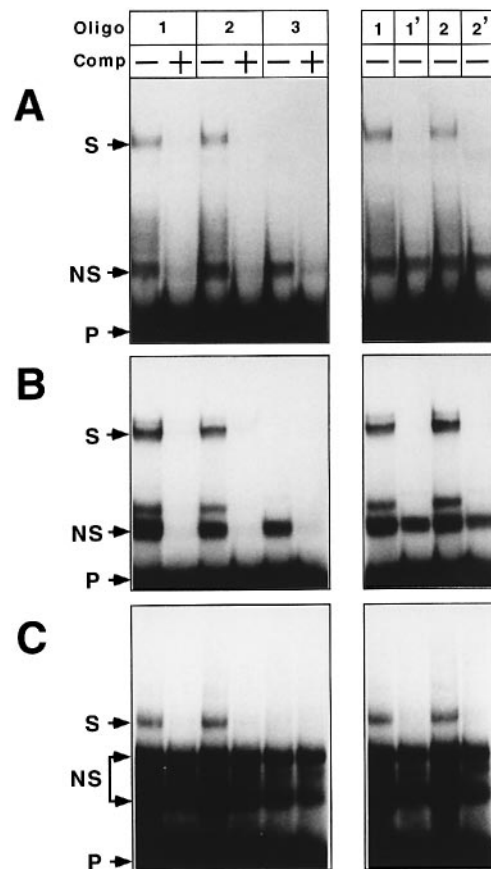


Fig. 6. *Axl* and HNF3 β bind to *shh* promoter sequences. Gel retardation analysis was carried out with partially purified zebrafish *Axl*-GST (A), rat HNF3 β -GST (B) or whole cell extract from 24 h old zebrafish embryos (C). Oligonucleotides 1 and 2 containing HNF3 β consensus elements HNF3 β 1 (from -103 to -74) and HNF3 β 2 (from -126 to -97) formed specific complexes (S, arrow) while oligonucleotide 3 (from -143 to -112) was not shifted. Retarded bands were abolished by addition of a 100-fold excess of cold competitor oligonucleotide (+ lanes). Oligonucleotides containing a cluster of point mutations in the HNF3 β 1 (Oligo 1') or HNF3 β 2 (Oligo 2') sites did not form specific complexes. S, specific complex; NS, complex due to non-specific binding; P, unbound oligonucleotide; Oligo, oligonucleotides.

(Figure 9). In summary, these data suggest that the *shh* promoter can be regulated by RARs in response to t-RA.

Discussion

We present evidence that *shh* is responsive to misexpression of *axl* in a wide variety of distinct cell types, suggesting that *axl* is a crucial determinant of *shh* expression. By analysis of the *shh* promoter in HeLa cells, two *Axl*/HNF3 β -binding sites were found to be required for transactivation of the *shh* promoter, strongly suggesting that *Axl*/HNF3 β is a direct regulator of the *shh* gene. We furthermore identified a RARE in the *shh* promoter which is functional in the HeLa expression system, indicating that the *shh* promoter is under direct transcriptional control by RARs.

Despite the fact that direct proof by mutation is lacking in the zebrafish embryo, several lines of evidence strongly argue in favour of the notion that *axl* indeed controls *shh* expression *in vivo* during development of the axis in the

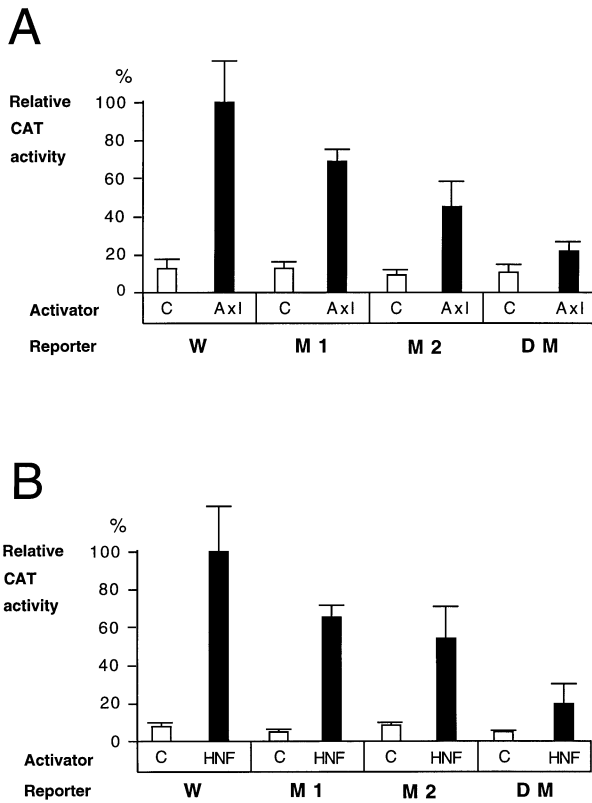


Fig. 7. Mutation of HNF3 β -binding sites abolishes transactivation of the *shh* promoter by AxI and HNF3 β . -563shhCAT fusion constructs in which the HNF3 β -binding sites HNF3 β 1 (M1) or HNF3 β 2 (M2) alone or both together (DM) had been mutated were transfected into HeLa cells with either pCMVaxI (A, AxI) or with CMVHNF3 β (B, HNF). In controls (C) pCMVaxI or CMVHNF3 β were replaced by CMV β . Bars represent the average of the results from three transfections carried out in parallel. CAT activity obtained from the wild-type promoter construct (-563shhCAT) was set to 100%, and results from mutant constructs were normalized accordingly. Standard deviations are indicated.

zebrafish embryo. First, *axl* expression precedes that of *shh* in the embryonic shield, and the two genes have overlapping expression patterns (Krauss *et al.*, 1993; Strähle *et al.*, 1993, 1996). Second, ectopic expression of *axl* leads to strong activation of *shh* in cells that normally do not express *shh*. Third, *shh* and *axl* expression are affected similarly in midline-defective mutant embryos (Krauss *et al.*, 1993; Strähle *et al.*, 1993, 1996; Talbot *et al.*, 1995). In the mouse embryo, the effects of targeted mutation of HNF3 β are in agreement with our findings in the zebrafish embryo. HNF3 β ^{-/-} mouse embryos show loss of the notochord, lack of *shh* expression and impaired patterning of the ventral neural tube (Ang and Rossant, 1994; Weinstein *et al.*, 1994). It is, however, not clear from these studies in the mouse whether loss of *shh* expression and disturbed neural patterning is a direct effect of the mutation in HNF3 β or whether it is an indirect consequence of impaired notochord development. Our results show that AxI (HNF3 β) activates *shh* expression in both the mesoderm and the neuroectoderm, indicating a function of AxI (HNF3 β) in regulation of *shh* in both structures. Ectopic activation of *shh* in the neuroectoderm in response to misexpression of *axl*/HNF3 β has also been noted in *Xenopus* embryos (Ruiz i Altaba *et al.*,

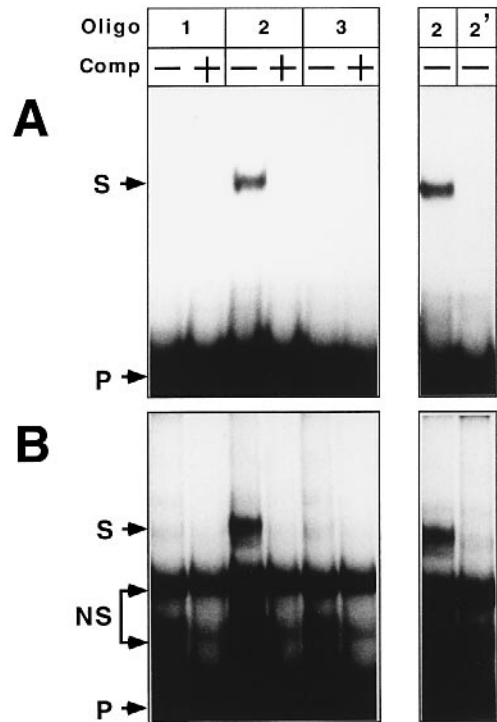


Fig. 8. RAR/RXR complexes bind to the DR5 RARE in the *shh* promoter. Oligonucleotides were incubated with purified recombinant mouse RAR α /RXR α or whole cell extract prepared from 24 h old zebrafish embryos. Oligonucleotide 2 (from -265 to -236) harbouring the DR5 RARE of the *shh* promoter region showed specific complex formation (S, arrow) which was competed by a 100-fold excess of cold oligonucleotide 2 (+ lanes). Oligonucleotides 1 (from +31 to +60) and 3 (from -490 to -461) which contain an imperfect single repeat of the hexamer motif characteristic of RAREs did not form complexes with RAR α /RXR α or proteins present in whole cell extract. Mutation of the RARE sequences by a cluster of point mutations (Oligo 2') abolished specific complex formation of Oligo 2 with recombinant proteins (A, right panel) and proteins from whole cell extract (B, right panel). S, specific complex; NS, complex due to non-specific binding; P, unbound oligonucleotide; Oligo, oligonucleotide.

1995a). Ectopic activation of *shh* in the mesoderm, however, has not been reported in these studies. It is not clear whether this is a peculiarity of *Xenopus* embryos. HNF3 β is expressed later in the axis of *Xenopus* than of zebrafish and mouse embryos, and another related, but distinct factor *pintallavis*/XFKH1/XFD1 has been proposed to substitute functionally for the lack of early HNF3 β expression (Ruiz i Altaba and Jessel, 1992).

We noted ectopic activation of *shh* in many different cell types of injected embryos. There are, however, cells in an unmanipulated embryo that express *axl* but not *shh*. For example, cells scattered in the ventrolateral hypoblast during gastrulation express *axl* weakly but do not show detectable *shh* expression (Strähle *et al.*, 1996). Also, expression of *shh* in the spinal cord of the 24 h old embryo is restricted to the floor plate whereas *axl* expression is found in addition in cells on each side of the floor plate (Strähle *et al.*, 1996). Expression levels of *axl* may be critical for *shh* activation; in the gastrula, ventrolateral hypoblast cells express *axl* at levels lower than in the axis. Alternatively, the response of *shh* to *axl* could be subject to cell-specific modulation that has been suggested

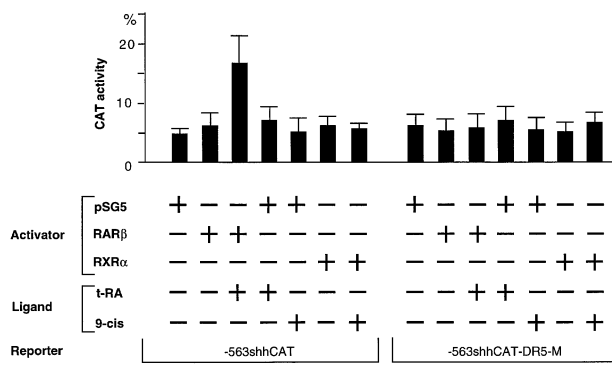


Fig. 9. The DR5 RARE renders the *shh* promoter inducible by retinoic acid. The wild-type -563shhCAT or a mutant derivative -563shhCAT-DR5-M, in which the DR5 RARE at position -258/-242 had been mutated by a cluster of nucleotide changes, were transfected into HeLa cells in combination with either plasmid RAR β , RXR α or pSG5 without receptor cDNA inserted. Cells were treated with either 10^{-7} M all-*trans* retinoic acid (t-RA), 10^{-7} M 9-*cis* retinoic acid (9-cis) or ethanol vehicle alone (-). Bars indicate the percentage conversion of chloramphenicol to its acetylated forms with 400 μ g of protein extracted from cells 48 h after transfection. Bars represent the mean of three independent transfections carried out in parallel. Standard deviations are indicated. The experiment shown is representative of three experiments carried out independently.

to account for the inability of *axl*-expressing cells lateral to the floor plate to express *shh* (Ruiz i Altaba *et al.*, 1995a). In agreement with these restrictions, we found cells in injected embryos which expressed Axl ectopically but did not show detectable *shh* expression.

There are sites in the embryo where *shh* expression is established independently from *axl*. The ventral diencephalon, the posterior endoderm and the posterior region of the fin bud express *shh* but do not express *axl* at detectable levels (Krauss *et al.*, 1993; Strähle *et al.*, 1993, 1996; MacDonald *et al.*, 1994). Other closely related members of the winged-helix family are expressed in the vertebrate embryo in a partially overlapping pattern (Dirksen and Jamrich, 1992; Knöchel *et al.*, 1992; Ruiz i Altaba *et al.*, 1992, 1993; Ang *et al.*, 1993; Monahagan *et al.*, 1993; Sasaki and Hogan, 1993; Dirksen and Jamrich, 1995) and may replace *axl* (*HNF3* β) in cells where it is not co-expressed with *shh*. Alternatively, distinct molecules may control *shh* in regions not expressing *axl/HNF3* β or other closely related winged-helix transcription factors. The presence of a DR5 RARE bound by RAR/RXR heterodimers in the *shh* promoter that is functional in the HeLa cell system and the responsiveness of *shh* to ectopic application of RA in the chicken wing and zebrafish fin buds (Riddle *et al.*, 1993; Akimenko and Ekker, 1995) indicate RARs as further direct regulators of *shh* expression. Conclusive evidence for a requirement for RARs and RXRs for *shh* expression is missing from RAR or RXR single and double knockouts, possibly due to functional redundancy (Luo *et al.*, 1995; Sucov *et al.*, 1995; Krezel *et al.*, 1996). However, evidence from vitamin A-deficient embryos indicates an involvement of retinoids in *shh* expression (Bavik *et al.*, 1996; Maden *et al.*, 1996). Also, inhibition of RA synthesis in the chicken limb bud causes loss of *shh* expression, an effect that can be reversed by ectopic application of RA (Stratford *et al.*, 1996). Whether the *HNF3* β elements and the DR5 RARE identified in the HeLa cell system are indeed

relevant for the *in vivo* regulation of *shh* remains to be determined by mutational or transgenic analysis in embryos.

There is also evidence that *shh* regulates expression of *axl/HNF3* β . Ectopic expression of *shh* results in activation of *axl/HNF3* β in the neuroectoderm of zebrafish, *Xenopus* and mouse embryos (Echelard *et al.*, 1993; Krauss *et al.*, 1993; Ruiz i Altaba *et al.*, 1995a). Exposure of chicken neural plate explants to recombinant Shh leads to activation of *HNF3* β expression in the presence of cycloheximide, suggesting that *HNF3* β is an immediate response gene to the Shh signal in the neuroectoderm (Martí *et al.*, 1995; Roelink *et al.*, 1995; Ruiz i Altaba *et al.*, 1995b). These data and our analysis of the *shh* promoter are consistent with the previously proposed model of the functional interdependence of the two genes (Strähle and Blader, 1994; Ruiz i Altaba *et al.*, 1995): initially, *axl/HNF3* β activates *shh* in the notochord. Secretion of Shh from the notochord leads then to induction of *axl/HNF3* β in floor plate cells and concomitant activation of *shh* also in the floor plate. Thus, *axl/HNF3* β appears to act both upstream and downstream of *shh* in the developmental pathway controlling specification of the floor plate.

Materials and methods

Fish stocks and embryo production

Wild-type zebrafish were purchased from a local dealer. Fish were bred and raised as described (Westerfield, 1995).

Cloning of the *shh* gene and mRNA start site mapping

An EMBL3 zebrafish genomic library (Molven *et al.*, 1991) was screened with the zebrafish *shh* cDNA (Krauss *et al.*, 1993) using standard procedures (Sambrook *et al.*, 1989). Restriction sites were mapped as described (Rackwitz *et al.*, 1984). *SalI* fragments were subcloned into Bluescript SK II (Stratagene). Exon-intron boundaries were mapped by sequencing with primers complementary to the cDNA.

RNA preparation and RNase protection analysis using uniformly 32 P-labelled antisense RNA probes was described previously (Strähle *et al.*, 1993). Mapping of the start sites by RNase protection was carried out with a probe spanning the *shh* promoter region between -563 and +221 and 20 μ g of total RNA prepared from 24 h embryos. Start sites were confirmed by the 5' RACE procedure (Frohman, 1988) and sequencing of amplified fragments.

Plasmid construction

Cloning was carried out following standard procedures (Sambrook *et al.*, 1989). pCMVaxl was cloned by excising the *axl* cDNA from p6T1N (Strähle *et al.*, 1993) with *NotI* and inserting it into the *NotI* sites of CMV β (MacGregor, 1989), replacing the *lacZ* coding region. pCMVmyc-axl was generated by inserting the *axl* coding region in-frame, downstream of the six *myc* epitopes in pCS2mt (Turner and Weintraub, 1994). -2200shhCAT and -563shhCAT were constructed by subcloning the 2.5 kb *SalI-XhoI* and 0.8 kb *PstI-XhoI* fragments, respectively, from genomic phage λ shh7 into Bluescript SK II (Stratagene). Subsequently, the CAT coding region including a polyadenylation site, which was excised from expression plasmid pBLCAT2 (Luckow and Schütz, 1987), was inserted downstream of the *shh* sequences into the *XhoI-KpnI* sites. 5' Deletion constructs were generated by PCR using different 5' primers with an artificial *XbaI* site (-469shhCAT, 5'-GTTTCAGTCTAGAGGCTGATGGGACAACAAG-3'; -364shhCAT, 5'-ATTAATCTAGAGATGGTGAACGCGCACAT-3'; -267shhCAT, 5'-AATGACTCTAGAAATAATGGTTCATTAGCG-3', -155shhCAT, 5'-TTCACATCTAGACGCGTCCCTCCCCTGAAA-3') and a common 3' primer (5'-CGCTGCGGTGTTCTCGAGCGGTCTGTCTAG-3') including the *XhoI* site at position +216. Amplified fragments were cloned as *XbaI-XhoI* fragments upstream of the CAT gene replacing the 0.8 kb *XbaI-XhoI* fragment in -563shhCAT.

Mutations of *HNF3* β -binding sites and the DR5 RARE in -563shhCAT were generated by PCR using oligonucleotides in which the binding sites had been replaced by a restriction enzyme recognition sequence.

Sequences upstream and downstream of the mutated binding site were amplified using common primers, 5'-GATTTACTGCAGGAAGC-ACAATAAATTTGATG-3' (*Pst*I site underlined) and 5'-CGTGC-GGTGTTCTCGAGCGGTCTGTCTAG-3' (*Xho*I site underlined) in combination with complementary oligonucleotides containing the mutated binding sites priming upwards and downwards, respectively. HNF3 β -binding sites 1 and 2 were mutated by changing the HNF3 β consensus sequence to a *Not*I site (*Not*I upward, 5'-ACACTTCTCGCGCCGCCACAAAACAGACGCGCAA-3'; *Not*I downward, 5'-TTTGTGGGGCGGCCGAGAAGTGTTCCTCCCGTCA-3') and to an *Apa*I site (*Apa*I upward, 5'-CCCACGGGGCCCCCGCGCAA-AAAACCTTTAAA-3'; *Apa*I downward, 5'-GCGCGGGGGCCCCCGTGGGATAACATCAGAAGT-3'), respectively. -563shhCAT-DM harbouring mutations in both HNF3 β sites was generated with a primer spanning both binding sites replacing element 1 and 2 with an *Apa*I and *Not*I site (*Not*I + *Apa*I upward, 5'-ACACTTCTCGCGCCGCC-CACGGGGCCCCCGCGCAA-3') and the *Not*I downward primer (5'-TTTGTGGGGCGGCCGAGAAGTGTTCCTCCCGTCA-3'). The DR5 RARE in -563shhCATDR5-M was mutated by introducing an *Apa*I site (*Apa*I upward, 5'-CGCTAAGGGCCATTATTTCTCAAGT-CATTTG-3'; *Apa*I downward, 5'-ATTAGCGGGCCCCACACGG-ATGTAAGAAAGTG-3'). Amplified fragments were digested with the corresponding restriction enzymes and were cloned into -563shhCAT, replacing the wild-type 0.8 kb *shh* fragment.

axl-GST encoding full-length *Axl* as a fusion with GST was generated by amplifying the *axl* coding region with appropriate primers (*Sa*II, AAAC TTTTGTTCGACATGCTCGGTGCTGTCAAAAT; *Not*I, ACA-AGTCCGCGGCCGCTTGAAGAGATTCAGGAT) and insertion into *Sa*II, *Not*I sites of plasmid pGEX4T3. All constructs generated by PCR were sequenced to exclude the introduction of errors during amplification.

Microinjection

Plasmid DNA was purified over two CsCl gradients, diluted to 50 μ g/ml in 100 mM KCl, 0.1% phenol red. In co-injection experiments of pCMVax1 with CMV β , plasmids were injected at equal concentrations, giving a total concentration of 80 μ g/ml plasmid DNA. Embryos were dechorionated using Pronase E (Westerfield, 1995) and transferred to agar-coated plastic dishes containing 10% Hank's solution (Westerfield, 1995) and 100 μ g/ml gentamycin. Microinjection was carried out using standard gas-driven microinjection equipment. Individual blastomeres were injected up to the 4-cell stage. Dead embryos and unfertilized eggs were removed after 3 h of development. Healthy embryos were grown on fresh agar-coated dishes at 28.5°C until fixation at 12 or 24 h.

In situ hybridization and sectioning

Digoxigenin whole mount *in situ* hybridization was carried out as described (Strähle *et al.*, 1993, 1996). Embryos were embedded in 1% low melting point agarose for orientation prior to embedding in paraffin, and 8 μ m sections were cut as described (Godsave *et al.*, 1988). Double labelling of embryos by β -galactosidase enzymatic activity or by horseradish peroxidase immunohistochemistry and subsequent *in situ* hybridization were carried out as described previously (Strähle *et al.*, 1993, 1997).

Transfection and CAT assays

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% calf serum. RA-depleted calf serum (Biomedica) was used in RAR/RXR transfection experiments. Transfection by calcium phosphate co-precipitation was carried out as described (Gorman *et al.*, 1982). Briefly, 1 μ g of transactivator plasmids pCMVax1, CMVHNF3 β (Pani *et al.*, 1992), hRAR β (Zelent *et al.*, 1989) or hRXR α (Elder *et al.*, 1992) co-precipitated with 5 μ g of CAT reporter plasmid and 8 μ g of Bluescript SK II (Stratagene) as carrier were applied to a 9 cm Petri dish. In controls, transactivator plasmids had been replaced by 1 μ g of CMV β (MacGregor, 1989) or pSG5 (Green *et al.*, 1988). In RA induction experiments, the medium was replaced 24 h after transfection by fresh medium containing 10⁻⁷ M t-RA, 10⁻⁷ M 9-*cis* RA or ethanol carrier (0.1%) alone. Transfected cells were harvested 48 h after transfection and cell extract was prepared by three cycles of freezing and thawing in 200 μ l of extraction buffer (0.25 M Tris-HCl pH 7.5, 5 mM EDTA). CAT assays using constant amounts of extract protein were performed as described (Gorman *et al.*, 1982; Webster *et al.*, 1989). Reaction products were separated by thin-layer chromatography, and conversion of chloramphenicol to its acetylated forms was determined with a Fuji BAS 2000 PhosphorImager. Each transfection experiment was repeated at least three times with different plasmid preparations.

Band-shift assays

HNF3 β -GST (Overdier *et al.*, 1994) encoding the amino-terminal and DNA-binding domain of rat HNF3 β or Axl-GST comprising full-length Axl were overexpressed in *E.coli* and partially purified by glutathione-Sepharose 4B (Pharmacia). Purified recombinant mouse RAR α and RXR α which lack the amino-terminal domains A and B were a gift from H.Gronemeyer. For whole cell extracts, 24 h old zebrafish embryos were squeezed through a syringe in Tris-buffer [20 mM Tris-HCl pH 7.5, 2 mM dithiothreitol (DTT), 20% glycerol] and centrifuged (4°C, 10 min, 10 000 r.p.m.) to remove the yolk proteins. An equal volume of extraction buffer (20 mM Tris-HCl pH 7.5, 2 mM DTT, 100 mM KCl, 20% glycerol) was added and cells were lysed by three cycles of freezing and thawing. The supernatant obtained by centrifugation (4°C, 10 000 r.p.m., 10 min) was used without further purification in the binding assays.

Synthetic oligonucleotides were end-labelled with T4 kinase, annealed and purified (Sambrook *et al.*, 1989). Each protein-DNA binding assay used 100–200 ng of recombinant protein (Axl-GST, HNF3 β -GST, RAR α /RXR α) or 2 μ l of cell extract and was carried out as described previously (Chen *et al.*, 1994; Overdier *et al.*, 1994). Poly(dI-dC) was omitted from binding assays with cell extract. In competitor experiments, a 100-fold excess of cold oligonucleotide was added. Sequences of oligonucleotides in HNF3 β band shifts were the following [homology to HNF3 β consensus core sequence (Overdier *et al.*, 1994) and base changes in mutants are underlined]: Oligo1 (HNF3 β 1), TTGTGGATAAACATCAGAAAGTGTTCGTC (–103/–74); Oligo2 (HNF3 β 2), TAAAGGTTTTTGGCGCTCTGTCTTTGTTGGG (–126/–97); Oligo3, CTGAAACCCACCTTCTTAAAGGTTTTTTG (–143/–112); Oligo1' (HNF3 β 1 mutated), TTGTGGGGCGGCCGAGAAGTGTTCGTC (–103/–74); Oligo2' (HNF3 β 2 mutated), TAAAGGTTTTTGGCGGGGGGCC-CCGTGGG.

The oligonucleotides employed in RAR/RXR band shifts were the following (RARE hexamer half-sites and base changes in the mutant are underlined): Oligo 1, TTTTCGAGGTCAGTCTTACCTTTTCGATCCC (+31/+60); Oligo 2, AAATAATGGTTCATTAGCGGGTACACACG (–265/–236); Oligo 3, CTGTAACCGAGTTGTTTCAGGTTAGAGGCTG (–490/–461); Oligo 2' (DR5 mutated), AAATAATGGGGCCACACGG-ATGTAAGAAA.

Accession number

The DDBJ/EMBL/GenBank accession number of the sequence reported here is AF004987.

Acknowledgements

We thank A.Molven, F.Stewart, H.Gronemeyer, R.H.Costa and B.Chatton for advice and materials. We are grateful to the IGBMC cell culture facility staff, to the photographers and to the sequencing and oligonucleotide synthesis group. We thank H.Gronemeyer for critically reading the manuscript. B.-E.C. is a recipient of a scholarship from the government of the Republic of China. P.B. is supported by the EC. U.S. is a recipient of a fellowship from the Deutsche Forschungsgemeinschaft. This work was supported by the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Centre Hospitalier Universitaire Regional, ARC and GREG. We also thank the ICRF for support in the initial stages of the project.

References

- Akimenko, M.A. and Ekker, M. (1995) Anterior duplication of the Sonic hedgehog expression pattern in the pectoral fin buds of zebrafish treated with retinoic acid. *Dev. Biol.*, **170**, 243–247.
- Ang, S.L. and Rossant, J. (1994) HNF-3 β is essential for node and notochord formation in mouse development. *Cell*, **78**, 561–574.
- Ang, S.L., Wierda, A., Wong, D., Stevens, K.A., Cascio, S., Rossant, J. and Zaret, K.S. (1993) The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. *Development*, **119**, 1301–1315.
- Barth, A.K. and Wilson, S.W. (1995) Expression of zebrafish nk2.2 is influenced by sonic hedgehog/vertebrate hedgehog-1 and demarcates a zone of neuronal differentiation in the embryonic forebrain. *Development*, **121**, 1755–1768.
- Bavik, C., Ward, S.J. and Chambon, P. (1996) Developmental abnormalities in cultured mouse embryos deprived of retinoic acid by inhibition of yolk-sac retinoid binding protein synthesis. *Proc. Natl Acad. Sci. USA*, **93**, 3110–3114.

- Berrodin,T.J., Marks,M.S., Ozato,K., Linney,E. and Lazar,M.A. (1992) Heterodimerization among thyroid hormone receptor, retinoic acid receptor, retinoid X receptor, chicken ovalbumin upstream promoter transcription factor, and an endogenous liver protein. *Mol. Endocrinol.*, **6**, 1468–1478.
- Boshart,M., Weber,F., Jahn,G., Dorsch-Häsler,K., Fleckenstein,B. and Schaffner,W. (1985) A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell*, **41**, 521–530.
- Bugge,T.H., Pohl,J., Lonnoy,O. and Stunnenberg,H.G. (1992) RXR alpha, a promiscuous partner of retinoic acid and thyroid hormone receptors. *EMBO J.*, **11**, 1409–1418.
- Chen,Z.P., Shemshedini,L., Durand,B., Noy,N., Chambon,P. and Gronemeyer,H. (1994) Pure and functionally homogeneous recombinant retinoid X receptor. *J. Biol. Chem.*, **269**, 25770–25776.
- Chiang,C., Lititung,Y., Lee,E., Young,K., Corden,J.L., Westphal,H. and Beachy,P. (1996) Cyclopia and defective axial patterning in mice lacking sonic hedgehog function. *Nature*, **383**, 407–413.
- Concordet,J.-P., Lewis,K.E., Moore,J.W., Goodrich,L.V., Johnson,R.L., Scott,M.P. and Ingham,P.W. (1996) Spatial regulation of a zebrafish patched homologue reflects the roles of sonic hedgehog and protein kinase A in neural tube and somite patterning. *Development*, **122**, 2835–2846.
- Currie,P.D. and Ingham,P.W. (1996) Induction of a specific muscle cell type by a hedgehog-like protein in zebrafish. *Nature*, **382**, 452–455.
- Dirksen,M.-L. and Jamrich,M. (1992) A novel, activin-inducible, blastopore lip-specific gene of *Xenopus laevis* contains a fork head DNA-binding domain. *Genes Dev.*, **6**, 599–608.
- Dirksen,M.-L. and Jamrich,M. (1995) Differential expression of fork head genes during early *Xenopus* and zebrafish development. *Dev. Genet.*, **17**, 107–116.
- Echelard,Y., Epstein,D.J., St-Jacques,B., Shen,L., Mohler,J., McMahon,J.A. and McMahon,A.P. (1993) Sonic hedgehog, a member of a family of putative signalling molecules, is implicated in the regulation of CNS polarity. *Cell*, **75**, 1417–1430.
- Ekker,S.C., Ungar,A.R., Greenstein,P., von,K.D., Porter,J.A., Moon,R.T. and Beachy,P.A. (1995) Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. *Curr. Biol.*, **5**, 944–955.
- Elder,J.T., Astrom,A., Pettersson,U., Tavakkol,A., Griffiths,C.E., Krust,A., Kastner,P., Chambon,P. and Voorhees,J.J. (1992) Differential regulation of retinoic acid receptors and binding proteins in human skin. *J. Invest. Dermatol.*, **98**, 673–679.
- Ericson,J., Muhr,J., Placzek,M., Lints,T., Jessell,T.M. and Edlund,T. (1995) Sonic hedgehog induces the differentiation of ventral forebrain neurons: a common signal for ventral patterning within the neural tube. *Cell*, **81**, 747–756.
- Evan,G.L., Lewis,G.K., Ramsey,G. and Bishop,J.M. (1985) Isolation of monoclonal antibodies specific for human *c-myc* proto-oncogene product. *Mol. Cell Biol.*, **5**, 3610–3616.
- Fan,C.-M. and Tessier,L.M. (1994) Patterning of mammalian somites by surface ectoderm and notochord: evidence for sclerotome induction by a hedgehog homolog. *Cell*, **79**, 1175–1186.
- Fan,C.-M., Porter,J.A., Chiang,C., Chang,D.T., Beachy,P.A. and Tessier-Lavigne,M. (1995) Long-range sclerotome induction by sonic hedgehog: direct role of the aminoterminal cleavage product and modulation by the cyclic AMP signalling pathway. *Cell*, **81**, 457–465.
- Frohman,M.A., Dush,M.K. and Martin,G.R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl Acad. Sci. USA*, **85**, 8998–9002.
- Godsave,S.F., Isaacs,H.V. and Slack,J.M.W. (1988) Mesoderm-inducing factors: a small class of molecules. *Development*, **102**, 555–566.
- Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell Biol.*, **2**, 1044–1051.
- Green,S., Issemann,I. and Sheer,E. (1988) A versatile *in vivo* and *in vitro* eukaryotic expression vector for protein engineering. *Nucleic Acids Res.*, **16**, 369.
- Hauptmann,G. and Gertser,T. (1996) Complex expression of the *zp-50 pou* gene in the embryonic zebrafish brain is altered by overexpression of *sonic hedgehog*. *Development*, **122**, 1769–1780.
- Helms,J., Thaller,C. and Eichele,G. (1994) Relationship between retinoic acid and sonic hedgehog, two polarizing signals in the chick wing bud. *Development*, **120**, 3267–3274.
- Hynes,M., Poulsen,K., Tessier-Lavigne,M. and Rosenthal,A. (1995) Control of neuronal diversity by the floor plate: contact-mediated induction of midbrain dopaminergic neurons. *Cell*, **80**, 95–101
- Johnson,R.L., Laufer,E., Riddle,R.D. and Tabin,C. (1994) Ectopic expression of Sonic hedgehog alters dorsal–ventral patterning of somites. *Cell*, **79**, 1165–1173.
- Kaufmann,E. and Knöchel,W. (1996) Five years on the wings of fork head. *Mech. Dev.*, **57**, 3–20.
- Kliwer,S.A., Umehono,K., Mangelsdorf,D.J. and Evans,R.M. (1992) Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. *Nature*, **355**, 446–449.
- Knöchel,S., Lef,J., Clement,J., Klocke,B., Hille,S., Köster,M. and Knöchel,W. (1992) Activin A induced expression of a fork head related gene in posterior chordamesoderm (notochord) of *Xenopus laevis*. *Mech. Dev.*, **38**, 157–165.
- Krauss,S., Concordet,J.-P. and Ingham,P.W. (1993) A functionally conserved homolog of the *Drosophila* segment polarity gene hedgehog is expressed in tissues with polarizing activity in zebrafish embryos. *Cell*, **75**, 1431–1444.
- Krezel,W., Dupé,V., Mark,M., Dierich,A., Kastner,P. and Chambon,P. (1996) RXR γ null mice are apparently normal and compound RXR α ^{+/−}/RXR β ^{−/−}/RXR γ ^{−/−} mutant mice are viable. *Proc. Natl Acad. Sci. USA*, **93**, 9010–9014.
- Lai,E., Prezioso,V.R., Tao,W., Chen,W.S. and Darnell,J.E. (1991) Hepatocyte nuclear factor 3a belongs to a gene family in mammals that is homologous to the *Drosophila* homeotic gene fork head. *Genes Dev.*, **5**, 416–427.
- Laufer,E., Nelson,C.E., Johnson,R.L., Morgan,B.A. and Tabin,C. (1994) Sonic hedgehog and Fgf-4 act through a signalling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell*, **79**, 993–1003.
- Lee,J.J., von Kessler,D.P., Parks,S. and Beachy,P.A. (1992) Secretion and localized transcription suggest a role in positional signalling for products of the segmentation gene hedgehog. *Cell*, **71**, 33–50.
- Leid,M. *et al.* (1992) Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell*, **68**, 377–395.
- Levin,M., Johnson,R.L., Stern,C.D., Kuehn,M. and Tabin,C. (1995) A molecular pathway determining left–right asymmetry in chick embryogenesis. *Cell*, **82**, 803–814.
- Luckow,B. and Schütz,G. (1987) CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. *Nucleic Acids Res.*, **15**, 5490.
- Luo,J., Pasceri,P., Colon,R.A., Rossant,J. and Giguere,V. (1995) Mice lacking all isoforms of retinoic acid receptor β develop normally and are susceptible to the teratogenic effects of retinoic acid. *Mech. Dev.*, **53**, 61–71.
- MacDonald,R., Xu,Q., Barth,A.R., Mikkola,I., Holder,N., Fjose,A., Krauss,S. and Wilson,S.W. (1994) Regulatory gene expression boundaries demarcate sites of neuronal differentiation in the embryonic zebrafish forebrain. *Neuron*, **13**, 1039–1053.
- MacDonald,R., Barth,K.A., Xu,Q., Holder,N., Mikkola,I. and Wilson,S.W. (1995) Midline signalling is required for Pax gene regulation and patterning of the eyes. *Development*, **121**, 3267–3278.
- MacGregor,G.R. and Caskey,C.T. (1989) Construction of plasmids that express *E.coli* β -galactosidase in mammalian cells. *Nucleic Acids Res.*, **17**, 2365.
- 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.
- Marks,M.S., Hallenbeck,P.L., Nagata,T., Segars,J.H., Appella,E., Nikodem,V.M. and Ozato,K. (1992) H-2RIIBP (RXR beta) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes. *EMBO J.*, **11**, 1419–35.
- Marti,E., Bumcrot,D.A., Takada,R. and McMahon,A.P. (1995) Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. *Nature*, **375**, 322–325.
- McKnight,S.L. and Kingsbury,R. (1982) Transcriptional control signals of a eukaryotic protein-coding gene. *Science*, **217**, 316–324.
- Mohler,J. and Vani,K. (1992) Molecular organization and embryonic expression of the hedgehog gene involved in cell–cell communication in segmental patterning of *Drosophila*. *Development*, **115**, 957–971.
- Molven,A., Njolstad,P.R. and Fjose,A. (1991) Genomic structure and restricted neural expression of the zebrafish *wnt-1 (int-1)* gene. *EMBO J.*, **10**, 799–807.
- Monaghan,A.P., Kaestner,K.H., Grau,E. and Schütz,G. (1993) Postimplantation expression patterns indicate a role for the mouse forkhead/HNF3 α , β , γ genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. *Development*, **119**, 567–578.

- Münsterberg,A.E., Kitajewski,J., Bumcrot,D.A., McMahon,A.P. and Lassar,A.B. (1995) Combinatorial signalling by sonic hedgehog and *wnt* family members induces myogenic bHLH gene expression in the somite. *Genes Dev.*, **9**, 2911–2922.
- Overdier,D.G., Porcella,A. and Costa,R.H. (1994) The DNA-binding specificity of the hepatocyte nuclear factor 3/forkhead domain is influenced by amino-acid residues adjacent to the recognition helix. *Mol. Cell. Biol.*, **14**, 2755–2766.
- Pani,L., Overdier,D.G., Porcella,A., Qian,X., Lai,E. and Costa,R.H. (1992) Hepatocyte nuclear factor 3b contains two transcriptional activation domains, one of which is novel and conserved with the *Drosophila* Forkhead protein. *Mol. Cell. Biol.*, **12**, 3723–3732.
- Rackwitz,H.R., Zehetner,G., Frischauf,A.M. and Lehrach,H. (1984) Rapid restriction mapping of DNA cloned in lambda phage vectors. *Gene*, **30**, 195–200.
- Riddle,R., Johnson,R.L., Laufer,E. and Tabin,C. (1993) Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell*, **75**, 1401–1416.
- Roelink,H. *et al.* (1994) Floor plate and motor neuron induction by *vhh-1*, a vertebrate homolog of hedgehog expressed by the notochord. *Cell*, **76**, 761–775.
- Roelink,H., Porter,J.A., Chiang,C., Tanabe,Y., Chang,D.T., Beachy,P.A. and Jessell,T.M. (1995) Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell*, **81**, 445–455.
- Roux,J., Pictet,R. and Grange,T. (1995) Hepatocyte nuclear factor 3 determines the amplitude of the glucocorticoid response of the rat tyrosine aminotransferase gene. *DNA Cell. Biol.*, **14**, 385–396.
- Ruiz i Altaba,A. and Jessell,T.M. (1992) *Pintallavis*, a gene expressed in the organizer and midline cells of frog embryos: involvement in the development of the neural axis. *Development*, **116**, 81–93.
- Ruiz i Altaba,A., Prezioso,V.R., Darnell,J.E. and Jessell,T.M. (1993) Sequential expression of HNF3 β and HNF3 α by embryonic centres: the dorsal lip/node, notochord and floor plate. *Mech. Dev.*, **14**, 91–108.
- Ruiz i Altaba,A., Roelink,H. and Jessell,T.M. (1995a) Restrictions to floor plate induction by hedgehog and winged-helix genes in the neural tube of frog embryos. *Mol. Cell. Neurosci.*, **6**, 106–121.
- Ruiz i Altaba,A., Placzek,M., Baldassare,M., Dodd,J. and Jessell,T.M. (1995b) Early stages of notochord and floor plate development in the chick embryo defined by normal and induced expression of HNF3 β . *Dev. Biol.*, **170**, 299–313.
- Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sasaki,H. and Hogan,B.L. (1993) Differential expression of multiple forkhead related genes during gastrulation and axial pattern formation in the mouse embryo. *Development*, **119**, 579–595.
- Strähle,U. and Blader,P. (1994) Early neurogenesis in the zebrafish embryo. *FASEB J.*, **8**, 692–698.
- Strähle,U., Blader,P., Henrique,D. and Ingham,P. (1993) Axial, a zebrafish gene expressed along the developing body axis, shows altered expression in cyclops mutant embryos. *Genes Dev.*, **7**, 1436–1446.
- Strähle,U., Blader,P. and Ingham,P.W. (1996) Expression of axial and sonic hedgehog in wildtype and midline defective zebrafish embryos. *Int. J. Dev. Biol.*, **40**, 929–940.
- Strähle,U., Fischer,N. and Blader,P. (1997) Expression and regulation of a *netrin* homologue in the zebrafish embryo. *Mech. Dev.*, **62**, 147–160.
- Stratford,T., Horton,C. and Maden,M. (1996) Retinoic acid is required for the initiation of outgrowth in the chick limb bud. *Curr. Biol.*, **6**, 1124–1133.
- Sucov,H.M., Izpisua-Belmonte,J.C., Ganan,Y. and Evans,R.M. (1995) Mouse embryos lacking RXR α are resistant to retinoic-acid-induced limb defects. *Development*, **121**, 3997–4003.
- Talbot,W.S., Trevarrow,B., Halpern,M.E., Melby,M.E., Farr,A.E., Postlethwait,J.H., Jowett,T., Kimmel,C.B. and Kimelman,D. (1995) Requirement for the homeobox gene *floating head* in zebrafish development. *Nature*, **378**, 150–157.
- Turner,D.L. and Weintraub,H. (1994) Expression of *achaete-scute* homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.*, **8**, 1434–1447.
- Webster,N.J., Green,S., Tasset,D., Ponglikitmongkol,M. and Chambon,P. (1989) The transcriptional activation function located in the hormone-binding domain of the human oestrogen receptor is not encoded in a single exon. *EMBO J.*, **8**, 1441–1446.
- Weinstein,D.C., Ruiz i Altaba,A., Chen,W.S., Hoodless,P., Prezioso,V.R., Jessell,T.M. and Darnell,J.E., Jr (1994) The winged-helix transcription factor HNF3 β is required for notochord development in the mouse embryo. *Cell*, **78**, 575–588.
- Westerfield,M. (1995) *The Zebrafish Book*. 2nd edn. University of Oregon Press.
- Westerfield,M., Wegener,J., Jegalian,B.G., DeRobertis,E.M. and Püschel,A.W. (1992) Specific activation of mammalian Hox promoters in mosaic transgenic zebrafish. *Genes Dev.*, **6**, 591–598.
- Yu,V.C. *et al.* (1991) RXR beta: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell*, **67**, 1251–1266.
- Zelent,A., Krust,A., Petkovich,M., Kastner,P. and Chambon,P. (1989) Cloning of murine alpha and beta retinoic acid receptors and a novel receptor gamma predominantly expressed in skin. *Nature*, **339**, 714–717.
- Zhang,X. K., Hoffmann,B., Tran,P.B., Graupner,G. and Pfahl,M. (1992) Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature*, **355**, 441–446.

Received on December 20, 1996; revised on April 4, 1997