Retinoids in Embryonal Development

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elements of their promoters are summarized. The fundamental role of homeobox genes in embryonal development and the actions of retinoids on their expression are discussed. The similarity of the effects of retinoid ligand knockouts to effects of compound retinoid receptor knockouts on embryogenesis is presented. Although much remains to be clarified, the emerging landscape offers exciting views for future research.

I. INTRODUCTION

Vitamin A and its derivatives (the retinoids) are essential for both normal embryonic development (87) and maintenance of differentiation in the adult organism (35, 36, 78), accounting for the intense interest in these compounds in biology and medicine. Embryo segmentation and growth fail, vascularization stops, and the embryo is eventually resorbed in the absence of sufficient vitamin A (151, 279, 297). Excess vitamin A, on the other hand, results in terata during embryogenesis and is membrandolytic and hepatotoxic in the adult organism (204). In the adult, epithelial differentiation requires vitamin A. Its deficiency causes squamous metaplasia, a preneoplastic lesion, which eventually also alters functional epithelial characteristics and leads to infection and death (36, 247, 306). These considerations make it obvious that maintenance of retinoid homeostasis is tantamount to maintenance of normal physiology of the intact organism.

The use of the retinoid ligand knockout models to study embryonic development has unequivocally linked the physiological function of vitamin A to development of the heart, the embryonal circulation, the central nervous system (CNS), and the normal left-to-right cardiac symmetry (321). Recent work has also emphasized that the developmentally regulated generation of bioactive retinoids is fundamental to the control of embryonic development (47, 321).

Remarkable progress has occurred in the past 10 years in our understanding of the mode of action of vitamin A and its derivatives, the retinoids (37). The discovery of the nuclear receptors for retinoic acid and other retinoids has provided a conceptual basis to explain how these compounds preside over a large network of gene activation processes (35).

It is the purpose of this review to present a balanced synopsis of the actions of retinoids in embryonal development and to provide suggestions for future studies.

II. RETINOID STRUCTURE

Figure 1 shows the chemical structure of the salient, physiologically important retinoids so far identified. The parent compound retinol and its oxidation product, retinoic acid (RA), are shown in the stretched-out all-trans-configuration at the top of the figure. Derivatives of retinol include 4-hydroxy-retinol, 4-oxo-retinol, and 14-hydroxy-4,14-retro-retinol. Of the RA derivatives, 4-hydroxy-RA, 4-oxo-RA, 3,4-didehydro-RA (ddRA), and the retinoid receptor (RXR) ligand 9-cis-RA are shown. All-trans RA is the natural ligand for the retinoic acid receptors (RAR) (71, 221), and 9-cis-RA for the RXR (137), although the latter compound binds to both receptor families. The specificity of interactions would also suggest the possibility that various retinoids, both natural and synthetic, may specifically be useful as drugs to combat diverse diseases.

III. RETINOID REQUIREMENT DURING EMBRYOGENESIS

A. Introduction

Retinol (also referred to as ROL and/or vitamin A) is the only retinoid known to be capable of sustaining all vitamin A functions, including development, growth, vision, and reproduction (Fig. 2). On the other hand, RA maintains differentiation and growth of the adult organism; it, however, is insufficient to support gestation of the embryo. Because RA cannot fulfill all vitamin A functions, it is obvious that several retinoids, in addition to RA and which are not RA metabolites, act in concert to maintain the health and well being of the entire organism. Also possible are limitations of cellular metabolism and transport, where cells require RA but must transport retinol across a tissue barrier. For a more detailed discussion of the different roles of vitamin A, the reader is referred to the following reviews (11, 263, 307).

As early as the 1930s it was realized that maternal insufficiency of vitamin A results in death of the fetus as well as congenital malformations (79, 172). Later, Wilson and co-workers (279, 305) defined congenital abnormalities resulting from vitamin A deficiency during gestation. Teratogenic targets of vitamin A deficiency were the heart, ocular tissues, and respiratory, urogenital and circulatory systems (321). These abnormalities were prevented by inclusion of vitamin A in the diet.

Excess dietary vitamin A, on the other hand, has been shown to also cause teratogenesis (26). Several other studies followed this original observation and are discussed in section VIII. Suffice it to say here that major excess vitamin A targets include the heart, the skull, skeleton, limbs, brain, eyes, CNS, as well as craniofacial structures (15, 110, 111, 189, 191, 203, 234, 252, 322).

Similarity of teratogenic responses between vitamin A deficiency and excess supports the concept that the same molecules are involved. It also suggests a fundamental role for this nutrient in embryonal development.
The work of Wellick and De Luca (297) has demonstrated that retinol is essential and RA alone appears insufficient to allow gestation in the rat to reach completion. In fact, vitamin A-deficient pregnant rats resorb their fetuses at about day 15 of gestation, even if given retinoic acid daily. Retinol, on the other hand, prevents this resorption when administered no later than day 10 of gestation. In fact, the administration of as little as 2 \( \mu \text{g} \) on day 10 is sufficient to allow continuation of gestation through parturition (298). Claggett-Dame recently proposed that extremely high RA doses may, in fact, rescue the dpc 14.5 gestational barrier identified by Wellick and De Luca (298). Whether this reflects rescue by traces of alternative retinoids in not known at this time. Countering this is Niederreither’s RALDH2 knockout, which is incompletely rescued by exogenous RA (206, 207).

The recommended dietary allowance (RDA) for vitamin A for nonpregnant and pregnant women is 800 \( \mu \text{g} \) RA, which is equivalent to ~2,700 IU (210). No increment of vitamin A is recommended during pregnancy (210, 214). For a discussion of the data which justify this recommended intake, the reader is referred to the 10th edition of the RDA (210).

Different retinoid requirements between the avian and mammalian systems have been highlighted and are summarized as follows: 1) ddRA and its precursor 3,4-didehydroretinol (ddROH) were undetectable in mouse limb buds, although prominent in chick limbs; 2) a relatively high concentration of retinyl esters (1.5 \( \mu \text{M} \)) is evident in chick limb buds but not in mouse; and 3) there is a higher concentration of cellular retinoic acid binding proteins (CRABP), especially CRABPII, than their ligands RA and ddRA in both species. Retinol and ddROH, on the other hand, were present in much higher concentration than cellular retinol binding proteins (CRBP) (245). The concentration of “free” RA was found to be near the range for the dissociation constant \( (K_d) \) value of the murine RAR.

Principal approaches to study the essential function of vitamin A during embryonal development are exposure to excess retinoid, retinoid receptor knockout studies, and finally retinoid-ligand knockout models. This last approach is discussed in section III B.
B. Retinoid Ligand Knockouts Models

The studies of Zile (321) have demonstrated the usefulness of the vitamin A-deficient (retinoid ligand knockout) avian embryo. These embryos display a strict dependence on vitamin A for their development of early vasculature. These avian embryos die at 3 days of embryonic life in the absence of vitamin A (43, 279). After the initial discovery by Heine et al. (83) that vitamin A is necessary for the establishment of the early vasculature, Zile (321) extended these studies to early development. Dong and Zile (53) and Chen et al. (25) had established that RA given to the hen is not transported to the egg. In this way a completely vitamin A-deficient embryo can be obtained from hens that are RA sufficient (53). This model was used by Zile and collaborators to define primary target tissues of vitamin A action during embryogenesis.

The vitamin A-deficient quail embryo presents with gross abnormalities in the cardiovascular system, head, CNS, hematopoietic organs, and trunk (43, 151, 152, 283, 321, 322). Bioactive retinoids can "rescue" the vitamin A-deficient embryo by preventing abnormal development when available at early development (43, 83, 115, 117, 279, 321). Because both retinoic excess as well as deficiency had similar teratogenic effects on the heart, Zile's group has launched an in depth investigation of the function of vitamin A in early heart development, using the vitamin A-deficient quail model (115–117). These studies are of particular interest because of the obvious relevance to human cardiovascular malformations, which may well be diet-related during pregnancy. Availability of vitamin A may be a relevant contributory element to the incidence of heart malfunction in developing countries, where vitamin A deficiency is a significant problem (261). Very little is understood of its etiology in the industrialized world, hence, the importance of better definition of etiological factors, especially dietary ones during pregnancy, since diet is likely to contribute the majority of xenobiotic as well as homobiotic factors to the physiology and pathology of the body. Vitamin A is also required for normal specification of heart left-right asymmetry. In a large percentage of vitamin A-deficient quail embryos, the heart appears on the wrong side (randomization) (281, 283, 284, 323). Retinoids, although not directly involved in assigning cardiac asymmetry genes to their asymmetry-specific sites, are absolutely essential for normal heart sidedness to occur (M. H. Zile, personal communication). Importantly, administration of vitamin A to deficient embryos as late as stage 8 (neurulation) prevents the anticipated vitamin A-deficient phenotype, including situs inversus (43, 115, 117, 323). They propose that the critical retinoid-requiring developmental window is at the four/five-somite stage during neurulation, when retinoid presence is absolutely essential for normal embryonic development to proceed. Excess retinoid resulting from implantation of retinoid impregnated pellets also causes cardiac abnor-
malities, including duplicate heart, cardia bifida, abnormal looping, and situs inversus (46, 215). Genes coding for extracellular matrix proteins appear important during heart embryogenesis and are regulated by retinoid status (267). Kostetskii and collaborators (116, 117) have found that RARβ2 expression in the retinoid ligand knockout quail model is reduced, as is RARa. They also reported that several retinoids, including all-trans, 9-cis, 4-oxo-, and didehydro-RA could rescue embryonic development, when provided to the developing embryo at up to the five-somite stage of development, but not later. These retinoids induced RARβ expression; 4-oxo-RA is least active.

Metabolic routes of retinoid activation and disposal are highly relevant to the occurrence of orderly developmental events and are covered separately in this review.

Rat embryos made deficient in retinoids during embryogenesis (gestational days 11.5–13.5) show specific cardiac, limb, ocular, and nervous system deficits (258). Many of these abnormalities also occur in retinoid receptor null mutants as discussed in section VI. Retinoid-ligand knockout models reveal additional defects, however, clearly supporting redundancy of the retinoid receptors (258).

Stratford et al. (267) have recently identified target genes in the development of the early limb bud of the quail, in the retinoid ligand knockout model. The retinoid-deficient quail embryos were found to develop to about stage 20/21. In a study of genes involved in anteroposterior axis, these authors found that Hoxb-8 was upregulated and its border was shifted anteriorly. In sharp contrast, shh and mesodermal bmp-2 were downregulated. These authors also studied the apical ectodermal genes and found that fgf-8 and ectodermal bmp-2 were not affected (267). A strong effect of retinoid deficiency was observed for genes involved in dorsoventral polarity. Wnt-7a was expressed in ventral ectoderm, although normally it is expressed exclusively in dorsal ectoderm. On the other hand, the corresponding dorsal mesodermal gene Lmx-1 was found to extend its expression into the ventral mesoderm. En-1 expression was absent from its normal expression site, the ventral ectoderm (267). This study exemplifies the usefulness of this model to study the role of retinoids in the regulation of gene expression during development.

Kostetskii et al. (115) have investigated the expression of cardiomyocyte differentiation genes. They have reported that vitamin A deficiency in the quail embryo fails to alter these genes including atrial-specific myosin heavy chain, ventricular-specific myosin, and sarcomeric myosin as well as the putative cardiomyocyte specification gene Nkx-2.5. However, the expression of the transcription factor GATA-4 was greatly reduced in the heart-forming region of stage 7–10 embryo in vitamin A deficiency. This downregulation occurs in the areas of the heart which eventually progresses to develop abnormally in the absence of vitamin A, the lateral mesoderm posterior to the heart. These authors also report that administration of retinol to the vitamin A-deficient embryos is able to restore GATA-4 expression and completely rescues the vitamin A-deficient phenotype. The authors conclude that their results indicate GATA-4 is a component of the retinoid-mediated cardiogenic pathway. They also conclude that this pathway appears unlinked to the differentiation of cardiomyocytes, and it is involved in the morphogenesis of the posterior heart tube as well as the development of the cardiac inflow tract, which does not form in the vitamin A-deficient embryo.

IV. METABOLISM

A. Introduction

RA regulates development by activating gene transcription in many different locations within the embryo. A particular cell will only respond to RA if 1) it expresses RA receptors and 2) the RA concentration lies within a range that is appropriate for its response. Because different genes are known to be activated by different RA levels (13), the effectiveness of RA as a developmental regulator involves the precise control of its distribution and concentration. Several mouse strains transgenic for a RA-activated reporter gene show that RA is not homogeneously distributed throughout the vertebrate embryo, but is instead localized to specific territories within a restricted number of developing organs in the embryo (8, 236). Studies on RA metabolism in the embryo indicate that the RA-synthesizing and catabolic enzymes are localized to subregions of developing tissues (175, 194, 206, 319), which resemble the activation patterns in the RA-reporter mice (8, 236). The pathways of synthesis and catabolism thus establish the local levels of RA and determine where and when RA regulation occurs. The most detailed description of this localization is available for the embryonic retina (54) and spinal cord (175). In addition, several other regions exhibit localized RA synthesis, both in the CNS, such as the substantia nigra and corpus striatum (176), and in mesoderm-derived tissues, such as the meninges (206), heart (194), and somites (206). Several regions in the developing CNS, e.g., the limb motor neurons in the spinal cord (319), express very high levels of RA-synthesizing enzymes, resulting in remarkably high RA concentrations locally, up to micromolar levels. The RA-rich regions can be located next to areas of high RA catabolism that contain little detectable RA, as is the case for the embryonic retina described in more detail below (181). The analysis of the distribution of RA synthetic and catabolic enzymes shows that several developing tissues contain compartments of high and low RA levels, a mor-
phogenetic RA pattern in addition to, and different from, the earlier model of patterning through RA gradients (280).

B. Retinol Dehydrogenase

The first step in RA synthesis is the reversible oxidation of retinol to retinaldehyde (22) (see Fig. 3). The full complement of enzymes that catalyze this reaction in the embryo remains to be determined. It is likely, though, that most of the retinaldehyde in the embryo is generated by alcohol dehydrogenases. A point of contention is, however, whether the medium-chain cytoplasmic alcohol dehydrogenases or the short-chain membrane-bound alcohol dehydrogenases are more important. Several members of the medium-chain, cytoplasmic class are competitively inhibited by ethanol, whereas the short-chain membrane-bound class is resistant to ethanol inhibition. The answer is important in reference to the hypothesis that the abnormalities evident in fetal alcohol syndrome result from the inhibition of RA synthesis by ethanol (58, 227, 315). This hypothesis has been proposed, because of the similarities between the embryonic abnormalities evident in RA teratogenicity and in fetal alcohol syndrome.

1. Medium-chain alcohol dehydrogenase family

This family of enzymes consists of at least four classes of zinc-dependent cytosolic enzymes: classes I, II, III, and IV (Adh1, -2, -3, and -4). Both Adh1 and Adh4 dehydrogenases can oxidize retinol to retinaldehyde, but only the Adh1 enzymes are inhibited by ethanol. In the adult, ethanol has been shown to inhibit RA synthesis in several tissues, including testes (289), liver (186), and cornea (94). Moreover, the alcohol dehydrogenase specific inhibitor 4-methylpyrazole has been shown to inhibit retinaldehyde synthesis in the testes (289). It is clear, however, that several retinol dehydrogenases are neither competitively inhibited by ethanol nor inhibited by 4-methylpyrazole. The alcohol dehydrogenase negative deermouse lacks all ethanol dehydrogenase activity but can oxidize retinol normally (226). Because this strain of deermouse is apparently normal, the ethanol-sensitive form of retinol dehydrogenase does not seem to be essential for normal development. The house mouse expresses only a single gene of the Adh1 group, and although its human homolog contains a RA response element, this is not the case in the mouse (292). In the mouse embryo, Adh1 is distributed in only a few of the regions known to synthesize RA, such as the developing kidneys (236, 292), and it is entirely absent from the CNS (292). It seems thus unlikely that Adh1, the only enzyme in the mouse competitively inhibited by ethanol, is important for RA synthesis during most of embryogenesis. Another candidate for embryonic retinol dehydrogenase is Adh4, since it oxidizes retinol to retinaldehyde and is expressed in several regions of embryonic RA synthesis (4). In particular, its expression commences at embryonic day 7.5, the time when RA synthesis begins (236). It is present in the early CNS and also in the tissue around the developing eye, both regions of high RA synthesis, as well as the otic vesicles and migrating neural crest (81). Adh4 is, however, absent from the embryonic retina at all times and from the entire embryo after embryonic day 10.5 (4); hence, retinaldehyde for embryonic development from this stage onward must be synthesized by another enzyme. In addition, null mutations for Adh4, as well as for Adh1, develop normally (34), indicating that, even at early

![Fig. 3. The metabolic flow from retinol to retinoic acid and its oxidation products is shown. Note that the oxidation of retinal to retinoic acid and further oxidation reactions are irreversible.](http://physrev.physiology.org/)

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\begin{align*}
\text{all-trans retinol (Vitamin A)} & \underset{\text{Retinol Dehydrogenase}}{\longrightarrow} \text{all-trans retinaldehyde} \\
\text{all-trans retinaldehyde} & \underset{\text{Cytochrome P450 Dependent}}{\longrightarrow} \text{all-trans retinoic acid} \\
\end{align*}
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\text{4-oxo all-trans retinoic acid}
developmental stages, redundancy in the retinol dehydrogenases exists.

2. Short-chain dehydrogenase/reductases and 9-cis-RA synthesis

At least five microsomal retinol dehydrogenases of the short-chain dehydrogenase/reductase are known to be expressed in the adult (270). These enzymes are typically resistant to inhibition by 4-methylpyrazole and ethanol. The type II microsomal dehydrogenase was reported to be associated with the cytochrome P-450/CYP2D1 oxidase which, combined with NADPH-P-450 reductase, promotes retinaldehyde oxidase activity (92). It is possible, then, that the short-chain dehydrogenase/reductases may be particularly important for retinaldehyde reduction to retinol, competing with the oxidation of retinaldehyde to bioactive RA. The embryonic distribution of the five dehydrogenases/reductases is largely unknown. Two recently identified short-chain dehydrogenase/reductases, however, have interesting properties; they are specific for cis-retinol isomers and do not catalyze all-trans-retinol oxidation (185, 233); they are named 9-cis-retinol dehydrogenases. One of the two was found to be broadly expressed in many regions of the embryo, including the CNS, eye, ear, and somites (233). The other one is identical to the 11-cis-retinol dehydrogenase required for regeneration of the visual chromophore in the retinal pigment epithelium (56, 256). In addition to the eye, it is expressed in several other tissues in the adult organism, including the testes, but its localization in the embryo is not yet known. The identification of a 9-cis-retinol dehydrogenases implies that 9-cis-RA, the only RA isomer that activates the RXR receptors, is generated from 9-cis-retinoid precursors, rather than by a RA isomerase from all-trans-RA. Such a route for 9-cis-RA synthesis is attractive, because a pool of 9-cis-retinol is known to exist, at least in the adult (124), and because a RA isomerase has remained elusive (285, 286). If RA isomerization does occur during normal development, then it may not be enzymatically catalyzed (285, 286), although tissues are known to differ in their ability to isomerize all-trans-RA to 9-cis-RA (131). It is also relevant that, so far, Xenopus is the only species in which 9-cis-RA has been identified in the embryo (118), although this may reflect lower levels of the isomer in embryos of other species.

The 9-cis/11-cis-retinol dehydrogenase is similar to the other short-chain microsomal enzyme in that it is insensitive to ethanol inhibition (185). An intriguing characteristic of type I, and perhaps other short-chain retinol dehydrogenases, is that its activity is stimulated by ethanol. This is consistent with the features of the mouse fetal alcohol syndrome (275, 295), which are more similar to the effects of a RA excess (252), than to the effects of vitamin A deficiency (305). We have evidence that some of the teratogenic actions of ethanol in the brain are due to the abnormal induction of RA synthesis (P. J. McCaffery and D. Ullman, unpublished observations).

C. Retinaldehyde Dehydrogenases

The embryonic enzymes that catalyze the irreversible oxidation of retinaldehyde to RA are predominantly of the cytosolic class I aldehyde dehydrogenase family (22, 177). Although retinaldehyde oxidases exist (163, 232) and a proportion of rat embryonic RA synthesis is believed to occur via oxidases (22), our work (175, 194, 206) demonstrates that the retinaldehyde dehydrogenases are almost totally responsible for the RA distribution observable in the mouse embryo.

In our search for retinaldehyde dehydrogenases we initially employed a zymographic technique (178). For this assay, native tissue extracts are separated by isoelectric focusing (IEF), and retinaldehyde dehydrogenases are identified with RA-reporter cells by their capacity to oxidize retinaldehyde to RA in a NAD-dependent reaction. This bioassay is highly sensitive, allowing the use of very low substrate concentrations and detection of synthesized RA levels similar to those present in vivo. This feature enabled the detection of two previously unknown retinaldehyde dehydrogenases in the embryo, which we initially termed V1 and V2. The V2 gene is now cloned and has been renamed RALDH2 (319), while V1 remains to be characterized at the molecular level. A third enzyme present in the eye had already been described as the predominant enzyme for RA synthesis in the adult mouse liver (133); this third enzyme is referred to as AHD2 in the mouse, following its gene locus name.

1. AHD2 and V1

The two enzymes AHD2 and V1 create the very high and spatiotemporally regulated RA levels in the embryonic retina. Apart from the embryonic retina, they are only expressed in a few restricted locations within the embryo, i.e., within the CNS. AHD2 is found only in a subpopulation of dopaminergic cells in the substantia nigra and adjoining ventral tegmentum, and within the head, V1 is only expressed in the maxillar regions of the developing face and the lateral ganglionic eminence of the brain (unpublished observations). In the retina (see Fig. 4), AHD2 is restricted to a dorsal (D) region, whereas V1 is expressed in the ventral (V) portion (179). AHD2 is relatively inefficient in synthesizing RA from the low concentrations of retinaldehyde present in the retina. In contrast, V1 shows a much greater efficiency at these retinaldehyde concentrations, and the net result is greater RA synthesis in the ventral than dorsal retina (179). It would be expected that the boundary region between the two enzymes contains an intermediate concentration of RA.
and that a ventral-to-dorsal gradient extends across the entire retina. Instead, between the two RA-synthesizing enzymes lies a horizontal stripe of the RA catabolic enzyme CYP26 (181). This arrangement creates three regions along the dorsoventral axis of the embryonic retina, a dorsal zone of intermediate RA concentration, an intervening region of very low RA and a ventral zone of high RA concentration (Fig. 4). Gradients of RA concentration may exist within each region. This is the first description of a mechanism by which zones of RA concentration can be created. Inhibition of RA synthesis by citral in zebrafish blocks the development of the ventral half of the retina in similar fashion to the effects of null mutations of the RA receptors (98, 140, 167). Thus the ventral retina is a zone distinguished by its expression of the V1 RALDH, by its high RA synthesis, and by its dependence on RA for development. The dorsal, medial, and ventral territories of differing RA levels are likely to regulate transcription of some of the genes with differential expression patterns along the dorsoventral axis of the retina. Such genes include the tyrosine receptor kinase EphB2 and its ligand ephrin B2, which are believed to influence the target selectivity of outgrowing retinal axons (164), and the green and blue cone photoreceptors in the mouse (276). The ligand ephrin B2 and the green cones are restricted to the dorsal retina, and the receptor EphB2 and the blue cones are located predominantly in the ventral retina. It is likely that other regions of the embryo contain a similar zonal patterning of RA, created by the juxtapositioning of RA synthetic and catabolic enzymes.

Another intriguing property of the class I aldehyde dehydrogenases, in addition to their enzymatic function, is their affinity for several ligands of the nuclear receptor family. The promoter of the human ALDH1 gene contains a putative response elements for androgen (313), and the enzyme has been demonstrated to bind androgen (218). The corresponding cytosolic aldehyde dehydrogenase in Xenopus has been identified as the major thyroid-hormone binding protein in this species (312). We found that AHD2 in the embryonic dorsal retina and substantia nigra is present in amounts far exceeding the concentrations of the other retinaldehyde dehydrogenases (180). This suggests this enzyme may have a dual function, both synthesizing RA and also modulating the local concentration of other nuclear receptor ligands that can influence RA signaling in the embryonic retina.

2. RALDH2

The retinaldehyde dehydrogenase RALDH2 exhibits the greatest substrate specificity of the three dehydrogenases, and its distribution provides the most accurate guide to the localization of all-trans-RA in the embryo. RALDH2 is essential for normal development, and its knockout results in a complete failure of embryo survival and early morphogenesis (207). The trunk of the embryo is severely shortened, and the neural tube remains open, whereas the anterior region is less affected. Unfortunately, the embryos die before the effect on more differentiated structures can be observed. Studies on these structures await the creation of conditional knockouts.

A guide to the pattern of all-trans-RA signaling in the embryo is provided by the RA-reporter mice, a strain transgenic for β-galactosidase controlled by a sensitive RA response element (RARE-lacZ) (27, 236). The expression of β-galactosidase in a particular cell is the result of the balance of RA synthesis and catabolism in that region, as well as the capacity of the cell to respond to RA depending on its expression of RA receptors, coactivators, and corepressors. Given the number of factors on which β-galactosidase expression depends, it is surprising that the distribution of RALDH2 (206) matches quite well with the distribution of β-galactosidase in the RA reporter mice (236), including expression in the spinal cord, motoneurons, and developing eye. When examined in greater detail, there was a very good correlation, for instance, in the embryonic heart (194). This suggests that a significant proportion of the patterning of RA signaling in the embryo may rely on localized RA synthesis. Regions do exist, however, in which β-galactosidase distribution does not correlate with RALDH2 expression, and this includes the RARE-lacZ induction in the telencephalic vesicles and nasolachrimal groove. This is likely to be the result of RA synthesis by other RALDH. Analysis of RA distribution by HPLC also indicates a pattern similar to that of RALDH2 (90). With the exceptions of the restricted regions of V1 and AHD2 expression, which include the retina, face, and the striatum (176), RALDH2 appears to be responsible for
retinaldehyde oxidation in most other regions of early embryonic all-trans-RA synthesis. Within the embryonic CNS, the limb motor neurons in the spinal cord (175, 260, 319) represent major sites of RALDH2 expression, whereas most of the remaining CNS shows lower RA synthesis. It is of note, however, that the meninges surrounding the brain contain extremely high levels of RALDH2, and this potent source of RA may be important in brain development and plasticity (unpublished observations). For the developing cerebellar region, the meninges are likely to represent a major source of RA required for the differentiation of granule cells in the adjacent external granular layer. RALDH2 does not discriminate between all-trans- and 9-cis-retinaldehyde (M. Warren, personal communication); hence, this enzyme does not appear to influence the all-trans- and 9-cis-RA ratios.

### D. Synthesis of Other Bioactive Retinoids in the Embryo

1. Didehydro-RA

   Although RA is the predominant active retinoid in the embryonic mouse, this is not the case in several other vertebrate classes. In the chick, didehydro-RA was found to be the principal active retinoid in the developing limb bud (278), as well as in the developing spinal cord and the somites (156). Moreover, the likely precursor didehydro-retinol has been detected in the embryonic chick. The distribution of didehydro-RA in the chick embryo is similar to the distribution of all-trans-RA in the mouse. Furthermore, the patterns of RALDH2 expression in both mouse (206) and chick (10) are similar. It is thus likely that RALDH2 will oxidize didehydro-retinaldehyde to didehydro-RA, although this has still to be demonstrated directly. Didehydro-RA is also present in both *Xenopus* and zebrafish (29, 30) but has yet to be detected in mouse (90).

2. 4-Oxo-RA, 4-oxo-retinaldehyde, and 4-oxo-retinol

   In the embryonic *Xenopus* (223), all-trans-4-oxo-RA influences the development of the anteroposterior body axis in a fashion that is unresponsive to all-trans-RA. Both retinoids act through RA receptors, and it is assumed that they activate differing configurations of receptors. Similarly, the 9-cis-isomer of 4-oxo-RA differs from 9-cis-RA in receptor selectivity, since it is able to specifically activate RAR-RXR heterodimers and not RXR-RXR homodimers (222). Presumably, both isomers of 4-oxo-RA can be synthesized from their respective RA isomers via a cytochrome P-450-linked oxidase, such as CYP26. Another potential source of 4-oxo-RA is 4-oxo-retinaldehyde, and this retinoid has been identified in early *Xenopus* embryos (12). Surprisingly, 4-oxo-retinaldehyde itself can activate the RAR, and it is the major bioactive retinoid in *Xenopus* (12). Another transactivator of RAR is 4-oxo-retinol, a compound present in both *Xenopus* and the mouse F9 teratocarcinoma cell line (12). The existence of such active ketone derivatives of the retinoids suggests the potential importance of cytochrome P-450-linked oxidases in activating retinoids, in addition to their role in retinoid catabolism (see sect. vF).

### E. Influence of Substrate Concentration and Distribution

In embryonic mouse (90) and human (119), retinol is present at micromolar concentrations, whereas retinaldehyde levels are barely detectable. This contrasts with several cold-blooded organisms including zebrafish (29) and *Xenopus* (7), in which high levels of retinaldehyde are present, bound via the labile Schiff-base linkage to vitellogenin. High retinaldehyde concentrations in the zebrafish embryo place even greater emphasis on the retinaldehyde dehydrogenases as the determinant of RA synthesis (29). The substrates all-trans-retinaldehyde, all-trans-retinol, and didehydro-retinol all appear to be localized to specific regions of the early *Xenopus* embryo (118), a distribution that is likely to influence the distribution of RA and didehydro-RA. We have found this to be the case for the retinaldehyde distribution in the zebrafish trunk (170). The distribution of the retinyl esters has not been investigated in the developing embryo, but both retinyl acetate and retinyl palmitate have been identified in cultured cells from the chick retina (266).

### F. RA Catabolism

A number of pathways of RA catabolism have been described, which may be tissue or species specific (6, 70, 113, 118, 254). These routes include oxidation, isomerization, and formation of glucuronides and taurine conjugates. Recently, a new member of the cytochrome P-450 family, CYP26 (or P-450RAI), has been identified as an enzyme in the embryo that specifically mediates RA oxidation (68), and it is presumed to mediate RA catabolism. CYP26 was first identified in the adult zebrafish as a RA-inducible catabolic enzyme (301) and was later cloned in mouse and human cells by several laboratories (230, 300). Its predominant metabolites have been described as either 4-hydroxy-RA, 18-oxo-RA, and 4-oxo-RA (230, 300), or as 5,8-epoxy-RA (68). The CYP26 promoter contains a RA response element; however, RA exposure of the mouse embryo induces expression only in a limited region (68), indicating the existence of other transcriptional regulators for this gene. Its normal expression pattern in the embryo is suggestive of a function in regulating RA distribution. In the mouse (68), at embryonic day 7.25, it is
present in a posterior to anterior gradient in the mesoderm. High expression at the posterior end continues through embryonic day 8.5 to be present in the posterior neural plate and mesoderm of the hindgut and tailbud at embryonic days 9.5–10.5. Localized areas of anterior expression also exist, with high levels in the neural crest cells of the hindbrain which migrate to the cranial ganglia. In the CNS, expression is also present in the otic vesicle and eye (68). As described above, the presence of CYP26 in the retina is particularly revealing since it sits as a midline stripe subdividing the two RA synthetic enzymes in the dorsal and ventral (181). This suggests a possible role for CYP26 in boundary formation, acting as a perimorphic enzyme.

H. Hypothesis: CRABP Captures RA for Cells That Lack RA Synthesis

RA can function as an embryonic morphogen when synthesized by discrete organizer regions to create gradients of transcriptional activation. This is likely to occur in several regions of the embryo including the early eye anlage (55). The observations reviewed here demonstrate another mode of patterning in the form of zones of differing RA concentration. These territories are created by the ordered patterning of RA synthetic and catabolic enzymes in the embryo, and they have been observed in several regions during development, including the early heart (194) and the retina at later stages of embryonic development (181). A sharp drop off in RA levels occurs also between the spinal cord and the hindbrain in the embryonic mouse (175) and chick (156), and it was noted that gradients are not evident in the RA reporter mice (236). It appears that the zonal patterning of RA levels may be an important form of organization in addition to RA gradients. Several regions of retinaldehyde dehydrogenase expression show exceptionally high levels of enzyme activity, including the spinal cord and the retina (54, 175). The resulting RA concentration in the ventral retina probably exceeds 1 μM, far beyond the concentrations necessary for the activation of the RA receptors. Because the ventral retina is bounded by the catabolic enzyme, this high concentration cannot serve to create a continuous RA gradient across the entire retina. One possible explanation for the high retinaldehyde dehydrogenase levels in neurons may relate to the observations that high enzyme levels are not restricted to the cell bodies but extend also into the axons. Very high AHD2 concentrations are present in the retinal axons (180) and in the dopaminergic axons innervating the corpus striatum (176), and RALDH2 is found in axons of the motoneurons projecting from the embryonic spinal cord (10). Axons from the retina, the ventral tegmentum, and the spinal cord innervate regions that do not contain synthetic enzyme but do express RA receptors. If the axon terminals represent sources of RA, then high enzyme activities would be required in the soma region, to provide amounts for the transport through the thin axons which are sufficient for target activation. Territories of high RA may also represent RA sources for neurons whose processes make contact or pass through these regions, because RA diffuses readily through cell membranes. Fibers innervating the spinal cord motoneurons, or passing by in their vicinity, are exposed to the
very high RA levels present here. Cells of the neural crest, which require RA (151) but do not express RALDH2 (10), migrate through mesenchyme which expresses high levels of RALDH2 (206). Such exposure may be a necessary part of their maturation. Cell types that do not express retinaldehyde dehydrogenases but that respond to external sources of RA may have to be efficient at RA capture. This may be a function of CRABP-I, since it is almost exclusively expressed in cells that respond to RA but that do not themselves synthesize RA. Examples include the neural crest (104), cerebellum (311), and interneurons of the spinal cord (253). The normal response of these tissues to low concentrations of RA might explain why these regions are so sensitive to RA teratogenicity (9, 287); the exposure to abnormally high RA levels will disturb their normal sequence of gene expression. The axons of several neurons are known to express CRABP-I (147, 154, 253), and the binding proteins may assist in the capture of RA, allowing for its transport back to the cell body. The polarization of neurons into soma and far-reaching axonal projections makes it likely that axonal transport plays a role in the localized capture and release of RA. There can be little doubt that the developing CNS will be an interesting area of investigation for the interactions between retinoid metabolism, signaling, and patterning of gene expression.

V. RETINOID RECEPTORS/FUNCTION

A. Introduction

The cloning and characterization of the retinoid receptors represent a landmark in our understanding of the physiology of the retinoids. The discovery was a direct consequence of basic work in the field of steroid and thyroid hormone receptors. A characteristic of these receptors is their modular structure with six different domains (A to F) that fulfill different functions (21, 160). The DNA-binding domain or DBD, also known as the C domain, is very highly conserved between these different receptors. Retinoid receptor cloning was possible because of the common 24-nucleotide probe within this C region used to identify new orphan receptors (71, 221). These approaches permitted the characterization of three genes (α, β, and γ) for RAR and three genes for RXR (159). The latter class of receptors specifically bind 9-cis-RA, whereas RAR bind both RA and 9-cis-RA (35, 137, 159).

Genes containing retinoic acid response elements (RARE) in their promoters are known to be involved in diverse and yet interconnected biological processes, such as embryogenesis, growth, and differentiation (35, 160, 161). The complexity of interactions becomes greater if one considers that RXR, in addition to forming homodimers (159), also form heterodimers with other receptors (135, 159, 166, 314), including RAR, the thyroid hormone receptor TR, the 1,25-dihydroxy-vitamin D3 receptor VDR (314), the peroxisomal proliferator activated receptor PPAR (105, 108, 282), NGFI-B, NURR1 (220), and COUP-TF (106). A distinctive characteristic of the RXR is their ability to interact with other nuclear receptors of the superfamily resulting in their binding to their respective DNA response elements in the promoters of different target genes (107). Gene transcription responses are RXR partner receptor dependent as well as dependent on the presence of the ligand of their heterodimeric partner, e.g., vitamin D3 for the VDR/RXR combination, thyroid hormone for the TR/RXR combination, and so on. It is therefore easy to conceptualize how RXR may control a complex network of hormone-dependent pathways. Complexity increases if one also considers that there are some 18 isoforms of the RAR and probably just as many RXR formed through the use of different promoters or alternative splicing (134).

In addition to the activation function AF-1 in the NH2-terminal A/B regions of the receptors, the use of various reporter gene assays has made it possible to identify a transcriptional activation function (AF-2), which overlaps the ligand binding domain (LBD) in the E region of the RAR and RXR. RAR AF-2 are activated similarly by all-trans- and 9-cis-RA and their 3,4-didehydroderivatives (Fig. 1), whereas RXR AF-2 are only efficiently activated by 9-cis-RA and by 9-cis-3,4-ddRA (see Ref. 134 and references therein).

A third level of complexity accrues from interactive elements on different promoters. For instance, response elements containing direct repeats (DR) of the canonical sequence AGGTCA with a spacer of two nucleotides (DR2) or five (DR5) are known to occur in the homeobox b1 (169, 269) and RARβ genes. In addition, the actual sequence of the direct repeats and the types of flanking bases appear to be important determinants for RAR and RXR binding efficiencies (157). Moreover, interactions with other proteins, which may either function as transcriptional repressors (73, 89, 122, 316) or ligand-dependent activators (20, 23, 97), such as the cAMP response element binding protein (CBP), have also been reported and may add another level of complexity as well as permit a variety of interactive pathways to regulate specific gene expression.

B. Speculations on Orders of Receptor Interactions

These considerations suggest different orders of interactions that may lead to diverse biological outcomes. We suggest that effects of interactions between RXR-RAR heterodimers and target gene promoters are the most immediate and would be the first to respond to retinoid
TABLE 1. Natural retinoic acid response elements

<table>
<thead>
<tr>
<th>Element</th>
<th>Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) hLactoferrin composite</td>
<td>5’-(59)GGCGAGTTCAAGAAAGTTTACGCCAG(34)-3’ (136)</td>
</tr>
<tr>
<td>2) hRARα2</td>
<td>5’-(69)GGCGAGTTCAAGAAAGTTTACGCCAG(33)-3’ (174)</td>
</tr>
<tr>
<td>3) mRARβ2</td>
<td>5’-(57)GGAGGGTTCAAGAAAGTTTACGCCAG(32)-3’ (273)</td>
</tr>
<tr>
<td>4) hRARβ2</td>
<td>5’-(57)GGAGGGTTCAAGAAAGTTTACGCCAG(32)-3’ (45, 86)</td>
</tr>
<tr>
<td>5) hRARγ2</td>
<td>5’-(401)GGCCGGGGTTCAAGAAAGTTTACGCCAG(35)-3’ (174)</td>
</tr>
<tr>
<td>6) hADH3</td>
<td>5’-(280)ACAGGGTTCACTTCAAGTTTACGCCAG(30)-3’ (59)</td>
</tr>
<tr>
<td>7) mCRBPⅡ</td>
<td>5’-(1015)TATAGGTCACAAAAAAGTTTACGCCAG(903)-3’ (250)</td>
</tr>
<tr>
<td>8) mLamBl</td>
<td>5’-(432)GGAGGTTCACTGGTTAAGCCCTTAGAAAGGGTCAAA(468)-3’ (290, 291)</td>
</tr>
<tr>
<td>9) hapoAI</td>
<td>5’-(192)AGGGCGAGTTTACGCCAGTTTACGCCAG(217)-3’ (238)</td>
</tr>
<tr>
<td>10) mCRH</td>
<td>5’-(147)AGCCGGTTCACTCCAGGGCATAGM(122)-3’ (196)</td>
</tr>
<tr>
<td>11) rCRBPⅡ</td>
<td>5’-(341)GGGTGTTCAACTTTCTTCTCCGGTTAAGTTGAAAG(308)-3’ -3’</td>
</tr>
<tr>
<td>12) hMCAD</td>
<td>CACCAAACTGGAAAGAGGCCCCATTTCCACTTCC(5)-228)</td>
</tr>
<tr>
<td>13) hLactoferrin composite</td>
<td>5’-(351)AAGGTTCAGCAAGTTAACCAC(326)-3’ (132)</td>
</tr>
<tr>
<td>14) mCRABPⅡ</td>
<td>5’-(1162)CCACAGTTCCACCGGTAGGCT(140)-3’ (61)</td>
</tr>
<tr>
<td>RARE</td>
<td>5’-(457)CTGTGACCTCTGCCCCCTC(639)-3’ (61)</td>
</tr>
<tr>
<td>15) rPEPCK</td>
<td>5’-(451)TGACCTTTGCGGCTGCA(434)-3’ (143)</td>
</tr>
<tr>
<td>RARE</td>
<td>5’-(351)GATCGTCCGGCCGACCGTTCTCTGACCCACCTGACAATTAGGCAAGG(290)-3’ (244, 274)</td>
</tr>
<tr>
<td>16) mTσase (mTGRREⅠ)</td>
<td>5’-(1703)CATGGGGGGGTGTTACATGAGAGTGTACCAGTGAGGTGTTGCGAGATTA(1751)-3’ antisense (199)</td>
</tr>
<tr>
<td>17) mHox1.6</td>
<td>3’-enhancer 5’-(Sac-74)CGAGAGGTCAAGGAAGTTCAAGSac-55)-3’ (128)</td>
</tr>
<tr>
<td>18) mHox1b-1</td>
<td>3’-enhancer 5’-CATTAGGGTTAAGAGTTTACGCCAG(169-269)</td>
</tr>
<tr>
<td>19) mHoxb-1</td>
<td>5’-repressor 5’-AGGGCAGAGATTTCAC(169, 269)</td>
</tr>
<tr>
<td>20) mSTAT1</td>
<td>5’-(274)TCAGAGTTGCGGAGTCTCACAGTTAATG(252)-3’ (114)</td>
</tr>
<tr>
<td>21) rDopamine D2 receptor</td>
<td>5’-(71)CCTCGTGCCGGAGTTTGACCACCC(51)-3’ (288)</td>
</tr>
</tbody>
</table>

Consensus shown are the natural retinoic acid response elements (RARE) from the promoters of the following genes: 1) mouse (m) RARα2 (136); 2) human (h) RARα2 (174); 3) mRARβ2 (273); 4) hRARβ2 (45, 86); 5) hRARγ2 (174); 6) human alcohol dehydrogenase 3 (ADH3) (50); 7) mouse cellular retinol binding protein I (mCRBPⅡ) (250); 8) mouse laminin B1 (mLb1) (290, 291); 9) human apolipoprotein AI (238); 10) complement factor H (mCp-H) (196); 11) rat cellular retinol binding protein II (rCRBPⅡ) (162); 12) putative RARE of the human medium chain acyl-CoA dehydrogenase (hMCAD) (228); 13) composite element containing an RARE (RXRE) and ERE from the human lactoferrin promoter (132); 14) two RARE from the mouse cellular retinoid acidic protein (mCRABPⅡ) (61); 15) two RARE from the rat phosphoenolpyruvate carboxykinase (rPEPCK) (143, 274); 16) mouse transglutaminase type II (mTσase) (199), which contains three hexad repeats (DR5 and DR7 motif) capable to bind both RXR homodimers as well as RXR-RAR heterodimers; 17) repeated hexad motifs in the 3’ enhancer of the mouse Hox1.6 (mHox1.6) (128); 18) mouse Hoxb1 (169, 269); 19) mouse Hoxb1 5’-repressor (169, 269); 20) mouse STAT1 (114); and 21) rat dopamine D2 receptor (288). Key motifs (157) are highlighted. Reference numbers are given in parentheses.

Depletion or excess. These reactions would activate the characteristic immediate response, if the RARE is located in the promoter of the target gene itself. This is the case for the RARβ2 gene, which is therefore “first-order dependence gene.” Other first-order dependence genes would be the RARγ2 and α2 and all the genes that contain a RARE in their promoters. Some of these are listed in Table 1.

First-order dependence would also be observed with ligands activating RAR-RAR or RXR-RXR homodimers, such as for 9-cis-RA in the activation of the CBPⅡ gene (162). RXR cognate receptors other than RAR, such as VDR, TR (17, 107, 166, 318), COUP-TF (106), and PPAR (282), would mediate second-order types of retinoid responses, in that their responses, although possibly dependent on 9-cis-RA (188), are also dependent on other hormonal ligands such as vitamin D3, thyroxine (314), as well as xenobiotic agents.

We suggest that genes belonging to the “second-order retinoid dependence” are probably much more numerous than those of first-order dependence. These genes control most gene activation processes dependent on thyroid hormone, vitamin D3, and other important hormonal and xenobiotic ligands, which also depend on heterodimer formation with RXR. We call “third-order retinoid-dependent genes” all those genes that would be activated as the result of secondary transcriptional events. An example of this type of dependence is genes that depend on AP-1 complexes. It is well known that transcriptional repression occurs at the AP-1 site concomitant with RA-

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mediated gene activation on various RARE, especially when RA is in excess. Genes containing 12-O-tetradecanoylphorbol-13-acetate (TPA) responsive elements (TRE), such as the stromelysin or the collagenase genes, are negatively regulated by RA. This negative regulation was thought to be due to binding of the AP-1 Jun-Fos protein to the RAR, thus removing them from interactions with the TRE. More recent results indicate that trans-repression is the result of limiting concentrations of the cAMP response element binding protein (CREB) and the protein that binds to CREB or CBP. This CBP apparently binds with high affinity to CREB as well as to RAR, estrogen receptor (ER), and possibly a variety of other receptors (97). Whereas the binding and transcriptional activation by the CREB homodimer is independent of ligands, CBP binding to RAR and other hormone receptors and consequent transcriptional activation is ligand dependent (97). Because CBP is present in limiting amounts, AP-1 trans-repression normally occurs when RAR or the ER concentrations are increased. When CBP is nonlimiting, trans-repression is not observed. Therefore, the concentration of CBP and one of the other ligand-dependent coactivators, as well as that of corepressors, add another level of transcriptional control. Recent reviews on the important aspects of structural configuration and space-filling models (159) of the retinoid receptors and on the genetic knockout (99) of their genes have recently been published.

C. Receptors and Embryogenesis

Data from in situ hybridization studies show that different RAR and RXR subtypes are widely expressed during embryogenesis and that each subtype has its own individual expression patterns that may or may not overlap with the other subtypes. Each retinoid receptor subtype can give rise to different isoforms, each with their own unique expression patterns during embryogenesis (62). Retinoid nuclear receptors of different types appear to be expressed along the entire anterior/posterior (A/P) axis in the CNS. The data suggest that individual receptor subtypes have specific and probably multiple functions. The RARα subtype is expressed in a general fashion during murine embryogenesis, whereas RARβ and RARγ are more restricted (52, 183, 239–241). In the hindbrain, RARβ has a rostral limit of expression at the rhombomere 6/7 boundary (149), and RARγ is expressed in the open neural tube before the fusion of neural folds (239). Among the RXR, murine RXRβ is expressed in a general fashion, but RXRα and RXRγ have more restricted patterns during embryogenesis (48, 158).

The patterned expression or suppression of developmental genes (e.g., the homeobox b1 gene) has been demonstrated to be strictly retinoid responsive, in specific rhombomeres of the mouse hindbrain (168). In fact, RARE have been demonstrated both 5’ as well as 3’ of the gene with the 5’-RARE functioning as a suppressor and the 3’-RARE as an inducer of transcriptional activity for the homeobox b1 gene (38, 168, 269). The developmental time dependency of the function of the two RARE permits and controls the switching on and off of the homeobox β1-gene expression and termination of expression at the border of different rhombomeres, possibly controlling segment identity. It is also evident that a highly ordered retinoid delivery system must be operative to ensure the regulated series of developmental events. Availability of excess retinoids or their deficiency may lead to teratogenesis and/or resorption of the embryo.

VI. RETINOID RECEPTOR KNOCKOUT MUTANTS

A. Isoform-Specific Knockouts

Much effort has been applied over the past few years to the identification of the specific functions of the different retinoid receptors during embryogenesis. One strategy has been to evaluate the phenotype of mice lacking the gene for a retinoid receptor or receptor subtype. Although the different receptor isoforms have unique distributions during development, knocking out one specific isoform revealed a surprising redundancy between members of each receptor subtype. Mice homozygous for either RARα1, RARβ2, or RARγ2 mutation were viable and did not display phenotypic abnormalities (99). Since these initial studies, a stepwise progression of mutant mice from single isoform retinoid receptor mutants to RAR subtype mutants to RAR/RXR compound mutant mice have been created to examine the in vivo function of these receptors (Table 2).

B. Single Subtype Knockouts

Developmental malformations can arise as a result of knocking out the entire gene of a single retinoid receptor. For example, homozygous RARα mutant mice, which express no RARα isoform transcripts, exhibited early postnatal lethality and testis degeneration (145). Of the RAR single-type null mutations, RARγ knockout mice showed malformations (138). RARγ mutant mice exhibited several abnormalities previously associated with vitamin A deficiency, including homoeotic transformations of the cervical vertebrae and occipital region of the skull, fusion of the first and second ribs, and irregularities of the tracheal rings, among others (138).

Because of their interaction with several members of the steroid/thyroid hormone receptor superfamily, the ab-
sence of a RXR would be expected to have more serious consequences during embryogenesis. One such gene knockout, RXRβ, resulted in morphologically normal mice, with the exception that males were sterile (101). Homozygous RXRa null mutant mice died between gestation days 13.5 and 16.5 (271). The embryonic lethality in RXRa-deficient mice was due to hypoplastic development of the ventricular chambers of the heart, which resulted in a very thin ventricular wall and defects in ventricular septation. RXRa null mutants showed abnormalities of the eye (e.g., shortening of the ventral retina) as also observed in vitamin A deficiency (Table 2).

None of the phenotypes resulting from single knockouts, however, was expected on the basis of expression patterns of retinoid nuclear receptors during embryogenesis. Although some specific aberrations have been found to be associated with the lack of certain nuclear receptor subtypes, these studies seem to suggest a high degree of functional redundancy among these receptors. The results of single RAR and RXR null mutants suggest that embryogenesis is well protected by a system that apparently allows most or all functions of an absent receptor to be substituted by another.

### C. Double/Compound Mutants

#### 1. RAR

Unlike RAR single mutants, RAR double or compound mutants died either in utero or shortly after birth (139, 140, 146, 184). Histological and anatomical analyses of these transgenic mice revealed many defects characteristic of fetal vitamin A deficiency (Table 2). Multiple eye abnormalities were found in various RAR double mutant fetuses that were similar to those previously seen in vitamin A-deficient fetuses. A majority of these abnormalities recapitulated those observed in the fetal vitamin A-deficient syndrome, initially described more than 40 years ago (Table 2) (305). A number of additional malformations not described in vitamin A deficiency studies, however, were also observed (140, 184). These findings probably reflect the difficulty of achieving severe vitamin A deficiency by dietary deprivation (99). Taken collectively, these results demonstrate that RAR are essential for vertebrate development and that, most likely, retinoic acid is the active retinoid, required at several stages of the development of numerous tissues and organs (Table 2). In contrast to the apparent necessary role for RAR in transducing the RA signal, CRABPI and CRABPII are probably not critical to these processes, since CRABPI/CRABPII double null mutant mice were found viable and morphologically normal (127).

#### 2. RXR

Double or triple mutations of RXR would be expected to reproduce the defects associated with inactivations of RAR/RXR heterodimers or, at least, produce defects similar to the RAR double mutant defects. Strikingly, RXRβ/−/ RXRγ−/− double and RXRa+/− RXRβ−/− RXRγ−/− triple mutant phenotypes were viable, displaying no obvious congenital or even postnatal abnormalities, except a marked growth deficiency and male sterility due to loss of function of RXRβ (121). Therefore, it ap-
pears that one copy of RXRα is sufficient to perform most of the functions of the RXR.

3. RAR/RXR double mutants

Kastner and co-workers (99, 100) performed a massive undertaking to determine the functional significance of RXR/RAR heterodimeric signaling in embryogenesis. These investigators created compound mutant fetuses bearing null alleles in one RXR (α, β, or γ) and one RAR (α, β, or γ) subtype or isoform gene (100). The data presented show synergy between the effects of RXRα and RAR mutations, but no such synergy was observed when RXRβ or RXRγ mutations were combined with RAR mutations. It is also noteworthy that all defects of fetal vitamin A deficiency are recapitulated by the various RXRα/RAR compound mutations (Table 2). Some of the abnormalities were specific to one type of RXRα/RAR mutant combination, whereas others were seen in several types of RXRα/RAR double mutants. As an example of specificity, the study of RAR double mutants has suggested that RARα and RARβ2, as well as RARα and RARγ, are functionally redundant for the formation of the aorticopulmonary septum (184). In all RXRα/RARα double mutants, a complete absence of this septum was observed. This suggests little functional redundancy between the RAR subtypes, since RARα can never be functionally replaced by either RARγ or RARβ2 in a RXRα mutant background. The case for multiplicity of function may indicate that more than one RXRα/RAR pair is normally involved in the underlying developmental process, which may require the expression of several RA target genes. These data confirm the role of RXR/RAR heterodimers as the functional units that transduce the retinoid signal for a large number of RA-dependent processes. These results further implicate RXRα as the main RXR in the developmental functions of RAR. However, it is noted that a tight functional specificity of RXRα for heterodimerization with RAR is unlikely, because many RAR-dependent developmental processes proceed normally in mutants that express RXRβ as the only RXR (121).

D. Retinoid Receptor Mutants and Limb Malformations

Mouse embryos lacking RXRα have normal limbs and display resistance to limb malformations normally induced by teratogenic RA exposure (272). RA treatments that cause limb defects in 100% of wild-type embryos failed to elicit malformations in RXRα null homozygotes, implicating RXRα as a component in the teratogenic process in the limbs. Furthermore, heterozygous embryos are intermediate in their sensitivity to RA, suggesting the importance of RXRα gene dosage in limb teratogenesis. These investigators, however, found that expression of the RA-inducible gene RARβ2 was equivalent between wild-type and homozygous RXRα embryos after RA treatment. The spatial expression of sonic hedgehog (Shh) and Hoxd12 was also similar for both wild-type and RXRα embryos, following RA treatment. Hoxd12 expression, however, was elevated in RXRα embryos. These observations indicate that transcriptional processes, which are inappropriately influenced in the mouse limb by exogenous RA, require RXRα for their execution.

Results from double mutant mice provide additional information for the role of RA in limb patterning (100). Because both RARα and RARγ transcripts are uniformly expressed in the early stage mouse limb bud (51), it was surprising that neither RARα nor RARγ single knockout mice produced limb malformations (138, 145). These observations, however, suggested a functional redundancy between these two receptors. This may well be the case since limbs from RARα/RARγ double mutants consistently exhibited malformations, including size reduction of the scapula, perforated scapula, radius agenesis, and abnormal digit number (140). Many of these limb defects appear to be fairly restricted to the forelimb skeleton and may reflect a requirement of RA to generate the proper amount of limb mesenchyme, since a deficit of mesenchyme leads to preferential loss of anterior skeletal elements in frog hindlimbs and preferential loss of posterior skeletal elements in salamander hindlimbs (3). The defects do not appear to result from an early zone of polarizing activity (ZPA) defect because limbs displayed a clear A/P asymmetry. The investigators suggest this does not exclude a role for RA in normal A/P limb patterning, since RARβ transcripts are expressed in a region that overlaps with the ZPA and appear to be unaffected in the limbs of RARα/RARγ double mutants (140). The observations from the limb defects in RARα/RARγ double mutants suggest that either RA plays different roles in forelimb and hindlimb development, or the observation is related to events occurring at different developmental time periods for the two limbs. It has also been suggested that inactivation of all three RAR might result in more dramatic effects on limb patterning (99).

E. Retinoid Receptor Mutants and Malformations of the Vertebral Column

RARγ and occasionally RARα single mutants show homeotic transformations and malformations of vertebrae (138, 140). These studies establish that RA plays an important role in patterning of the main body A/P axis. Penetration and expressivity of cervical anterior transformations observed in RARγ null mice increased in a graded manner, with subsequent loss of RARα1 and RARα2 isoforms from the RARγ background (140). Furthermore,
concomitant inactivation of all RARα and -γ isoforms resulted in severe degeneration of the cervical vertebrae (140). RARβ2 may also be involved in axial patterning, because RARαβ2 (but not RARα) double mutants displayed a high frequency of anterior transformations of the sixth and seventh vertebrae (140). Cervical region patterning has also been examined after inactivation of one RXRα allele within several RAR mutant backgrounds (100). Of these, the RARα -/-, RARγ +/-, as well as RXRα +/- -RARα +/- new-borns exhibited a high frequency of defects in the cervical region. The nature of the vertebral abnormalities in RAR single and double mutants as well as RXRα/RAR mutant mice is of significance in view of the interactions between RA and Hox gene signaling. In the somitic mesoderm of the future vertebral column, each vertebral level has a specific Hox gene specification (18, 193). The Hox code in this region can be modulated by either altered expression of Hox genes or by overexpression or mutational analyses or by excess RA (193). Furthermore, several of the homeotic transformations observed in RAR mutants (single and double) or RXRα/RAR mutants appear similar to those described for Hox null mutants (229). The homeotic transformations, observed in RAR and RXR/RAR null mutants, suggest that retinoids may play a role in Hox gene specification in the early vertebral column. The results from the RXR/RAR mutant studies suggest a synergism between RXRα and RARα or RARγ mutations for generating defects during cervical vertebrae morphogenesis (100).

F. Response of Mutants to Excess RA

Both RARα and RARβ null mutants show the same teratogenic responses to RA excess as wild-type mice (193). Furthermore, RARα -/- RARβ double mutant mice were also susceptible to the teratogenic effects of RA, demonstrating that RARα1 and RARβ isoforms, singly or in combination, do not play a major role in RA-induced craniofacial malformation and limb deformities (146).

The response of RARγ null mice to RA excess, however, is significantly different from wild-type mice (138). RA treatment at 8.5–9.0 dpc results in craniofacial abnormalities and axial defects (truncations of the lumbarosacral spine and fusions of the lower ribs) in wild-type mice. The RARγ null mutants show the same RA-induced pattern of craniofacial abnormalities as the wild-type mice. Interestingly, RA administration to RARγ null heterozygotes shows a mild version of the axial abnormality. Therefore, RARγ may be required for the teratogenic RA signal in the generation of the trunk, but not for craniofacial skeletal abnormalities. The toxic effects of repeated doses of RA and two synthetic retinoids, Ro-15–1570 and Ro-40–6055, on RARγ null mice have also been examined (141). Surprisingly, homozygous mutant mice were resistant to fourfold higher doses of RA than wild-type mice as well as to elevated doses of the synthetic retinoids. The results indicated that RARγ may have a major role in mediating retinoid toxicity. Furthermore, these findings may have practical implications for reducing the toxicity of synthetic retinoids in clinical use. Mouse embryos lacking RXRα have been described as resistant to RA-induced teratogenicity, in particular for limb defects (272). RA treatments, which caused limb defects in 100% of wild-type embryos, failed to elicit malformations in RXRα homozygotes, implicating RXRα as a component in the teratogenic process in the limbs.

G. Conclusions of Mutant Mice Studies

Much knowledge has been obtained on the possible function of retinoid receptors during embryogenesis by analyzing their expression patterns and the effects of “knocking out” receptor classes. It is not, however, within the scope of this review to describe all the various phenotypes arising from the several retinoid receptor mutant mice; instead, we provide a few of the highlights. The defects are diverse, as might be expected from the complex expression patterns of the different RAR and RXR, and some of the effects are common in the offspring of vitamin A-deficient mice. Simple knockouts of a given receptor do not appear to result in the unambiguous characterization of the receptor’s role in vivo, most likely because it involves a family of receptors and because it functions as a heterodimer with other receptors. Additionally, many of the mutations may not reveal the functions of a given receptor, because they result in either a lethal phenotype or agenesis of that organ. Characterization of the receptor’s role in vivo, most likely because it involves a family of receptors and because it functions as a heterodimer with other receptors. Additionally, many of the mutations may not reveal the functions of a given receptor, because they result in either a lethal phenotype or agenesis of that organ. Expression patterns of RAR/RXR during embryogenesis suggest conserved specialized functions for particular receptor subtypes. Genes that appear to be functionally redundant from knockout studies, however, may not be redundant in normal development. The studies of single and double RAR mutations reveal that a given RAR subtype can exert some of the functions of another RAR subtype, only when the latter, which performs these functions and hinders the activity of the former under physiological conditions, has been knocked out. Such artifactual redundancies generated by knockouts of genes that belong to a multigene family may limit our ability to deduce the in vivo functions of a given gene product. The RXRα/RAR double mutants, however, provide some information on functional specificity among retinoid receptors. Specific phenotypes do arise in these double mutants, indicating that the different receptor types probably possess different functions during development. Furthermore, the observation that developmental malformations arising from RXR/RAR double mutants are similar to malformations in vitamin A-deficient mice suggests that the RXR/RAR heterodimer most likely functions to express the retinoid signal in vivo.
In the past it has been widely accepted that the teratogenic effects of exogenous RA reflect a physiological role for endogenous RA in embryogenesis. The findings from RAR and RXR mutant mice, however, cast some doubt on the normal physiological relevance of RA excess studies. In the two cases for which the involvement of a given RAR or RXR in the mediation of a teratogenic event was demonstrated, the same receptor was not required for development of the same structure during normal embryogenesis [e.g., RARγ in the vertebral (138) and RXRα in the limbs (272)]. Therefore, it appears that specific teratogenic processes, as well as specific normal developmental processes under vitamin A control, occur through individual members of the RXR and RAR families and that these receptor-specific processes appear to be different in the excess RA situation, compared with the normal condition.

VII. HOX GENES AND RETINOIDs IN DEVELOPMENT

A. Hox Genes in Development

Homeobox genes have fundamental roles in the organization of developmental pattern in both invertebrate and vertebrate embryos (182). Vertebrate homeobox genes of the Hox family are expressed during segmental patterning of the mammalian hindbrain as well as in axial patterning of the limb (77). Hox genes contain homeoboxes, which encode a helix-turn-helix motif that functions to bind DNA (76). These DNA-binding proteins are thought to control pattern formation by providing positional cues that determine organization within the developing embryo (75, 76, 255). The mammalian genome contains up to 38 Hox genes subdivided in four clusters (Hox A, B, C, and D). These Hox gene clusters have been identified in mouse and human genomes, each cluster on a different chromosome. A Hox gene cluster contains at least nine genes organized in a homologous linear arrangement of ~100–150 kb. Furthermore, all Hox genes share the same transcriptional orientation. During early development, Hox genes display colinearity with respect to their temporal order of activation. Generally, the 3’-genes are activated earlier in development than the 5’-genes (2). In addition to temporal order of expression, the Hox genes are expressed in sequential zones along the A/P axis in the hindbrain and trunk regions. Hox genes show A/P colinearity of expression, such that progressively more 5’-genes are expressed in progressively more posterior zones (74, 120, 182). Therefore, Hox genes at the 3’-ends of the clusters are expressed earlier in embryonic development and in more anterior regions, whereas Hox genes closer to the 5’-ends of the clusters are expressed at later times in development and in posterior regions of the embryo.

B. Retinoids and Hox Genes

Retinoids have been shown to affect the expression of Hox genes in vitro, in teratocarcinoma cells, and in early embryos (62). Recent investigations have identified RARE in the enhancer regions of certain Hox genes. In F9 cells, Langston and Gudas (128) have shown that at ~5 kb 3’ of the Hoxa1 gene transcriptional start site there is an RA-inducible enhancer that contains an RARE of the DR5 type (RAIRD5) that specifically binds RAR. A similar enhancer was also found in the murine and chick Hoxb1 gene and was shown to be required for expression in the developing foregut, which gives rise to various organs including the esophagus, lung, stomach, liver, and pancreas. This enhancer is necessary and sufficient for RA activation of the Hoxa1 gene, the 3’-most gene in the A cluster. Retinoid response elements have also been identified for the human and murine Hoxb1 (169, 213, 269, and described below) and murine and human Hoxd4 genes (190, 225). From expression analysis of Hoxb1-lacZ transgenic mice, Gudas and collaborators (91) have shown that a wild-type (wt) transgene with 15 kb of Hoxb1 genomic DNA, which includes the Hoxb1 3’-RAIRD5, displays a pattern of localization and developmental expression similar to that of the endogenous Hoxb1 gene. Introduction of a point mutation in the DR5 RARE caused it not to be expressed in the developing foregut (91). Exogenous RA also resulted in enhanced expression of the wt transgene in the gut, with an anterior expansion of expression in this tissue. Therefore, these studies conclude that Hoxb1 is expressed in the developing gut and that this expression is regulated through the DR5 RARE. The authors suggest a role for Hoxb1 in the A/P axis patterning of the gut and a critical role for endogenous retinoids in early gut development.

The Hox B cluster of genes is expressed in the developing hindbrain and spinal cord in head to tail order with a 3’ to 5’ sequence of expression. Some of the genes in the Hox B cluster may help establish pattern and positional identity in neural development. However, the Hoxa1 is expressed before Hoxb1 and is thought to be involved in Hoxb1 initiation (268). Interest in Hoxb1 is based on the observation that this gene is among the early genes to be expressed in the mesoderm and neuroectoderm of primitive streak and presomite embryo, before its expression in hindbrain rhombomeres (67, 197, 198, 304). In mice, the expression of the Hoxb1 gene varies at different times of development. The initial expression takes place at 7 dpc in a diffuse manner starting at the r3/r4 boundary and reaching caudally to the end of the developing spinal cord. Hoxb1 continues to be expressed in the posterior hindbrain and trunk as well as r4 until very late in development. Excess exogenous RA can cause craniofacial malformations and malformations in the hindbrain region. The ability of RA to alter the segmental organiza-
tion of the developing hindbrain may result in part from its ability to alter the pattern of expression of Hox genes (28). In fact, both the early broad pattern and the later restricted pattern of the Hoxb1 expression are regulated by RA through RARE (169, 269).

Regulatory elements required to generate the early neuroectodermal and mesodermal phase of Hoxb1 expression were localized to the 3′-flanking region of the gene (169). Two separate enhancers were revealed in this 3′-flanking region, each regulating different aspects of the early phase of Hoxb1 expression. The enhancer regulating the expression of Hoxb1 in neuroectoderm was found to contain a RARE. Point mutations within this 3′-RARE prevented the establishment of neural expression of Hoxb1 in transgenic mice. The position of this Hoxb1 3′-RARE (DR2 type) is similar to that seen with the Hoxa1 RAIDR5 (91, 128). The demonstration of 3′-RARE enhancer regions in both Hoxa1 and Hoxb1, the first genes of the Hox A and Hox B clusters, respectively, suggests that RA has important molecular functions during morphogenesis. The restriction of Hoxb1 expression to r4 during later stages of hindbrain development is due to two DNA sequences located upstream, i.e., 5′ of the Hoxb1 gene (269). A combination of activation and repression determines the limitation of Hoxb1 to r4. An enhancer sequence at the 5′-end of the Hoxb1 gene is necessary for expression in r4 as well as in r3 and r5 (265). However, next to this 5′-enhancer sequence is another DNA sequence that sharply limits Hoxb1 expression to r4 (269). This sequence was found to turn off Hoxb1 expression in r3 and r5. Furthermore, the sequence of this repressor element contains a DR2-RARE. Mutations in this sequence abolish its repressive activity. Therefore, the Hoxb1 5′-RARE is an essential component of the repressor region and cooperates with the r4 enhancer to refine Hoxb1 expression in the hindbrain. Gudas and collaborators (130) have discovered an enhancer containing a DR5 RARE (RAIDR5) located at a DNase I-hypersensitive site 3′ of the murine Hoxb1 gene. This enhancer regulates the RA responsiveness of the Hoxb1 gene and is different in location and sequence from the RA-regulated 3′ Hoxb1 enhancers described by Marshall et al. (169) but is similar in sequence to the Hoxa1 RARE enhancer discovered previously by the same group (128). These 3′-enhancers may be important in the activation of the entire Hox loci as well as in the regulation of Hoxa1 and Hoxb1. A targeted mutation in the Hoxb1 3′-RARE showed that its expression is required to attain early high levels of Hoxb1 in neural ectoderm. Double mutant analysis with this (3′-RARE) allele and other targeted loss of function alleles from both Hoxa1 and Hoxb1 revealed synergy between these genes. Consistent with these studies is the recent report that shows a synergy between Hoxa1 and Hoxb1 in patterning the hindbrain, cranial nerves, and the second pharyngeal arch (69). In the absence of both genes, tissue formed in the region of r4 lacks the earliest r4 marker, Eph tyrosine kinase receptor EphA2. This suggests a failure to initiate rather than maintain the specification of the r4 identity by both Hoxb1 and Hoxa1. This genetic analysis shows that individual members of the vertebrate labial-related gene family have multiple roles in different steps governing segmental processes in the developing hindbrain (268). However, studies on mice lacking expression of Hoxb1 showed a very limited phenotype, which is not consistent with a quantitatively major role of this gene in embryogenesis (69, 268). Even the double a-1/b-1 mutants show little change in phenotype from the single a-1 mutant (69, 268). Even though this change may be crucial for the development of certain organs like the ear, it does not appear to support a role for b-1 in hindbrain segmentation or overall hindbrain neural arrangement. It appears, therefore, that the role of b-1 is mainly at the level of a single segment identity. Importantly, the normality of trunk development in the a-1/b-1 double mutants argues against a requirement for these genes for initiation of more posterior Hox genes (69, 268).

A comparison of Hox misexpression experiments with the results of RA teratogenesis suggests that retinoids influence development via Hox gene regulation. Exposure of embryos to exogenous RA, which effectively increases the levels of retinoids along the body axis, extends the anterior boundaries of some of the Hox genes (129) and may cause transformation of anterior rhombomeres into morphologically posterior rhombomeres (168, 192). Interestingly, transgenic overexpression of Hoxa1 transformed anterior rhombomeres 2 and 3 (normally not expressing Hoxa1) into rhombomeres that resemble rhombomere 4 (the most anterior boundary of Hoxa1 in normal embryogenesis) (317). Expression of Hoxb1 occurred in the neuroepithelial cells in r2 following overexpression of Hoxa1, which suggests possible interactions between Hox genes. With the exception of Hoxb1, however, the expression patterns of other Hox genes were unaffected by the overexpression of Hoxa1. Furthermore, abolishing Hoxa1 expression by introduction of a null mutation reduces some of the segmentation of the hindbrain leading to the loss of rhombomeres 4 and 5 (50, 144, 165). Interestingly, the r4-restricted pattern of Hoxb1 expression was maintained in Hoxa1 mutant mice (165). The similarities in phenotypes between retinoid teratogenicity and models generating Hox gene mutations suggest that endogenous retinoids may establish the A/P graded expression of the Hox genes and thus influence A/P identity along the axis.

In an attempt to describe some of the physiological requirements for vitamin A in development, Maden and co-workers (151, 152) characterized neural defects using the vitamin A-deficient quail embryo as a model. Among the defects observed in the vitamin A-deficient quail system is the absence of posterior rhombomeres. In addition
to observing hindbrain morphology, these investigators confirmed the loss of posterior hindbrain by utilizing expression patterns of Hoxa2, Hoxb1, Hoxb4, Krox20, and FGF-3 as markers and found that segmentation of the myelencephalon (rhombomeres 4–8) was disrupted (151). Expression patterns of other early developmental genes revealed the presence of rhombomeres r1, r2, and r3. Maden et al. (153) later observed that the absence of the posterior hindbrain was caused by a highly localized region of cell death occurring as the hindbrain becomes respecified at the seven-somite stage. The authors suggest that a posterior regression of Hox gene expression may have resulted in the absence of the posterior hindbrain (152). The results imply that Hox genes, or other developmental genes, play a role in the A/P patterning of the CNS and are under the control of RA in the early embryo. Using the vitamin A-deficient quail model, Maden and co-workers (151, 152) also observed that neural crest cells need RA for normal development and survival and that the neural tube failed to extend neurites into the periphery. The influence of RA on the expression of HoxD (Hox4) genes during limb and vertebrae morphogenesis has been reviewed (87).

C. Conclusions on the Induction of Hox Genes by Retinoids

A direct molecular link between RA, RAR, and the activation of specific homeobox genes has been observed during embryogenesis. The availability of retinoid response elements in Hoxa1, Hoxb1, and Hoxd4 genes suggests that retinoids act as an early developmental signal, possibly conditioning the posterior-to-anterior gradient in the gastrula and providing positional specification of the A/P axis in the developing vertebrate embryo. How other Hox genes located in more 5'-positions of the cluster are regulated by RA is not known. The Hoxa1 and Hoxb1 genes appear to be modulated directly via the RARE, and the protein products of these genes may activate the proximal 5'-genes in the clusters, that is Hoxa2 and Hoxb2, respectively. This scenario would allow for a sequential activation of Hox genes. It may also be that downstream targets of Hox genes, as well as other developmental genes regulated by RA, are involved in the regulation of the Hox genes located in more 5'-positions.

Specific combinations of Hox proteins, expressed for example at different axial levels in the hindbrain, appear to result in expression patterns of target genes and unique tissue identity.

Mechanisms in normal development and RA-induced malformations may further be elucidated by determining downstream targets of the homeobox proteins. The target genes for both the homeobox proteins and retinoid receptors are a current area of investigation in embryogenesis. For example, evidence suggests that genes involved in cell shape determination are targets of the Hoxa1 homeobox protein (75).

In conclusion, Hox gene regulation is mediated partly by positional information supplied by retinoids, and the effects of excess RA on axial patterning are partly mediated through the disruption of Hox gene expression in the hindbrain and spinal cord. Studies suggest, however, that RA, along with its nuclear receptors, is an important signaling molecule in this developmental process.

VIII. TERATOLOGY/EXCESS RETINOIDS

A. Introduction

Embryonic exposure to either an excess of retinoid or a deficiency of vitamin A leads to abnormal development (149, 204). From these results, it was understood very early that the embryo requires a precisely regulated supply of retinoids. Although the spectrum of malformations that have been observed due to either vitamin A deficiency or excess appears to overlap, there are examples of selective responses to only deficiency or excess. No single mechanism is likely to explain the action of retinoids in normal or teratogenic development. It is more likely that each tissue utilizes RA in a unique process during development. Much work has been performed to relate the mechanism of malformations from excess exogenous retinoids to the role that retinoids play in normal developmental processes. Here we summarize some of the important work that has been performed utilizing excess retinoids, as well as a summary of epidemiologic evidence for human teratology from exposure to retinoids. This provides an historical context for our current thinking on retinoid action in embryogenesis and also reflects our interest in teratogenesis resulting from retinoid toxicity.

B. Retinoids and Limb Morphogenesis

The developing chick limb bud has been extensively used to study morphogenesis (87); cells in the limb bud differentiate into muscle, cartilage, and bone cells of the mature wing (66). The digits of the wing are oriented along the A/P axis of the wing bud, and they are ordered by the ZPA in the posterior region of the bud. A mirror image duplication of digits (296) can result from either grafting the ZPA to the anterior region of the bud or by the local application of RA to this area. This implied that RA could mimic the behavior of the cells of the ZPA. It is possible that high concentrations of RA may give rise to a new ZPA region adjacent to an RA implant (inert carrier material impregnated with RA). In support of the hypoth-
esis that RA may induce a ZPA region is the observation that retinoid receptor antagonists blocked the formation of the ZPA when applied to the chick limb bud (85, 111).

The intercalary interaction model of pattern formation has also been postulated to explain the action of RA in limb bud duplication (87). In this model, RA or the ZPA influences short-range cell-to-cell interactions, and each cell has a positional characteristic that represents its spatial coordinate within an embryonic tissue. In the RA implant-induced digit duplication experiments, it is thought that RA “converts” cells next to the implant into posterior cells. These posterior cells interact with existing adjacent anterior cells, giving rise to cells with progressively greater anterior positional values, eventually resulting in digit duplications.

Molecular evidence suggests that the Hox4 genes may encode positional information (93, 212). The Hox4 homeobox genes have been found to be coordinately expressed in partially overlapping domains during chick wing development. Local application of RA has been shown to induce transcription of Hox4 genes in a mirror-image pattern of expression, which correlated with the subsequent development of mirror-image patterns of digits. Additional molecular evidence suggests that the product of the sonic hedgehog (Shh) gene may be the morphogen in limb bud duplication and that RA may function indirectly in limb patterning by inducing Shh (209, 231, 257). Chen et al. (25), however, have demonstrated that the expression of Shh in Hensen’s node is not affected by the endogenous vitamin A status in chick embryos, further complicating the role that RA plays in limb morphogenesis (25). Stratford et al. (267) find diminished Shh expression in vitamin A-deficient quail limb. Additional evidence suggests that other factors, e.g., fibroblast growth factors (FGF), play a role in generating and maintaining ZPA activity and limb patterning (209). It was found that Shh gene expression was activated in limb mesenchyme by RA plus FGF-4, but, once induced, Shh expression was maintained by FGF-4 alone (209). It can be hypothesized from these developments that retinoids may encode positional information (93, 212). The Hox genes, such as the Hox D complex (Hox4, according to the older nomenclature), are expressed in the mouse limb bud in a spatial and temporal pattern (49). Because homeobox genes have been shown to be regulated by RA in certain tissues, current work continues to explore RA’s regulation of Hox genes during mouse limb bud morphogenesis as well as the teratogenic effect of RA on Hox gene expression. Exogenous retinoids are also known to influence patterning of other structures, such as the anteroposterior body axis which is reviewed elsewhere (87).

C. Animal Teratogenic Models

The teratogenic effects of high doses of vitamin A in pregnant rats were first described by Cohlan in 1953 (26; see Ref. 231; as reviewed in Ref. 5). Cohlan demonstrated that feeding pregnant female rats 35,000 IU/day of a “natural vitamin A” preparation (most likely a retinyl ester preparation) on gestation days 2–16 resulted in a large number of fetal anomalies. These anomalies included exencephaly, cleft lip, cleft palate, brachygnathia, and eye defects. RA was later shown to be a more potent teratogen than retinol in several animal models (109, 252). Similar anomalies to those described above have been observed in embryos of many species of experimental animals including monkeys, rabbits, rats, mice, and hamsters after excess retinoid administration (63, 95, 96, 103, 109, 112, 252). In addition to malformations in the offspring, the treatment of pregnant female experimental animals with excessive amounts of vitamin A (retinyl acetate or palmitate) or one of its metabolites, e.g., RA or 13-cis-RA, has resulted in dramatically increased rates of fetal resorptions and stillbirths. Generalizations concerning retinoids in teratogenesis have emerged from these and other studies. First, the anomalies observed in animals in response to retinoids are stage dependent. Early postimplantation exposure [gestational days (GD) 8–10] typically leads to craniofacial and overt CNS defects, whereas exposure on GD 12–14 is often associated with limb and genitourinary defects (103). Second, the teratogenic response for each retinoid is dose dependent (1, 246). Higher doses increase the frequency and severity of
malformations and are more likely to be lethal to the embryo (109). When both stage and dose dependency are considered, it is noted that relatively lower doses ≤10 mg/kg are toxic early in the critical phase of development, whereas higher doses (≥100 mg/kg) are required later in the critical phase of organogenesis (1). Third, some retinoids are more potent than others. In mice, for example, 13-cis-RA and retinol are ~20 and 4 times less potent teratogens than RA (262), respectively. Furthermore, the potency of the retinoid can vary with different animal models. Humans, for example, are more sensitive to 13-cis-RA than monkeys and rabbits, whereas mice and rats are relatively insensitive to this retinoid (201, 202). Species differences in response to the teratogenic potency of 13-cis-RA can be explained by variation in pharmacokinetics (204). In many of the animal studies described above, the dosages tested were generally far in excess of likely human exposures. However, the spectrum of malformations observed in animal models are similar to those observed with human exposure to pharmacological doses of 13-cis-RA and, although seen less frequently, retinol (251).

D. Molecular Mechanism of Excess Retinoids in Teratogenesis

One approach to unravel the molecular events in RA-induced teratogenesis is to examine the spatial distribution of active retinoids during embryogenesis. For example, radiolabeled RA administered to pregnant mice at postimplantation stages (approximately GD 8–11) was shown to accumulate in restricted areas along the neural tube (39, 41). These areas were identified as rhombomeres of the hindbrain, neural crest cells, and neural crest-derived structures, putative targets for the teratogenic action of retinoids (126, 294, 303). Other work suggested the distribution of RA was found to be correlated with expression patterns of CRABPI (40, 42), suggesting that CRABPI may be an integral part of the teratogenic effect of RA. For example, CRABPI may become increasingly saturated upon “dosing” the embryo with exogenous RA, resulting in an enhanced availability of unbound RA for nuclear translocation and consequent gene modulation. Horton and Maden (90) had an interesting result, where the regional accumulation of teratogenic RA had no relationship to the distribution of CRABPI or -II.

Other approaches have been utilized to study the molecular events in RA-induced teratogenesis. Determining the pattern of expression of retinoid nuclear receptors in embryos exposed to teratogenic doses of RA is one such approach. Studies using GD 11 mouse embryos demonstrated that mRNA levels of the RARβ2 isoform were elevated after a teratogenic dose of RA and distributed in regions of the mouse embryo that are most susceptible to the effects of RA (80). For example, RARβ2 mRNA levels were elevated 12-fold in limb buds (highly sensitive), 8-fold in the head (moderately sensitive), and only 3-fold in the remainder of the body (relatively insensitive) after treatment of the pregnant dams with teratogenic doses of RA. Furthermore, on GD 14, when the mouse embryo is no longer sensitive to the teratogenic effects of retinoids, the elevation in RARβ2 mRNA levels in all embryonic tissues was only two- to threefold (80). These observations are consistent with the possibility that elevation in the mRNA level of specific RAR (or RXR) isoforms may mediate abnormal development in RA-treated embryos (262). Furthermore, an alteration in the relative amounts of one receptor could have a cascadelike effect and perhaps result in wide-ranging effects on the expression of a variety of genes.

Regionalized synthesis of RA creates regions of high and low RA (see Fig. 4). Teratogenic RA may ablate these high/low differences and disrupt the patterning created by these. Regions of low RA content (which generally express CRABPI) would be most sensitive (54, 55).

Altered expression of specific homeobox genes has been associated with RA-induced teratogenesis. For example, alterations in Hoxb1 expression have been linked to defects in the hindbrain in embryos treated with excess RA (28, 169). These studies suggest a link between RA, CRABP, RAR/RXR, and transcriptional modulation of homeobox genes during teratogenesis.

E. Prescription Oral Retinoids as Teratogens in Humans

A relationship between high levels of retinoids and human birth defects has been known for some time (82 and reviewed in Ref. 217). The teratogenic effects of retinoids become manifest during the first trimester of embryonic development, and many defects likely arise from the abnormal migration of cranial-neural crest cells or a defect in early axial patterning. The threshold dosage of retinoids required to cause these malformations is unknown (204). Maternal ingestion of 13-cis-RA (isotretinoin or Accutane), prescribed for the treatment of severe cystic acne, has resulted in spontaneous abortions and the manifestation of severe malformations in the offspring (126). Abnormalities, such as microtia/anotia, micrognathia, cleft palate, conotruncal heart defects and aortic-arch abnormalities, thymic defects, retinal or optic-nerve abnormalities, and CNS malformations (e.g., hydrocephalus), were observed in the fetuses of women ingesting therapeutic doses of 13-cis-RA (0.5–1.5 mg/kg) during the first trimester of pregnancy, and these retinoids are, thus, contraindicated for use during pregnancy (126). The pattern of malformations arising from exposure to isotretinoin in humans is thought to closely resemble those ab-
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<td>Martinez-Frias and Salvador (171)</td>
<td>≥10,000 IU of retinol or retinol palmitate alone or in combination with other vitamins</td>
<td>Controls matched to cases on sex; hospital; considered the effects of dose and time of exposure during pregnancy; interviewed within the first 3 days after delivery about exposure</td>
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<td>Khoury et al. (102)</td>
<td>Retinol in supplements and combination of retinol in supplements and food</td>
<td>Mothers consuming &gt;10,000 IU from supplements (compared with ≤5,000 IU) increased prevalence of cranial neural crest tissue birth defects (OR: 4.8; CI: 2.2–10.5)</td>
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<td>Khoury et al. (102)</td>
<td>Vitamin A (preformed) supplement plus multivitamin supplement</td>
<td>No increased risk among women who regularly took both vitamin A supplements plus multivitamins (OR: 0.54; CI: 0.22–1.33)</td>
<td>Confounding effects considered; collected information on exposure variable before knowledge of birth outcome</td>
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<td>Shaw et al. (250)</td>
<td>Single supplements of vitamin A (preformed)</td>
<td>No increased risk for orofacial clefts with vitamin A supplement use (OR: 0.55; CI: 0.21–1.35); no increased risk for conotruncal heart defects</td>
<td>Malformations associated with neural crest cells-vitamin A target tissue</td>
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<td>Mills et al. (187)</td>
<td>Preformed vitamin A in fortified cereals and dietary supplements; also asked yes/no for consumption of organ meat</td>
<td>No association at doses &gt;8,000 IU (OR: 0.79; CI: 0.4–1.53) or &gt;10,000 IU (OR: 0.73; CI: 0.27–1.96) compared with doses &lt;5,000 IU</td>
<td>56.8% supplied dose information from the vitamin bottle; detailed info on types and doses of vitamin supplements and cereals; short time interval between organogenesis and interview; geographically based nature of sample; blinded interviews</td>
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normalities produced in animal studies of retinoid teratogenesis. Because 13-cis-RA is a metabolite of vitamin A, the possibility exists that retinol, in sufficiently large doses, is also teratogenic. Furthermore, 13-cis-RA occurs physiologically in very small amounts, and it shares many metabolic and binding properties with all-trans-RA, and therefore 13-cis-RA may be involved in the teratogenicity of preformed vitamin A. This is further strengthened by the hypothesis that the teratogenic potency of vitamin A is thought to be mediated by its metabolism to active retinoids (204).

Synthetic retinoids have also been implicated in teratogenesis. For example, human exposure to etretinate, a synthetic, aromatic retinoid prescribed for the treatment of psoriasis, has resulted in spontaneous abortions or the manifestation of severe malformations (e.g., craniofacial and skeletal) in offspring (235). One case of etretinate embryopathy occurred in an infant conceived 1 yr after termination of etretinate, which unlike isotretinoin is stored in maternal adipose tissue (125). Although there appears to be a characteristic set of birth defects with ingestion of these retinoids, the pattern of malformations is too complex to reconcile, at present, with the known effects of retinoids on morphogenesis or with the molecular mechanisms elucidated to date (110).

### F. Human Epidemiological Evidence: Intake of Vitamin A and Risk of Birth Defects

The embryo can be exposed to teratogenic doses of vitamin A when a pregnant woman consumes a chronic dietary intake of foods that contain high concentrations of vitamin A, either an acute large dose or chronic high doses of vitamin A dietary supplement products, or prescription drugs that contain retinoids. One case report of a single dose of supplemental vitamin A in early pregnancy (500,000 IU in the second month of pregnancy) provided evidence for the human teratogenicity of vitamin A (195, 235). In this case report, the fetus presented with preauricular appendices and eye defects. Less massive doses taken daily for several weeks or longer have been shown to produce defects such as facial dysmorphism, absence of external genitalia and of anal and ure-
of adverse pregnancy outcomes associated with supplemental vitamin A intakes of 25,000 IU or more per day have also been documented (235). Case reports of the earlier case-control studies did not question participants about vitamin A consumption until a few years after the diagnosis of a birth defect (250). In addition to supplemental vitamin A, this study also examined the consumption of breakfast cereals with added vitamin A and queried as to whether the women consumed organ meat. Neither breakfast cereal nor organ meat consumption changed the conclusions of the study. It is important to note that in studies evaluating dietary intake, provitamin A sources (e.g., β-carotene) have not been associated with an increased risk of birth defects. This may be due to the inefficient absorption and conversion of β-carotene to retinol.

Two prospective studies have been conducted (173, 237) (Table 3). These studies are important in that investigators collect information on vitamin A exposure before knowledge about birth outcome, which minimizes potential recall bias. In one prospective study, history of vitamin A supplementation use was obtained before pregnancy outcome on more than 22,000 pregnant women (237). One of the most significant findings of this study was an association of the consumption of >10,000 IU vitamin A/day from supplements and an increased risk of birth defects of all types. The consumption of more than 10,000 IU of vitamin A from supplements was also associated with an increased risk of birth defects originating from cranial-neural crest tissue, defects thought to be strongly correlated with high intakes of vitamin A. The results of this study raise concerns about the safety of high doses of vitamin A during early pregnancy. However, there are a number of methodological questions concerning the study that prevent reaching the conclusion that the dosages of vitamin A (>10,000 IU) examined in the study cause certain types of birth defects. One of the limitations of this study was the broad definition used for cranial-neural crest defects. Thus some birth defects arose in sites unlikely to be of true cranial-neural crest origin, which may have led to a birth defect being attributed to vitamin A, when in reality it was the result of some other factor. This misclassification problem, however, does not influence the finding that consumption of >10,000 IU of vitamin A daily from supplements was associated with increased risk of birth defects of all types.

In a more recent study, pregnant women exposed to 10,000 IU/day or more of vitamin A during the first 9 wk of pregnancy were identified prospectively and their pregnancy outcomes were compared with two control groups: 1) pregnant women exposed to high vitamin A exposure later in pregnancy or 2) pregnant women exposed to nonteratogenic agent exposures (173). The birth prevalence rate of major malformations in the study group compared with either of the control groups did not provide evidence for an increased risk of major malformations from 10,000 IU/day or more of vitamin A during organogenesis. Although timing and dose of vitamin A use

<table>
<thead>
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<th>Dose of Vitamin A</th>
<th>Odds Ratio</th>
<th>95% Confidence Interval</th>
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<tbody>
<tr>
<td>&lt;10,000 IU/day</td>
<td>0.7</td>
<td>0.2–2.3</td>
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<tr>
<td>10,000 IU/day</td>
<td>2.2</td>
<td>1.0–5.0</td>
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<tr>
<td>&gt;10,000 IU/day</td>
<td>3.3</td>
<td>1.5–7.2</td>
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was collected prospectively, limitations of this study include the representativeness of the study population to healthy women of childbearing age and the liberal definition of vitamin A use in the study group, i.e., \( \geq 10,000\) IU/day for at least 1 wk during the first trimester. Furthermore, confounding factors such as diet and other maternal factors were not controlled for in this study.

The results from randomized, placebo-controlled studies can potentially provide important information in assessing a relationship because the design minimizes the effects of confounding and bias. A randomized controlled trial in Hungary compared a multivitamin containing 4,000 or 6,000 IU of vitamin A (in addition to 0.8 mg folic acid) with a placebo. The study found a lower incidence of spina bifida, anencephaly, and other birth defects among the infants of multivitamin users (31, 57) (Table 3). This study, however, was undertaken to determine whether folic acid in a multivitamin supplement reduced the occurrence of neural tube defects rather than as an examination of the association between vitamin A supplement use and birth defects. Following this trial these same authors utilized a large population-based, case-control data set, the Hungarian Case-Control Surveillance of Congenital Abnormalities (1980–1994), to explore whether cases of congenital abnormalities were more likely to be exposed to high vitamin A intake than healthy controls (32). These authors report that maternal vitamin A use was significantly associated with pregnancies resulting in healthy newborn infants. Smoking, alcohol, and dietary intake were not assessed in this case-control study.

Preformed vitamin A may be teratogenic; however, the teratogenic threshold in humans has not yet been determined. The teratogenicity in humans of doses above 10,000 IU/day of preformed vitamin A has been reported, but not yet confirmed. Before the Rothman study, the lowest maternal dose reported to be teratogenic in humans was \( \sim 25,000\) IU (235). It is clear, however, that exposure to therapeutic doses of certain analogs of vitamin A, e.g., 13-\(c\)-RA (0.5–1.5 mg/kg), results in substantially increased risk of birth defects. As the dose decreases, however, the strength of the apparent relationship between excess vitamin A ingestion and birth defects becomes more tenuous. In many of the studies cited above, it was rare for women to take more than 10,000 IU of supplemental vitamin A per day. This fact has impacted on the ability to detect an association between high potency vitamin A supplements and birth defects. It is encouraging that the frequency of exposure to high-potency vitamin A supplements is apparently rare.

To approximate safe levels of vitamin A intake during pregnancy, an assessment of vitamin A metabolites in plasma during pregnancy has been performed. In this study, endogenous plasma concentrations of vitamin A metabolites during pregnancy ranged from 0.26 to 7.72 ng/ml (302). The vitamin A metabolites and their concentrations reported in this study include the following: all-t\(r\)-RA (0.68–2.18 ng/ml), 4-oxo-RA (0.26–2.04 ng/ml), 13-cis-RA (0.72–4.72 ng/ml), and 4-oxo-13-cis-RA (0.84–7.72 ng/ml). The authors suggested that retinoid plasma levels in this range may be nonteratogenic because these pregnancies did not result in fetal malformations. Vitamin A intake from dietary sources was not reported in this population of pregnant women. The same investigators report plasma levels of vitamin A metabolites following daily vitamin A supplements of 4,000, 10,000, and 30,000 IU for 3 wk to nonpregnant women (302). Plasma levels were found to be in the range or slightly above the range observed in early pregnancy. Furthermore, after a 3-wk treatment with 30,000 IU of vitamin A/day, peak plasma levels of RA and isotretinoin were within or just slightly above the range of their physiological levels. These findings lend insight into possible safe upper intake levels of vitamin A.

Marginal vitamin A status of pregnant women in underdeveloped countries has been documented, and although the association of marginal deficiency status and risk of birth defects has been hypothesized, it has not been confirmed because of concurrent nutrient deficiencies in these populations. The vitamin A status of specific populations groups in the United States may also be at risk for marginal deficiency. The vitamin A status of low-income women during the third trimester of pregnancy in a population in Iowa was examined using the modified relative dose response test (MRDR) (60). Twenty-six percent of the study population was found to be in a marginal vitamin A status with MRDR values of \( \geq 0.03\), whereas 9% had values of \( \geq 0.06\). As vitamin A is depleted in an individual, the MRDR value increases. These cutoff values correspond to tentative cutoff points that have been set at 0.03 in the United States and at 0.06 in Indonesia. These findings, therefore, suggest that additional research is needed to examine the relationship between low vitamin A intake and risk of birth defects.

Because of the teratogenic potential of retinoids, women treated with large doses of oral retinoids for various skin disorders and some types of cancer should avoid pregnancy during this period. Furthermore, women using vitamin A supplements need to be aware of the teratogenic risk during early pregnancy. Multivitamins, in addition to their beneficial effects, remain a potential hazard because women may take large doses in the belief that more is better. Additionally, capsules containing 25,000 IU of preformed vitamin A are available on store shelves in the United States. Multivitamins targeted for adult consumption, and potentially used by women of childbearing age, may contain high doses of preformed vitamin A. Many multivitamins, however, also contain folic acid, which reduces the risk of neural tube defects and possibly other birth defects (211). Several randomized, controlled intervention tri-
als have been performed to investigate the use of multivitamin supplements containing folic acid, with or without vitamin A, during the periconceptual period for the prevention of neural tube defects. In many trials, the rate of malformations was significantly lower in the supplemented compared with placebo groups, or there was no effect. Because of the wide range of vitamin A intake in the human population, additional research, i.e., epidemiologic as well as mechanistic, is needed to determine whether excess consumption of vitamin A from supplements or foods, as well as diets deficient in vitamin A, or the therapeutic use of retinoids constitutes a public health problem.

IX. CONCLUSIONS AND FUTURE ASPECTS

The discovery, over a decade ago, of the retinoid receptor families has led to the present view of the retinoids as transcriptional activators, which regulate gene expression during embryonal development and differentiation. In addition to the important insights into mechanisms responsible for the control of morphogenesis at the molecular level, these discoveries have also offered suggestions as to how vitamin A deficiency or excess may disrupt the finely tuned developmental processes, perhaps through a disregulation of RAR and the consequent effects on homeobox genes, which might be either not expressed or overexpressed at the wrong place and time, finally resulting in teratogenesis.

An interesting area for future investigation is the characterization of the ligands and functions of the retinoid-related orphan nuclear receptors. Among these are the ROR/RZR (19, 72), RVR/Rev-erb (65), COUP-TF (106), and NGFI-B (220), and NURR1 (220). Some of these orphan receptors have demonstrated transactivation activity via the same responsive elements as the retinoid receptors. Future work may show that retinoid-like effects may be evoked by the activation of one or more of these orphan receptors (224).

This review has highlighted the retinoid ligand and retinoid receptor knockout models to understand their essentiality during embryogenesis. New models are being developed to study retinoid-dependent developmental events. The use of retinoid receptor antagonists is one example. A recent study reported that a single oral dose of an RAR antagonist, AGN-193109, given on 8 dpc produced severe craniofacial anomalies, including eye malformations (111).

An important focus of current research is the metabolism of retinoids during embryogenesis. This area of research is likely to lend additional insights into the normal process of embryonal development as well as of teratogenesis. It is known that many of the functions of vitamin A in embryogenesis are carried out through the active metabolite retinoic acid, formed by the oxidation of retinol in a two-step process. Human embryos undergo normal morphogenesis when converting a small amount of maternally derived vitamin A (retinol) into RA in a tissue-specific fashion, but may undergo teratogenesis, when responding to events (e.g., ethanol intoxication), which increase or decrease retinoic acid levels above or below the normal range. Antiepileptic agents (e.g., valproic acid) are other exogenous factors implicated as human teratogens. Recent evidence suggests that these compounds may disrupt RA metabolism (205). Additional work is necessary to clarify if, as well as how, disruption of retinoid metabolism affects fetal alcohol syndrome, and perhaps malformations in general.

More research is needed on the enzymes involved in the conversion of retinol to retinal and the consequent effects of increases or decreases of the retinol metabolites on gene expression during critical periods of morphogenesis. Mechanisms regulating expression as well as enzyme activity for both alcohol dehydrogenase and aldehyde dehydrogenase enzymes need to be better understood.

It also reasonable to suggest that vitamin A deficiency pauses a clinical risk similar to excess retinoids. In support of this concept, Olson and collaborators have identified a surprisingly profound deficiency in a Iowa woman enrolled in the Women Infants and Children Program (60).

Finally, it should be considered that research in embryogenesis and teratogenesis may have an important impact on our understanding of the molecular events taking place during carcinogenesis. This is because this process may reestablish, in adult tissues, the high growth potential characteristic of embryonic tissues. Therefore, genes that are involved in ontogenesis may also play fundamental roles during carcinogenesis. Particularly, homeobox genes may represent an interesting area of investigation. The findings that RAR are downregulated (33, 142) or mutated (44) during carcinogenesis and that retinoic acid is an effective differentiation agent in acute promyelocytic leukemia (248) indicates the involvement of the retinoid receptors in this process as well and offers clues for preventive approaches (24, 277).

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