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Retinoid signalling and axial patterning during early vertebrate embryogenesis

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Abstract. There are many indications that active retinoids are regulatory signals during vertebrate embryogenesis. Treating vertebrate embryos with retinoids can cause teratogenic defects, including specific derangements of the main body axis. Other data show that early vertebrate embryos contain physiologically relevant concentrations of active retinoids and express retinoid binding proteins and receptors; that knockouts of retinoid receptors can induce homeotic defects; and that relevant developmental control genes are regulated by retinoid response elements. Here, we discuss the possibility that retinoids are developmental signals which regulate axial patterning in the early vertebrate embryo.

Key words. Retinoid; Hox gene; *Xenopus*; retinoid receptor; axial patterning.

Introduction

It is well known that the vitamin A derivative, all-*trans*-retinoic acid (tRA), has teratogenic effects on early vertebrate embryos. Exposure of early embryos to tRA results in a range of defects, with the brain, eyes, cranio-facial structures, heart and limbs being particularly sensitive. Studies of limb development [1] and subsequently of the formation of the primary embryonic axis [2, 3] suggested that retinoids might function as morphogens in vivo, different concentrations specifying different positions along embryonic axes. In the limb, the in vitro effects of tRA resemble those expected of a morphogen, but evidence has recently been accumulating that limb patterning involves a hierarchy of other induction signals including sonic hedgehog, BMP.2 and FGF4. The way in which retinoids interact with this hierarchy is not yet clear (reviewed by Maden [4] and [5]), but many findings, including the expression pattern of retinoid receptors and binding proteins in the developing limb bud suggest strongly that retinoids have a role in limb patterning.

The effects of retinoid excess on the antero-posterior (a-p) patterning of the primary body axis suggest a role for a retinoid during a-p patterning in vivo. The aim of this review is to discuss some of the literature bearing on this possibility, laying particular emphasis on data obtained using *Xenopus laevis*.

Axial patterning and the effect of retinoid treatment during gastrulation

A degree of antero-posterior specification may occur in the blastula during mesoderm induction: this is suggested by the concentration-dependent induction of particular axial markers by different mesoderm-inducing factors [6–8]. However, processes occurring during gastrulation are clearly very important in ensuring development of the complete a-p axis. As shown initially by the pioneering experiments of Spemann and collaborators, the mesoderm in the dorsal blastopore lip of the gastrula is an organiser region which emits head- and tail-specific signals at sequential stages during gastrulation [9, 10]. It is possible to disrupt this process in several ways, one of which is the addition of tRA to gastrulae. *Xenopus* embryos are most sensitive to tRA at gastrula stages [2, 3]. The main effect is a concentration-dependent loss of head structures, and at high concentrations, there are also effects on tail formation.

tRA has been shown to have effects both on the neural plate and on the axial mesoderm. Its action on central nervous system patterning resembles that of a neural transformation signal proposed in the classical literature to be produced in the organiser in the posterior part of the late gastrula. This signal posteriorizes induced neural tissue but is not itself a neural inducer [11]. Treatment of whole *Xenopus* embryos with tRA increases the volume of the hindbrain and spinal cord at the expense of a decrease in the volume of the forebrain [2]. tRA treatment at the end of gastrulation can also induce the expression of posterior neural markers both in the presumptive forebrain region of the intact embryo [12] and in explants of presumptive forebrain tissue [13, 14]. Interestingly, early tRA treatments also appear to contract or delete the most anterior part of the developing hindbrain as well as disturbing hindbrain segmentation in *Xenopus* and Zebrafish embryos [15, 16]. These observations, together with data concerning the regulation of *Hox* gene expression in the developing hindbrain (see below), indicate that

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retinoids could have several roles during a-p specification of the central nervous system (CNS), including specific functions in the hindbrain.

In the axial mesoderm, tRA has been shown to cause a loss of expression of the anterior marker, c-otx-2, in anterior mesoderm in chick embryos [17] and to reduce the capacity of organiser mesoderm to induce the formation of head structures in Einsteck experiments in *Xenopus* [8, 18, 19]. tRA also clearly ventralizes mesoderm in growth factor-induced explants [8], although this tendency was not obvious in whole *Xenopus* embryos [19].

These results make it interesting to speculate that an endogenous retinoid may function as a posteriorizing morphogen, both in the neural plate and in the axial mesoderm in the early embryo.

The effects of tRA as a teratogen appear to be conserved. Gastrula stage treatments with tRA have similar posteriorizing effects in various vertebrate species. Interestingly, organisers from the gastrulae of chickens and mice are able to exert at least some of their organiser functions when transplanted to a *Xenopus* gastrula or when combined with *Xenopus* embryonic tissue [20, 21]. An organiser (Hensen's node) from a chicken gastrula also has ZPA activity in the chicken limb bud [22]. These findings indicate that different organiser regions use conserved signals. One of these conserved signals may be a retinoid.

Endogenous retinoids in the embryo

If retinoids function as morphogens, they should be available endogenously in the embryo and, considering the posteriorizing effect of tRA and its inducing effect on *Hox* gene expression (see below), one might also expect a source of retinoid to become localized posteriorly around the blastopore during gastrulation. Since the original discovery that all-*trans*-retinoic acid (tRA) is a naturally occurring active retinoid, a number of other natural retinoid metabolites have been shown to be biologically active. These are: 9-*cis*-RA, the 9-*cis* stereoisomer of retinoic acid and the ligand for the RXR family of receptors described below [23, 24]; all-*trans*-3,4-didehydro-retinoic acid (ddRA) [25]; all-*trans*-4-oxo-retinoic acid (4-oxo-RA) [26]; 14-hydroxy-4,14-retroretinol (14-HRR) [27] and anhydroretinol (AR) [28].

Investigations using HPLC analysis have now indicated that four of these active retinoids, tRA [29], 9-*cis*-RA [30], 4-oxo-RA [26], and ddRA [25], are available in developing embryos in vivo. Two of them have also already been reported as being localized, or synthesized locally, during embryogenesis. tRA has been reported as a posterior to anterior gradient in the developing limb bud [29]. tRA and 9-*cis*-RA have also been reported as being localized in *Xenopus* gastrula and neurula stage embryos. HPLC peaks for 9-*cis*-RA were highest in extracts of dorsal halves of gastrulae and neurulae and in the anterior and posterior thirds of neurula stage embryos. tRA appeared to be concentrated anteriorly, and ventrally rather than dorsally [30]. By contrast, tRA synthesis from retinol has been reported to be localized in the posterior region (Hensen's node organiser), in the early mouse embryo [31]. A different approach, using in vivo activation of a luciferase reporter construct driven by a retinoic acid-responsive element, has now also reported a gradient of active retinoids from the posterior (high) to anterior (low) in early neurula stage embryos of *Xenopus*. A significant increase in active retinoid concentration was also found during gastrulation specifically in the dorsal marginal zone, as the organiser assumes trunk and tail organiser properties [32]. Another study, using a reporter construct driven by RARE sequences, indicated the availability of active retinoid to be restricted to the posterior half of head fold and later stage mouse embryos, although they did not indicate the presence of a retinoid gradient [33]. Furthermore, a study using F9 teratocarcinoma cells transfected with a retinoid-responsive lacZ construct reported localized active retinoid secretion in the posterior part of the developing central nervous system of the later rat embryo [34].

Taken together, these investigations clearly leave some uncertainty about the localization of active retinoids in the early vertebrate embryo, although the majority of studies indicate a posterior localization in early neurula stage embryos. It should be borne in mind that HPLC investigation of the localization of endogenous retinoids is made difficult by the existence of overlapping retinoid peaks which hinder definitive identification, and that we do not yet know which are the most important active retinoids in vivo. Some of the studies using reporter constructs also employ the endogenous retinoid receptors in the embryo, and these can have localized availability (see below).

An interesting question concerning active retinoids is that of their specificities for different retinoid-sensitive processes in vivo. It is notable that tRA and 4-oxo-RA both apparently have qualitatively similar effects on *Xenopus* embryogenesis (inducing microcephaly if applied at the gastrula stage), but that 4-oxo-RA, which was initially characterized as an inactive tRA catabolite on the basis of its relatively weak activity in regulating growth and differentiation of cultured cells [35–37], is effective in disturbing patterning in the early embryo at 5-fold lower concentrations than tRA [26]. The basis of this biological specificity has not yet been accounted for, in terms of receptor specificity or other molecular differences. It is also notable that 9-*cis*-RA, which can work via a totally different receptor pathway than tRA or 4-oxo-RA (see below), apparently induces qualitatively similar morphological defects in *Xenopus* embryos

as these ligands (ref. 30, and our own unpublished observations). Explaining and characterizing such biological specificities will clearly require much more investigation, both at the embryological and at the molecular level.

Cellular retinoid binding proteins

Retinoids are lipid-soluble molecules which can enter cells by diffusion. It is predictable that one factor regulating the availability of active retinoids in the cell will be the availability of cellular retinoid binding proteins. Two types of cellular retinoic acid binding protein (CRABPI and II) have now been identified, and their genes have been cloned in mammals [38–41]. CRABPI and II are also available in birds [42], but each of two CRABP genes now cloned in *Xenopus* apparently encodes a new form (not obviously more homologous to CRABPI or to CRABPII [43, 44]. Besides CRABPs, there are also two forms of a cellular retinol binding protein (CRBPI and II) [45, 46], which will not be discussed further here. The cellular retinoid binding proteins are members of a family of small polypeptides which bind to low molecular weight hydrophobic substances [47, 48]. The CRABPs appear to have specific affinity for particular acidic retinoid ligands: they bind to the all-*trans* isomers of certain acidic retinoids (tRA, t-didehydro-RA, t-4-oxo-RA), but not to 13-*cis*-RA, nor to retinol or retinal (see ref. 49, reviewed in 40).

Expression data reveal that CRABPI and II are expressed during embryogenesis, and that they tend to be available in regions showing high retinoid sensitivity. These expression data have led to suggestions that CRABPs act both as positive and negative regulators of retinoid availability [43, 50, 51]. This issue has been tested more directly by overexpressing murine CRABPI in F9 teratocarcinoma cells; this led to 90% inhibition of tRA-induced differentiation and expression of RARβ (a tRA-inducible gene), while reduction of CRABPI expression via antisense CRABPI transfection increased the sensitivity of F9 cells to RA [52,53]. In contrast, overexpression of a *Xenopus* CRABP mRNA (xCRABP) during early *Xenopus* embryogenesis caused many teratogenic defects resembling those caused by RA administration. xCRABP overexpression also caused an increase in the expression of two *Hoxb* genes similar to that induced by treatment with high concentrations of RA. These results suggested indirectly that xCRABP might enhance the action of an endogenous retinoid signal.

The molecular functioning of CRABPs is still also obscure. Comparison with the related plasma retinol binding protein, which transfers retinol from the liver via blood to target cells, suggests that CRABPs might transport acidic retinoids to metabolic enzymes and/or to nuclear receptors (reviewed in refs 40, 54). Direct support for the first possibility was provided by the finding that CRABPI overexpression in F9 cells enhances metabolism of tRA to 4-oxo-RA [53]. Taken together with the high activity of 4-oxo-retinoic acid for teratogenesis in *Xenopus* embryos, but not for regulating growth and differentiation in teratocarcinoma cells [26], this finding sheds an interesting light on the different biological effects of CRABP overexpression in F9 cells and in *Xenopus* embryos.

The embryonic function of CRABPS has been investigated most directly by knocking out the murine CRABPI and II genes by homologous recombination. Surprisingly, CRABPI and CRABPII single and double mutant mice all appeared to be essentially normal except for minor defects in limb patterning in the CRABPII mutant and the double mutant, and they did not appear to show an increased sensitivity to RA [55–58], even though cytoplasmic extracts from the double mutant no longer showed any detectable cytoplasmic RA binding activity. These data show a discrepancy with the phenotypic abnormalities in CRABPI$^-$ F9 cells. They probably mean that CRABPs are not essential for retinoid signalling in otherwise normal mice, raised under laboratory conditions. However, their conserved nature, their distributions in the embryo and the effects of overexpression experiments suggest that CRABPs do have some function in early embryonic patterning. It is likely that CRABPs function in parallel with other regulatory mechanisms to help stabilize the availability of active vitamin A forms, under conditions where the total vitamin A supply varies due to the varying nutritional status of the mother.

Retinoid receptors

There is much evidence that most or all of the biological effects of acidic retinoids are mediated by members of the nuclear receptor super family of ligand-inducible transcription factors. Two types of nuclear retinoid receptors have been identified to date: the RARs and RXRs. Each receptor type comprises three closely related subtypes (α, β, γ) encoded by separate genes, and each receptor subtype gene also encodes several isoforms, via the use of different promoters and differential splicing. These receptor subtypes are all available in mammals, birds and amphibians, although the gene for the RARβ subtype has not yet been cloned in *Xenopus* (see below) (reviewed in ref. 59). The RAR receptor type is recognised with high affinity by several naturally occurring acidic retinoid ligands, namely tRA [60, 61], 9-*cis*-RA [23, 24], ddRA [62], and 4-oxo-RA [26]. The RXR receptor type has only one known natural ligand, 9-*cis*-RA [23, 57].

It is by no means certain that the complexity sketched above ends with RARs and RXRs, because two new

families of retinoid related orphan receptors (RORs/RZRs [63, 64], and RVR/Rev-erbβ [65, 66] which closely resemble RARs and RXRs in their DNA sequence, have now also been cloned. We note, however, that a ligand for RZRβ has very recently been identified as the non-retinoid hormone melatonin [67]. Another class of nuclear orphan receptors (COUP-TFs) are also relevant because they probably act as negative regulators of retinoid signalling [68–71].

At the molecular level, the RARs and RXRs function as transactivators or silencers of transcription by binding to response elements in the promoters of, or enhancers for, target genes. The most important role for RARs is probably to act in RAR-RXR heterodimers, by binding to response elements consisting of a direct repeat (DR) of AGGTCA or a closely related half site motif, spaced by one, two or five nucleotides (DR1, DR2 or DR5). The DR2 and DR5 response elements mediate transactivation via activated RAR-RXR heterodimers. The DR1 response element mediates silencing via activated RAR-RXR heterodimers, probably via competition with activated RXR homodimers [72; reviewed in 59], see also below. Interestingly, some RARs are themselves RA-inducible and RAREs have been found in the genes [14, 73–75].

Unlike the RARs, RXRs can function as homodimers, which activate gene expression via the DR1 response element. They also function very widely as promiscuous partners in heterodimers with several different nuclear receptors, including RARs, thyroid hormone receptors (TRs), vitamin D receptors (VDRs), peroxisome proliferator-activated receptors (PPARs), and COUP-TFs, with each heterodimer acting via its appropriate specific direct repeat response element [reviewed in 59, 76].

The nature of this mechanism makes it clear that retinoid signalling will interact with other signalling pathways. RXRs can function explicitly in heterodimers to transduce signals due to other non retinoid hormones. RARs have also been reported to form heterodimers with TRs [77] and VDRs [78]. RARs may also interact with other pathways indirectly by competing for the available pool of RXR receptors. It is possible that COUP-TFs inhibit retinoid signalling via RARs by the same mechanism. It is more likely, however, that COUP-TF homodimers [68] or COUP-RXR heterodimers [68, 70, 79] act by binding to the DR1 retinoid response element in competition with RXR homodimers. This connectedness obviously has far reaching implications. It cannot be excluded, for example, that at least some of the developmental effects observed after retinoid treatment are indirect, due to interference with a so far unidentified endogenous hormonal morphogen system. The existence of localized retinoid ligands with high specificity (above), of localized retinoid receptors (below) and of retinoid response elements in important target genes do, however, make this possibility unattractive, both from a functional and from an evolutionary point of view. The existence of this complex molecular machinery raises other interesting questions. One concerns the relationship between molecular and biological specificities. It seems probable that the different retinoid ligands may work principally via different receptor types and subtypes to regulate different biological processes and different target genes. The investigations in this area are still in their infancy, but are promising.

Studies using synthetic retinoids have identified ligands with quite high specificity for particular receptor subtypes [7, 80–82] and some specificities have also been identified for natural ligands, apparently depending on the assay used [83]. The most striking specificity so far identified for a natural ligand is that 9-*cis*-RA is the only known natural ligand for the RXRs. This fact has now been given added significance by the revealing discovery that only the RAR ligand binds to RAR-RXR heterodimers when these are bound to DNA [84]. It is likely that the RXR functions as a passive cofactor in RAR-RXR heterodimers and in some other heterodimers between RXR and hormone receptors, and that a main function of the RXR ligand is to activate RXR homodimers. 9-*cis*-RA is known to induce RXR homodimerization [85]. It is interesting to note that 9-*cis*-RA binding is able to activate RXR-PPAR heterodimers [86, 87], and that these, like RXR homodimers act via the DR1 response element. RXRs thus participate in two very different types of retinoid signal transduction pathway, mediating signals from RAR and RXR ligands respectively. A second aspect of retinoid receptor and ligand specificity concerns the point that, besides being transcription factors, which regulate gene expression specifically via retinoid-responsive DNA elements, RARs regulate gene expression via protein–protein interactions which have their own specificities. One such interaction which is rather well known is a retinoid-dependent inhibitory interaction with the AP-1 transcription complex [88]. Some very recent exciting findings using synthetic retinoids now show that this protein-protein interaction has its own unique retinoid ligand specificity [89]. Different functions of the same retinoid receptor may thus be regulated separately by different ligands. Relating these different molecular specificities to different biological functions of the retinoid signalling system in the embryo is thus an important challenge for the future. There is, so far, very little progress in this area. For example, the basis of the high specificities of 4-oxo-RA and 9-*cis*-RA for teratogenesis in the early embryo remains obscure.

Retinoid receptors and embryogenesis

Considering the molecular complexity sketched briefly above, it is hardly surprising that the importance of

individual retinoid ligands and receptors for pattern formation in the early embryo is still unclear. However, considerable data is accumulating about the expression patterns of known RARs and RXRs during embryogenesis and from functional analysis via gain and loss of function experiments.

Much is now known, from in situ hybridization studies and other approaches, about the mRNA expression patterns of different RAR and RXR subtypes in the mouse embryo. There are also limited data available in other vertebrates e.g. chicken [90], zebrafish [91, 92] and *Xenopus* [14, 93]. The data show that different RAR and RXR subtypes are widely expressed, both in the adult organism and during embryogenesis, and that they each have individual expression patterns, which may be overlapping with or exclusive of each other. Retinoid receptors of different types appear to be expressed along the entire antero-posterior axis in the central nervous system and in other cell types. These data suggest that individual receptor subtypes have specific, and probably multiple, functions. Among the murine RARs, the RARα subtype is rather generally expressed during embryogenesis while RARβ and RARγ are more restricted, sometimes being expressed in non-overlapping patterns [94–98]. An interesting example for axis formation is that RARβ is available only in the closed neural tube, and RARγ only in the open neural folds during murine neurulation [97]. Among the RXRs, murine RXRβ is rather generally available, while murine RXRα and especially γ have more restricted expression patterns during embryogenesis [99, 100; reviewed in 59]. Another aspect, which complicates the picture further, is that different isoforms of a receptor subtype can be expressed differently [53, and see below]. For details of these receptor expression patterns, the reader is referred to the original literature.

There is relatively little information available about the expression of retinoid receptor subtypes in *Xenopus*. The literature indicates that at least the RARα and γ and RXRα, β and γ subtypes are expressed with subtype specific timing in the early embryo. Of the RXRs, only RXRβ is expressed during gastrulation, maternal transcripts from RXRα and RXRγ being degraded at the late blastula stage [14, 93, 101, 102]. *Xenopus* RARβ has not yet been cloned. RARγ is, so far, the only subtype for which localized expression has been reported during gastrulation. At the beginning of gastrulation this subtype is expressed in ectoderm and mesoderm with slightly higher expression on the dorsal side, but by the late gastrula stage, transcripts have become more concentrated posteriorly in the dorsal lip of the blastopore with a second anterior expression zone in the mesoderm and archenteron roof [93]. These anterior and posterior zones persist into the neurula and tailbud stages, where isoform-specific patterns (γ1, γ2) are demonstrable [103]. Transcripts from another RAR isoform, RARα2, show localized expression within the neural tube by the late neurula stage when expression peaks [14]. These data are thus fragmentary, but they and the data from other vertebrates are consistent with specific functions for individual retinoid receptor subtypes and even isoforms during embryogenesis.

The functional significance of the different retinoid receptor subtypes, and of retinoid signalling, has been investigated by genetic manipulation in a number of different vertebrates. The most significant progress so far has been made by knocking out murine RARs and RXRs via germline homologous recombination in transgenic mice. Single gene knock-outs of the RARα1 or RARβ2 or RARγ2 isoforms all deliver an apparently normal phenotype [104–106]. Knocking out the RARα or γ or RXRα genes, however, delivered quite severe developmental abnormalities and crosses of these mutants (RARα, RARγ, RARβ2, RARα1, RXRα) to make double mutants delivered a much more severe phenotype in many cases [107–109]. It is not appropriate to describe all of the findings made in these experiments here; the reader is referred to the original publications for details. We note, however, that the defects obtained are complex, as would be expected from the complex expression patterns of the different RARs and RXRs, and that some of them duplicate defects observed in the offspring of vitamin A-deficient mice. We also note that the defects obtained include axial homeotic transformations, as would be expected if endogenous retinoids have a role in axial patterning. RARγ knock-outs thus generate anteriorizing homeotic transformations of the cervical axial skeleton [104], and other, more or less severe axial transformations are observed in several of the double mutants [108]. The results from these homologous recombination knock-outs are thus consistent with multiple roles for retinoids in embryogenesis, including a role in axial patterning. The increased severity of the double mutant phenotypes also indicates functional redundancy among retinoid receptors. Elucidating the functions of the individual receptors and the extent of the functional redundancy will require much more investigation. A very interesting in vitro study in RARγ-deficient F9 murine teratocarcinoma cells suggested a form that this redundancy might take. These cells now failed to express Hoxa-1 in response to RA, but overexpressed its paralogue Hoxb-1 [110].

The functional studies in *Xenopus* are, so far, not extensive and have delivered little insight into a possible role for a retinoid in axial patterning. They show that overexpression in pre-gastrula stages of intact *Xenopus* RARγ2, or of a truncated dominant negative version of RARγ2, by mRNA injection into the zygote, causes no obvious disturbance of embryogenesis, even though these treatments enhance tRA-induced teratogenesis

and suppress the tRA-induced expression of a retinoid responsive receptor construct [111]. Overexpression of *v-erb A* (a truncated version of the thyroid hormone receptor which can be predicted to interfere with retinoid signalling) did, however, induce developmental defects [112], but no obvious axial defects. These studies are clearly still in their infancy. The future will be interesting.

In conclusion, expression studies and gain and loss of function studies presently lead to no clear conclusions regarding the functioning of RARs and RXRs in early embryogenesis. The expression studies show patterns which are compatible with conserved specialized functions for particular receptor subtypes. The functional studies in the mouse indicate redundancy of function, and suggest multiple functions for retinoid signalling in embryogenesis. The studies in *Xenopus* are not extensive.

Hox genes and retinoids

Some of the effects of retinoids in modulating the embryonic main body axis are likely to be mediated via regulation of *Hox* gene expression. There are four clusters of *Hox* genes in vertebrates, which were originally identified because of their homology with *Drosophila* homeotic genes in the *antennapedia* and *bithorax* complexes [113, 114; reviewed in 115]. During early development, the *Hox* genes, encoding transcription factors, are expressed in sequential zones along the a-p axis in the hindbrain and trunk regions. They show a-p colinearity of expression, such that progressively more 5′ genes are expressed in progressively more posterior zones [13, 115–117]. As with the homeotic genes in *Drosophila*, these expression zones appear to be involved in providing a code for position along the a-p axis.

The *Hox* genes begin to be expressed during gastrulation, when a-p patterning is being established [13, 118–121] and in *Xenopus*, the characteristic sequence of *Hoxb* gene expression zones seen at tailbud stages appears to develop very early, by the late gastrula/early neurula stage [116]. These data suggest that the establishment of localized *Hox* gene expression is regulated by factors active in the gastrula, which may include retinoids.

There is substantial evidence to suggest that retinoids do indeed play a role in regulating *Hox* gene expression. The *Hox* genes are sensitive to retinoic acid both in vitro, in embryocarcinoma cells, and in early embryos. tRA induces transcription of 3′ *Hox* genes first and to a greater extent than that of progressively more 5′ genes [13, 122–125]. This colinearity in the response to tRA is what would be predicted if a gradient of a retinoid produced at the posterior end of the embryo was responsible for a sequential activation of *Hox* genes along the a-p axis. A gradient developing from a posterior source would also be expected to initiate *Hox* gene expression posteriorly with the expression spreading to an anterior border. In mouse embryos, spreading of *Hoxb* gene expression zones from the posterior end of late primitive streak stage embryos has been described. By the head fold/early somite stages, the expression of each *Hoxb* gene has reached an anterior expression border and from then on appears to be clonally transmitted [118, 121]. The 3′ *Hox* genes, which are most sensitive to retinoids would be expected to spread to more anterior borders than the less sensitive 5′ genes, as is the case. The idea that retinoids may regulate this process is supported by the finding that tRA treatment of early mouse embryos results in the expression of 3′ *Hoxb* genes extending to more anterior borders in the embryo [122, 126]. *Hoxb* gene expression can also be induced by RA in explants of anterior neural tissue from late gastrulae of *Xenopus* [13]. In the mesoderm, tRA also causes transformations of *Hox* gene expression domains in murine prevertebrae leading to homeotic transformations of vertebrae and to the idea of a Hox code for vertebral identity [127].

An involvement of retinoids in regulating *Hox* gene expression during embryogenesis is most directly supported by the recent finding of retinoid responsive elements (DR2 RAREs) in upstream and downstream *cis*-acting regulators for the human and murine *Hoxb-1* genes [128–130]. These RAREs appear to act in concert with each other, with retinoid inducible proteins and with autoregulatory elements (activated by *Hoxb-1* itself) to regulate different aspects of the *Hoxb-1* expression pattern. The *Hoxa-1* gene also contains a downstream enhancer with a RARE, a DR5 [131] and a RARE has been identified upstream of the murine and human *Hoxd-4* genes [132, 133]. Taken together with recent findings about interactions between *Hox* genes (see below), the finding of retinoid response elements for *Hoxa1* and *Hoxb1* suggests that one role for retinoids may be to trigger expression of the *Hoxa* and *b* clusters.

An interesting finding concerning RA-induced *Hox* gene expression in human embryonal carcinoma cells is that 3′ *Hox* gene expression may be required for the expression of more 5′ *Hox* genes. Inhibition of *HOXB1* or *HOXB3* causes a reduction in mRNA levels of more 5′ *HoxB* genes, and this effect increases towards the 5′ end of the cluster, a sort of 'domino effect'. In the case of *HOXB3* inhibition, it was shown additionally that more 5′ *Hox* genes in other clusters were also affected [134]. The cluster organization and regulation may therefore be important in the response to retinoids. Since, to date, only the most 3′ genes in the *Hoxa* and *Hoxb* clusters have been reported to contain RAREs, it is clearly a possibility that retinoids only directly activate the 3′ *Hox* genes and that more 5′ *Hox* genes become activated sequentially by more 3′ *Hox* gene

products. However, in vivo the picture is more complicated. Although the expression domain of *Hoxb-1* was found to be substantially reduced in *Hoxa-1* knock-out mice, the expression of a number of other *Hox* genes appeared to be unaffected [135]. It will be interesting to examine the expression of 5′ *Hox* genes in double knock-out experiments.

The effects of tRA on *Hox* gene expression are actually rather complex. For example, tRA has different effects on *Hoxb-1* expression before and after the onset of somitogenesis in mouse embryos. *Hoxb-1* expression is normally restricted to rhombomere 4 in the hindbrain. Treatment of embryos with tRA before somites start forming results in expression of *Hoxb-1* throughout the pre-otic (anterior) hindbrain, whereas treatment of older embryos leads to a duplication of the hindbrain segments r4, 5 and a second stripe of *Hoxb-1* expression in the pre-otic hindbrain [126, 136]. These findings suggest that retinoids may play several distinct roles in the patterning of the a-p axis, including a relatively late role in subdivision of the hindbrain. In further support of this idea is the finding of a RARE in a repressor responsible for restricting the expression of *Hoxb-1* to r4 in the hindbrain in later development [130]. Interestingly, transactivation of a DR2 RARE involved in regulating later expression may also be enhanced by a cofactor, a retinoid-inducible protein (RIP) which binds to an enhancer region close to the upstream RARE [129].

These data support the idea that *Hox* gene regulation is mediated partly by positional information supplied by retinoids, and that RA effects on axial patterning are partly mediated via effects on *Hox* gene expression in the hindbrain and spinal cord.

Conclusions

The findings above lead to a complex and inconclusive picture concerning the functioning of retinoids in early embryogenesis. The rather specific teratogenic effects of retinoids, the availability and localization of sufficient concentrations of active retinoid ligands in the gastrula embryo and the specific connections between retinoids and important developmental genes, like the *Hox* genes, and, particularly the availability of retinoid response elements in the *Hoxa-1*, *Hoxb-1* and *Hoxd-4* genes, suggest that a retinoid may act as an early developmental signal, possibly acting as a posterior to anterior gradient in the gastrula. The literature concerning the molecular mechanisms of retinoid signal transduction indicates complexity, the existence of multiple retinoid dependent pathways, and interactions with other signal transduction pathways. The relevance of these aspects for embryogenesis is still obscure. The gain and loss of function experiments with individual retinoid receptors so far show relatively mild effects on early embryogenesis. The results clearly indicate redundancy between different receptor subtypes (as demonstrated by the more severe phenotype of double receptor knock-outs). More extensive analysis is obviously required to elucidate the relevance of retinoid signalling for early embryogenesis.

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