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Retinoic Acid and Retinoic Acid Receptors in Development

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Abstract

The vitamin A derivative retinoic acid (RA) and related compounds (retinoids) are utilized as signaling molecules in a diverse array of developmental and physiological regulatory processes, including many important in the developing and mature nervous system. Retinoids function by interaction with high affinity receptors of the nuclear receptor family, which also mediate the effects of steroid and thyroid hormones and which act in the nucleus as transcription factors. This review summarizes current knowledge of the molecular mechanisms of retinoid action, the complex role of retinoid receptors in a variety of hormonal signaling processes, and illustrates current efforts to more fully understand the biological functions of retinoid receptors through analysis of downstream gene regulatory networks and studies of mouse gene knockout systems.

Index Entries: Retinoic acid; retinoic acid receptors; retinoids.

Introduction

Vitamin A (also known as vitamin A alcohol and as retinol) was identified in the early part of this century as a compound that could alleviate the symptoms of certain nutritional deficiencies, including night blindness. Subsequent purification, structural characterization, and biochemical studies have led to a fairly detailed understanding of vitamin A biochemistry and metabolism (reviewed in Blomhoff et al., 1990). Dietary retinol is absorbed in the intestine, esterified to long chain fatty aids, and transported via the circulatory system as retinyl esters in chylomicron particles to the liver. Dietary β-carotene, a precursor of retinol also known as provitamin A, is enzymatically processed in the gut into retinol and then to retinyl esters. Vitamin A is stored in vertebrates in the liver, and is mobilized by hydrolysis back to the free alcohol, complexed with a carrier protein, retinol binding protein (RBP), and exported via the circulatory system to the periphery. Cells take up retinol and locally

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metabolize it according to the pathway shown in Fig. 1. Retinol is converted in a reversible step to retinaldehyde (retinal), and then in an irreversible step to all-trans retinoic acid (RA). An isomerase activity or activities, as yet only crudely characterized, reversibly converts all-trans RA to 9-*cis* RA. The compounds in Fig. 1 are known collectively as retinoids, as are related natural and synthetic variants. A number of additional products are derived from retinoic acid by further metabolic conversion, some of which are also known to be biologically active (Pijnappel et al., 1993). Retinol is not solely a precursor of retinaldehyde and retinoic acid—at least one additional biochemical pathway from retinol has been described, leading to retro-retinol (in which the position of the conjugated double bonds are shifted over by one carbon) and then to additional retro-retinoids (Buck et al., 1991, 1993), and other pathways are possible as well (Blumberg, personal communication).

Night blindness, a consequence of vitamin A deficiency, is now known to be attributable to a deficiency of retinaldehyde. In what is perhaps the most popularly understood function of vitamin A, retinaldehyde is an essential component of the visual transduction system, isomerized in the eye by light from 11-*cis* to all-trans retinal, thus setting off a signal cascade that ultimately results in neural activation and cycling of the chromophore back to the 11-*cis* form (Rando, 1992).

This critical function, however, is only one unique and highly specialized use of vitamin A. Laboratory animals kept on a vitamin A-free diet (Wolbach and Howe, 1925; Thompson et al., 1964; Lamb et al., 1974; van Pelt and de Rooij, 1991), in addition to becoming blind, suffer from a large number of additional maladies, including epithelial keratinization, immunodeficiency, weight loss, lethargy, and male sterility, ultimately resulting in their death. However, supplementation of this diet with all-trans RA restores these animals to complete health, with three exceptions. First, these animals are still blind, a consequence of the unidirectional metabolic pathway that converts retinal to retinoic acid, but not back. Male sterility, via a defect in spermatogenesis, is a second defect of vitamin A deficiency that is also not rescuable with dietary all-trans RA, but for a different reason. The defect in spermatogenesis almost certainly reflects an underlying cellular requirement for retinoic acid (and not for retinol, retinaldehyde, or some other retinoid); however, retinoic acid cannot cross the blood–testes barrier, but is instead normally generated locally from circulating retinol that can cross into the testes (van Pelt and de Rooij, 1991; van Beek and Meistrich, 1992). Third, RA-supplemented vitamin A-deficient animals are viable but are immunocompromised. It has been speculated that retro-retinoid compounds, derived from retinol but not from retinoic acid, are essential for at least some aspects of immunocompetence (Buck et al., 1991, 1993), although there is direct experimental evidence to support a role for RA as well in the immune system *(see the following)*. The general conclusion, therefore, is that with the exception of a specific requirement for retinaldehyde in the visual transduction system, a likely requirement for retro-retinoids in the immune system, and possible functions for as-yet-uncharacterized retinoids, the remainder of the dietary requirement for vitamin A serves to generate all-trans RA, 9-*cis* RA, and compounds derived from their breakdown, and that these compounds have a number of diverse and necessary functions in adult physiology.

Retinoids in Development

In addition to a role in many aspects of adult homeostasis, retinoids are also implicated in embryogenesis. Laboratory rodents exposed prenatally to retinoids give birth to offspring with a severe but predictable spectrum of birth defects (Cohlan, 1953; Kalter and Warkany, 1961; Shenefelt, 1972; Kessel and Gruss, 1991). These defects include craniofacial malformations, abnormalities of the heart and thymus, skeletal dismorphogenesis, and nervous sys-

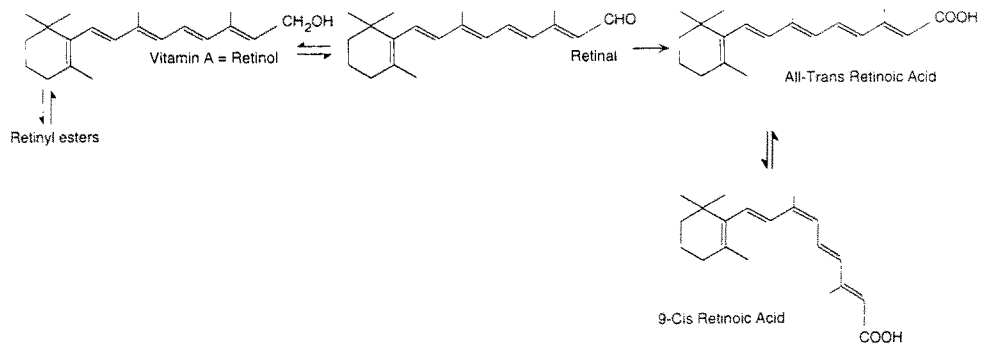

Fig. 1. Metabolism of retinoic acid. *See text* for details.

tem disorders. This is also a human clinical problem: Women who have taken oral retinoids (systemic retinoid therapy is used in the treatment of certain skin ailments, including acne [Hartmann and Bollag, 1993] and psoriasis [Haliona and Sanrat, 1990], as well as for more severe diseases) who became pregnant have given birth to children with similar embryopathies as demonstrated in the rodent studies (Lammer et al., 1985). A general inference from all of the teratology studies is that it is retinoic acid that is the teratogenic agent; whereas high doses of retinol and retinaldehyde also cause birth defects, this probably occurs by conversion to retinoic acid. An important observation is that embryopathies occur only during a defined window in development. In the rat this is between d 8 and 16 of gestation, in the mouse between d 7 and 14, and in humans between d 21 and 55 (Shenefelt, 1972; Agnish et al., 1990). For all species, this window corresponds to the period of organogenesis. Furthermore, for any individual tissue, the period of sensitivity is much narrower, and coincides with the time at which that tissue is undergoing differentiation. Significantly, embryos nurtured under vitamin A-deficient conditions, brought about by maternal vitamin A deprivation, develop a syndrome of defects that in many cases overlaps that of retinoic acid embryopathy (Wilson and Warkany, 1948, 1949; Wilson et al., 1953). Consequently, either an excess or a deficiency of vitamin A are detrimental to normal embryogenesis. This and other data described herein and elsewhere support the notion that endogenous processes utilize retinoic acid in the normal course of differentiation, and that an excess or deficiency interferes with the proper execution of these developmental programs. Furthermore, the spectrum of defects associated with embryonic vitamin A excess or deficiency define the biological processes that are likely to utilize retinoids in normal development.

Retinoids have been implicated in many aspects of neural differentiation. Depending on the time of RA administration, high teratogenic doses of RA cause defects in neural tube closure, including spina bifida (Alles and Sulik, 1990), exencephaly, and anencephaly (Yasuda et al., 1987). One of the more sensitive targets of teratogenic exposure is the neural crest (Webster et al., 1986; Morriss-Kay, 1991). For example, the most common defects seen in human cases of prenatal exposure are cardiac defects and craniofacial and ear defects (Lammer et al., 1995); the same defects are seen in rodent studies as well. Analysis of expression of appropriate hindbrain markers (Morriss-Kay et al., 1991; Marshall et al., 1992; Schneider-Maunoury, 1993), and direct experimental perturbation of neural crest differentiation (Kirby et al., 1983; Kirby and Waldo, 1990), support the interpretation that these RA-induced defects are attributable to an effect on the differentiation of the cardiac neural crest, and the hindbrain cranial neural crest, respectively. Finally, using indirect means, roles for

RA in organizing the floorplate of the neural tube (Wagner et al., 1990) and the developing olfactory epithelium (LaMantia et al., 1993) have been proposed.

In many if not most cases, embryogenesis is comparable both in morphogenic and molecular processes (where such have been analyzed) between all vertebrates, whether mammalian or not. Furthermore, nonmammalian systems offer considerable advantages in terms of experimental accessibility. One of the more influential systems studied has been the developing chicken limb. A region of tissue in the posterior portion of the developing limb bud, termed the zone of polarizing activity, or ZPA, was found to cause digit duplications when transplanted to the anterior portion of a recipient bud (Tickle et al., 1975). Chick digits, like human digits, are individually identifiable by morphology. Depending on the amount of tissue transplanted, the duplications range from a single digit in the mild case to a mirror image replication of the entire limb in the most severe case. The duplicated digits closest to the ZPA transplant were found to be more posterior digits rather than anterior. This suggested that the ZPA was capable of converting the anterior tissue at the site of implantation to a posterior identity, and suggested a model in which the bulk of the limb bud tissue is naive and unpatterned, and is organized along an anterior-posterior axis under the influence of the ZPA. It was then found that a positively charged bead soaked in retinoic acid and implanted to the anterior portion of the limb bud could cause the same duplications (Smith et al., 1989). Furthermore, the extent of digit duplication was found to be a function of the RA concentration in which the bead was soaked. This immediately led to speculation that the signal secreted by the ZPA in organizing the anterior-posterior axis was RA, acting in a concentration-dependent manner. More current evidence indicates that it is probably not RA itself that is directly responsible for directing the patterning of cells along the A-P axis (Noji et al., 1991; Wanek et al., 1991), but rather growth factor(s) that are induced by retinoic acid in cells adjacent to the ZPA (Echelard et al., 1993; Riddle et al., 1993; Laufer et al., 1994). Nonetheless, these and other comparable systems (Brockes, 1990; Mohanty-Hejmadi et al., 1992; Maden, 1993) introduced the concept that RA functions mechanistically in development as a signal between cells or tissues that can synthesize and secrete RA (i.e., the ZPA) and those that respond to this signal.

A number of tissue culture cell lines are known to differentiate after exposure to RA, providing a more expedient model system in which to study the role of retinoids in differentiation. For example, F9 embryonic carcinoma cells differentiate into visceral or parietal endoderm after exposure to RA or to RA plus cAMP, respectively, and P19 cells differentiate into muscle or neural phenotypes depending on the concentration of RA (McBurney, 1993). Several hematopoietic cell lines undergo differentiation after RA treatment, including HL-60 and U937 cells, which differentiate into granulocytes and monocytes, respectively. The human hematopoietic cell line NB4, derived from a patient with acute promyelocytic leukemia, differentiates to a granulocyte phenotype after RA exposure (Lanotte et al., 1991). This latter example is particularly interesting in that this type of leukemia is currently treated with RA chemotherapy (Huang et al., 1988; Castaigne et al., 1990; Warrell et al., 1991). In many or most of these cases, the differentiation process mimics known developmental events of the embryo or adult, suggesting that the sensitive cell line may have been derived and transformed from a precursor population whose differentiation is normally under retinoid control. Certainly, the inference is strong that these are valuable and valid models of in vivo differentiation. Because these diverse fates are all elicited by the same inducer (RA), it is clear that these cell lines are preprogrammed to respond to RA by differentiating, and in most cases, that RA has no instructional role but is merely the trigger to promote differentiation. The example of P19 cells, however, in which different pathways of differentiation are followed depending on the

RA concentration (McBurney, 1993), suggests that RA may in some cases bear informational content by its concentration.

Retinoid Receptors

A great advance in understanding the molecular mechanism of retinoid utilization in development and physiology came with the identification of specific receptors that mediate the effects of retinoic acid. These receptors belong to the nuclear receptor family of transcription factors (Evans, 1988; Mangelsdorf et al., 1993), so described because they function in the nucleus as direct transcriptional activators, as compared to cell surface receptors that transduce external signals to the nucleus via other proteins. The nuclear receptor family includes receptors for the six known steroid hormones (glucocorticoids, mineralcorticoids, estrogen, androgens, progesterone, and vitamin D_3), as well as for thyroid hormones and retinoic acid. A common mechanism of action for these receptors has been elucidated. The hormone enters the cell by diffusion, and interacts with its appropriate receptor. Some receptors, including the retinoid receptors, are constitutively nuclear, whereas others are cytoplasmic; this turns out to be a fairly trivial mechanistic distinction. After binding ligand the receptors undergo a conformational change (which also allows the cytoplasmic receptors to translocate into the nucleus), recognize and bind to specific sequences in the genome of the target cell, and cause the transcriptional activation of adjacent genes. The sequences bound by receptors are termed response elements. Different hormones have divergent effects in the body because their different receptors recognize different sets of response elements and transcriptionally activate the expression of different sets of genes. Mechanistically, the process is comparable for all of the nuclear hormone receptors.

This mechanistic similarity reflects an underlying structural similarity. Structure-function analysis has identified the specific domains of the receptor proteins responsible for DNA binding and ligand binding activities. The DNA binding domain is a characteristic and unique type of DNA binding motif, containing two so-called zinc fingers each coordinated by four cysteines. The level of homology between different members of the receptor family in this region is sufficiently high as to have allowed low stringency screening of cDNA libraries, resulting in the isolation of approx 30 additional members of this family. These "orphan" receptors are clearly members of the nuclear receptor family by virtue of the sequence of the DNA binding domain, yet for which cognate ligands have not so far been identified. A considerable effort is now being directed toward identifying ligands for these new receptors, since the known hormones that have been identified to date are clearly of considerable medical importance. Of course, it need not be the case that all or any of these orphans in fact have a ligand, although circumstantial evidence is strong at least for some. Orphan members of the nuclear receptor family have been found in a number of metazoan phyla, including *C. elegans* and *Drosophila* in addition to vertebrates, but have not been found in protozoa or in plants.

Retinoid receptors comprise two distinct subfamilies of the nuclear receptor family, the RARs and the RXRs, each of three members, α, β, and γ (Giguere et al., 1987; Petkovich et al., 1987; Mangelsdorf et al., 1990, 1992). There is an extremely high degree of homology in a comparison within the subfamily of the three RARs or of the three RXRs, especially in the DNA and ligand binding domains, but only marginally more homology between the RARs and RXRs than to other members of the nuclear receptor family, such as the two thyroid hormone receptors (TRs). In particular, the homology between the RAR, RXR, and TR receptors in the ligand binding domain is comparably low. Consequently, it came as a surprise when it was found that the RARs and the RXRs have overlapping ligand specificity: Both subfamilies are high affinity (in the nanomolar range) receptors for 9-*cis* RA, whereas all-trans RA is

a ligand only for the RARs (Heyman et al., 1992; Levin et al., 1992).

Receptors function as transcriptional activators by binding to specific sequences in the genome (response elements) and activating the expression of adjacent genes. By analysis first of naturally occurring hormone response elements, and subsequently by analysis of synthetic response elements, a general description of the nature of response elements has emerged (Umesono et al., 1991). Most receptors bind DNA as dimers, and consequently, response elements tend to be comprised of two so-called half sites, each representing the binding site of one monomer. The consensus retinoid response element half site sequence is (A/G)G(G/T)TCA, with some variation allowed; other hormonal pathways either overlap (e.g., the TRs) or vary considerably (e.g., the glucocorticoid receptor) from this consensus. These half sites can be oriented with respect to each other either as direct repeats, inverted repeats (palindromes), or everted repeats, and naturally occurring variants of all three types are now known. Specificity of a response element for a particular hormone pathway (i.e., for a particular hormone receptor complex) is derived from the orientation of the half sites, the sequence of the half sites, and the spacing between half sites. Examples are shown in Fig. 2. There are sequences that confer specific responsiveness to individual hormone pathways, and those that are recognized by multiple receptor subtypes. For any individual response element, specificity is rarely absolute—lower affinity recognition by alternative receptors is often seen, and under conditions of high receptor expression or high ligand concentration, these alternative pathways may be physiologically relevant. In particular reference to retinoid receptors, there are sequences that are uniquely RXR or RAR response elements, and those that are common to both (Mangelsdorf et al., 1991). It should also be noted that some retinoid response elements are comprised of greater than two half sites (the "complex" response elements), and that some receptors (not including the retinoid receptors) bind to DNA only as monomers, recognizing an extended half site sequence.

Once response element sequences were identified that conferred specific transactivation properties, it became possible to examine the biochemical nature of receptor-DNA binding. RAR purified from mammalian cells was found to bind to appropriate RA response elements (RAREs), but in vitro translated RAR, or bacterially expressed RAR, was not able to bind DNA with high affinity. However, this binding activity could be restored with a mammalian nuclear extract (Umesono et al., 1988), suggesting a required cofactor necessary for DNA binding. In fact, this turns out to be RXR: The binding complex that forms on RAREs is a heterodimer of RAR and RXR. A few other members of the nuclear receptor family, including the TRs, the vitamin D_3 receptor (VDR), orphan receptors activated by peroxisome proliferating chemicals (the PPARs), and other orphan receptors also require heterodimerization with RXR in order to bind DNA (Yu et al., 1991; Kliewer et al., 1992; Leid et al., 1992; Marks et al., 1992), although the estrogen and glucocorticoid receptors, for example, bind DNA as homodimers. The RXRs, therefore, are critically involved in several different hormonal signaling pathways in addition to retinoid signaling. In the heterodimer complex of RXR with the RARs, TRs, or VDR, it is these partners of RXR that are ligand-dependent in mediating transcriptional activation, and not RXR. That is, for the RXR-TR heterodimer bound to a thyroid hormone response element, presentation of 9-*cis* RA does not result in transactivation, and does not synergize transactivation with thyroid hormone. RXR itself can form homodimers in the presence of its cognate ligand 9-*cis* RA (Zhang et al., 1992), and this complex is transcriptionally active on appropriate RXREs. Furthermore, the RXR-PPAR heterodimer is also activated by 9-*cis* RA independently, as well as by peroxisome proliferating chemicals (Kliewer et al., 1992). The requirement for heterodimerization as a necessary component of hormonal signaling is not unique to these mammalian receptors—the

Response Elements of the AGGTCA Half Site Sequence

Type	Gene	Sequence	highest or best binding complex
Direct Repeats			
DR-1	CRBPII	**AGGTCA** A **AGGTCA**	RXR-RXR;
	AOx	**AGGACA** A **AGGTCA**	RXR-PPAR
DR-2	CRBPI	**AGGTCA** AA **AGGTCA**	RXR-RAR
	HoxB	**AGGTAA** AA **AGGTCA**	
DR-3	Osteocalcin	**GGGTGA** ATG **AGGACA**	RXR-VDR
	24-Hydroxylase	**AGGTGA** GTG **AGGGCG**	
	SSP-1	**GGTTCA** CGA **GGTTCA**	
DR-4	MHC	**AGGTGA** CAGG **AGGACA**	RXR-TR
	Malic enzyme	**GGGTTA** GGGG **AGGACA**	
DR-5	RARβ2	**GGTTCA** CCGAA **AGTTCA**	RXR-RAR
	RARα2	**AGTTCA** GCAAG **AGTTCA**	
	HoxA	**GGTTCA** CCGAA **AGTTCA**	
Inverted Repeats			
IR-0	synthetic	**AGGTCA TGACCT**	multiple
IR-3	Vitellogenin	**AGGTCA** CAG **TGACCT**	ER-ER
Everted Repeats			
ER-6	Lysozyme	**TGACCC** CAGCTG **AGGTCA**	RXR-TR
	MBP	**GGACCT** CGGCTG **AGGACA**	
ER-8	γF-crystallin	**TGACCC** TTTTAACC **AGGTCA**	RXR-RAR

Fig. 2. A selection of reported response elements are shown, with the receptor complexes of the highest affinity or of most physiologic relevance that recognize them. High affinity binding to the IR-0 sequence is seen with multiple complexes including RXR homodimers, RAR-RXR, TR-RXR, VDR-RXR, and ER homodimers. References: CRBPII (cellular retinol binding protein II): Mangelsdorf et al., 1991; AOx (Acyl coA oxidase): Kliewer et al., 1992; CRBPI: Smith et al., 1991; *Hox*B: Ogura and Evans, 1994; Osteocalcin: Terpening et al., 1991; 24-Hydroxylase: Ohyama et al., 1994; SSP-1 (osteopontin): Noda et al., 1990; MHC (myocin heavy chain): Fink and Morkin, 1990; Malic enzyme: Petty et al., 1990; RARβ2: de The et al., 1990; Sucov et al., 1990; RARα2: Leroy et al., 1991; *Hox*A: Langston and Gudas, 1992; synthetic IR-0: Umesono et al., 1988; Vitellogenin: Slater et al., 1991; Lysozyme: Williams et al., 1994; MBP (myelin basic protein): Williams et al., 1994; γF-crystallin: Tini et al., 1993.

Drosophila receptor for the insect molting hormone ecdysone requires heterodimerization with the product of the orphan receptor gene *ultraspiracle* to bind DNA (Yao et al., 1992), and in fact, both functional and sequence analyses indicate that *ultraspiracle* is a *Drosophila* homolog of mammalian RXR. In terms of heterodimerization and DNA binding, there is no indication that the three RARs, or the three RXRs, are different from one another in any way. That is, all three RARs require heterodimerization with RXR to bind DNA, and any of the three RXRs can fulfill this function.

Recent analyses have led to the identification of specific structural protein motifs that promote receptor dimerization and that

account for specificity in response element recognition (Kurokawa et al., 1993; Perlmann et al., 1993). Heterodimerization of RXR with an appropriate partner occurs through an interface in the ligand binding domain of each receptor. This interaction occurs in solution, is not hormone-dependent, and promotes moderately high affinity binding to response elements of the appropriate half site sequence. For response elements of the inverted or everted orientations, the interaction in the ligand binding domains is alone sufficient to promote DNA binding by the heterodimer, with each receptor's DNA binding domain independently recognizing one half site. Many (but not all) spacing options of the inverted or everted orientations are suitable as binding sites for RAR, TR, and VDR heterodimer partnerships with RXR, presumably subject to steric constraints. With half sites of the direct repeat organization, a further cooperative interaction between the DNA binding domains promotes not only higher affinity recognition of appropriate half sites but also confers specificity for unique spacings between half sites. In general, response elements of the inverted or everted repeat organization are more promiscuous in their recognition by different receptor complexes, whereas the direct repeat organization is more highly specific for individual receptor forms, and consequently more specific for uniquely mediating specific hormonal signaling pathways.

Negative Regulation and Cross-Coupling

Studies have indicated that certain members of the receptor family can function as negative regulators of transcription, in addition to the positively acting function described earlier. The TRs, when bound to DNA in a heterodimeric complex with RXR, in the absence of thyroid hormone are powerful suppressors of basal transcription. This suppression function is alleviated in the presence of hormone, converting the TR into a strong transactivator (Damm and Evans, 1993). This transition is almost certainly mediated by a conformational change in the ligand binding domain of the TR that in the presence of T3 masks a repression motif and uncovers a transactivation motif. The glucocorticoid receptor (GR), when bound to a unique sequence termed a negative GRE (nGRE), is a hormone-dependent repressor of transcription. In this case, it is the organization of the response element that promotes a receptor conformation that is inhibitory to transcription (Sakai et al., 1988). A third example of receptor-mediated negative gene regulation, separately described for the GR (Akerblom et al., 1988) and TR (Xu et al., 1993), involves the displacement of a required transcription factor from a gene promoter by receptor owing to an overlap in the two proteins' binding sites. Although these processes have not yet been described for the retinoid receptors, clearly the precedent for such a mechanism exists with other members of the receptor family, and such a process may turn out to be critical in retinoid regulation of certain genes as well.

These examples all require DNA binding activity by the receptor. A completely different mechanism of negative regulation, termed cross-coupling, has been described in which hormonal signaling interferes with signaling by the AP-1 pathway (reviewed in Schule and Evans, 1991). Surprisingly, this regulation does not require DNA binding by the receptor. AP-1 is a complex of the products of the two oncogene families *jun* and *fos*, and is a DNA binding activity that is required or important for the transcriptional activation of a number of genes. AP-1 activity is stimulated by a number of extracellular signals generally involved in mitotic signaling, such as growth factors or tumor-promoting phorbol esters. It was found that the GR (Schule and Evans, 1991), RAR (Nicholson et al., 1990; Schule et al., 1991), and the RXR (Salbert et al., 1993), in the presence of their cognate ligands are effective inhibitors of AP-1 dependent gene expression. Importantly, AP-1 activation is likewise inhibitory to hormone signaling, indicating a reciprocally antagonistic relationship between mitotic sig-

naling and hormonal signaling. Mutational analysis indicated that although the DNA binding domain of the receptor protein is necessary for this activity, it does not require DNA binding (as does, for example, the factor displacement mechanism described above), but instead is probably a solution interaction. Receptor hetero- or homodimerization does not seem to be a necessary aspect of cross-coupling. Interestingly, because synthetic retinoids have been identified that can dissociate transcriptional activation functions from anti-AP-1 function, it is likely that unique receptor conformations are involved in these different activities (Fanjul et al., 1994). Assays to demonstrate a direct interaction between receptor and AP-1 have not been successful, suggesting that a third component may be also involved. It is not yet known how many others of the nuclear receptor family participate in cross-coupling; it also remains possible, even likely, that other signaling pathways may intersect with hormonal signaling pathways in an analogous manner.

Cross-coupling between the mitogenic pathway and retinoid signaling may be of critical importance in differentiation. As described earlier, retinoids are implicated in a number of developmental processes, and glucocorticoids, although conventionally thought of as mediators of physiological homeostasis, are also implicated in a variety of differentiation processes as well. It is generally believed that proliferation and differentiation are opposing processes, in that differentiation requires cessation of the cell cycle, and cells forced to proliferate by oncogenic transformation fail to or are blocked in their ability to differentiate. The molecular basis for this behavior may in some cases lie in cross-coupling between the hormonal and AP-1 pathways.

Expression of Retinoid Receptor Genes

Assays of the mammalian expression patterns of the three RAR and three RXR genes have been reported, both by *in situ* hybridization in the mouse embryo (Dolle et al., 1990; Ruberte et al., 1991, 1993; Mangelsdorf et al., 1992) and by Northern blotting with adult tissues. Similar studies have documented expression patterns in nonmammalian systems, such as in the chick and *Xenopus* embryos, with comparable results. In developing neural tissue, RARα and RXRβ are expressed extensively in the fore- and midbrain, whereas RXRγ expression is specific to the pituitary and corpus striatum. In the hindbrain and spinal cord, RARβ is abundantly expressed along with RARα and RXRβ. All three RAR genes are expressed in neural crest-derived craniofacial domains. Outside the nrvous system, RARβ expression is seen in a variety of tissues, some of which are undergoing cell death (the interdigit region of the limb and the fusion region of the neural tube and the palate); RARγ is highly expressed in skin, bone, and cartilage; RXRα expression is abundant in visceral tissue, such as the liver, intestine, and kidney, as well as in skin and in most epithelium; and RXRγ shows abundant expression in the somites. In general, RARα and RXRβ are ubiquitously and abundantly expressed, and whereas the other four genes show more restriction in their expression pattern, these too are very widely expressed when assayed by more sensitive methods to detect lower expression levels.

Gene Knockouts

Given the variety of processes in which retinoids have been implicated, one appealing model is that this complexity is encoded by unique functional properties of individual receptor subtypes. Direct in vivo evidence by creation of gene mutations is only recently available to address this issue. Complete loss-of-function mutations of the RARα (Lufkin et al., 1993), RARβ (Giguere, personal communication), RARγ (Lohnes et al., 1993), and RXRα (Kastner et al., 1994; Sucov et al., 1994) genes have by now been established; surprisingly, this genetic analysis has indicated an extensive

degree of functional overlap between individual RARs.

Mutation of the RARa gene (Lufkin et al., 1993) allows for normal embryogenesis, with no apparent malformations, and yet causes a decreased postnatal viability for as yet unknown reasons The RARα gene is ubiquitously expressed, complicating efforts to focus on candidate tissues likely to account for this decrease in viability. In addition, the RARα mutation results in male infertility caused by a defect in spermatogenesis owing to testicular agenesis. This same phenotype is associated with adult vitamin A deficiency, and is of particular interest because supplementation of deficient diets with retinoic acid does not restore this function. The observation that mutation of a retinoic acid receptor results in this phenotype is one of the arguments that suggest (as described earlier) that the vitamin A requirement of spermatogenesis involves a retinoic acid-dependent pathway, apparently mediated at least in part through RARα.

Mutation of the RARβ gene (Giguere, personal communication) has no apparent effect. Homozygous embryos develop normally, and homozygous adults are viable and fertile. Although it remains possible that a late stage phenotype may emerge in these animals clearly most developmental and physiological processes appear to be normally executed in this mutant background.

Mutation of the RARγ gene (Lohnes et al., 1993) allows embryogenesis to proceed without gross perturbation, but results in a considerably decreased postnatal viability for unknown reasons. The RARγ gene is expressed abundantly in all bone, cartilage, and epithelium (and elsewhere; *see earlier*). For the most part, these appear normal in most mutant embryos and newborns. Sporadic malformations were seen in various skeletal, cartilagenous, and glandular epithelial structures; most individuals showed some malformation, and all mutants had one defect in a tracheal cartilage element. However, these defects are unlikely to account for the general inviability of animals carrying this mutation. The epithelial defects are of particular interest because similar defects are seen in adult animals with vitamin A deficiency. The most surprising phenotype associated with the RARγ mutation is resistance to a particular teratogenic effect of exogenous retinoic acid in embryogenesis. Normally, among other defects, teratogenic RA treatment causes truncations in the lumbosacral portion of the vertebral column; however, in RARγ mutant embryos no such malformation is evident. This clearly implicates RARγ as the mediator of the experimentally induced teratogenic effects of retinoids in this tissue. It still remains unclear what function the gene normally performs in the development of the lumbosacral region, if any, in that this region appears normal in untreated mutant embryos.

The three RAR genes (and likely the three RXR genes as well) are all organized at the 5' end of the gene with two major promoters, resulting in two major isoforms of each gene with unique amino terminal protein sequence, spliced to common sequences encoding the bulk of the receptor protein, including the DNA binding domain and the ligand binding domain (Leid et al., 1992). Mutation of individual RAR gene isoforms have been reported for RARα1 (Li et al., 1993; Lufkin et al., 1993), RARβ2 (Lohnes et al., 1994), and RARγ2 (Lohnes et al., 1993), and for all no phenotype is evident. By reference to the defects associated with mutations in the corresponding common regions of the RARα and RARγ genes, which eliminate all isoforms (mutation of the common region of the RARβ gene is nonphenotypic; *see earlier*), one inference is that individual isoforms of these genes appear to be functionally equivalent, at least to the extent of the observed phenotypes.

Of the three RXR genes, only the RXRα mutation has been reported to date (Kastner et al., 1994; Sucov et al., 1994). This mutation causes embryonic lethality—homozygous embryos die in midgestation from cardiac hypoplasia. This appears to be a result of a defect in the differentiation of ventricular cardiomyocytes specifically in the outer ventricular wall (the "compact zone"), rather than

of the cardiac neural crest that also populate the heart. Importantly, the same hypoplastic phenotype is associated with embryonic vitamin A deficiency (Wilson and Warkany, 1949), but not other nutritional deficiencies. This suggests that RXRα is an essential component of a normal retinoid-dependent signaling pathway in the ventricular portion of the heart, and excludes a thyroid hormone or vitamin D dependent pathway that might require RXRα as a heterodimeric partner. Because double mutation of the RARα and RARγ genes (*see the following*) causes a similar phenotype, it is likely that a RXR-RAR heterodimer pathway is involved in this normal process, rather than a RXR homodimer process.

Two independent mutant alleles of the RXRα gene have been established, both of which cause cardiac hypoplasia. In each case, an additional phenotype has also been noted—several related eye defects (Kastner et al., 1994) and a transient delay in liver development (Sucov et al., 1994). There may be leakiness in expression or strain-specific penetrance of phenotype that could account for these differences. The eye malformations are of interest in that these are also seen with vitamin A deficiency. The liver phenotype is not associated with retinoid signaling, and may therefore involve a signaling process in which RXRα functions as a heterodimeric partner for a different hormonal pathway.

The spectrum of tissues that utilize retinoid signaling, as evidenced by vitamin A deficiency and excess studies, is fairly broad. In principle, it should be possible to define every biological process that utilizes retinoic acid by the individual receptor gene products that mediate this signaling. However, the phenotypes of the described individual RAR gene mutations are surprisingly narrow. By combining mutations of two or more receptors, however, extensive developmental abnormalities emerge (Kastner et al., 1994; Lohnes et al., 1994; Mendelsohn et al., 1994; Sucov and Evans, unpublished observations; Sucov and Giguere, unpublished observations). Importantly, most if not all phenotypes associated with embryonic vitamin A deficiency can be recovered in these combinations. This is appealing, since receptor mutation should be equivalent to retinoid deficiency if receptors mediate retinoid signaling. Because most of these phenotypes emerge only by simultaneous mutation of two receptor genes, the functions of individual RAR and/or RXR genes must overlap and therefore appear to be redundant. A similar conclusion was reached in describing the absence of phenotype in the RAR isoform-specific mutations (*see earlier*). In general, the RARs and RAR isoforms are comparable in most functional (transfection-based) assays, as are the RXRs (clearly, however, the RARs are different in functional properties from the RXRs). However, some functional differences between different RARs in response element specificity and transcriptional activation profiles have been described (Husmann et al., 1991; Nagpal et al., 1992), and unique and different functional roles for RARα and RARγ subtypes in the urodele limb have also been characterized (Pecorino et al., 1994). It is likely that in most processes in which retinoic acid is involved, multiple receptors are coexpressed and equivalently capable of mediating appropriate signaling, i.e., functionally redundant. However, complete genetic redundancy is unlikely from a teleological standpoint (evolutionary conservation requires a unique selectable phenotype for a gene), suggesting some phenotype, perhaps very subtle, for animals homozygous for mutations in individual RAR isoforms and the RARβ gene. For the RARα, RARγ, and RXRα genes, where phenotypes from single gene mutations emerge, either in the affected tissues coexpression of other receptors does not occur, there may be a specific qualitative function for the missing mutated receptor that cannot be performed by other coexpressed receptors, or there might be a total quantitative requirement for receptor level of any subtype that is deleteriously reduced after mutation of one.

As described earlier, numerous aspects of neural and neural crest differentiation appear to be under retinoid control. Neural pheno-

types that have emerged in the various retinoid receptor gene mutations include failure of neural tube closure, exencephaly, and a variety of eye defects. Phenotypes likely to be caused by deficiencies in neural crest derivatives include craniofacial malformations, truncus arteriosis and aortic arch abnormalities, and agenesis or malformation of several tissues that receive contributions of neural crest progeny.

Retinoid Regulated Genes

Developmental processes that utilize retinoid signaling are not always sensitive to both deficiency and excess. For example, in the heart, vitamin A deficiency is associated with ventricular hypoplasia and also in defects in the cardiac outflow vessels. Teratogenic exposure to retinoids causes the same outflow vessel defects, but not ventricular hypoplasia, and RXRα mutation results in only ventricular hypoplasia and not in defects in the outflow tracts. Similarly, teratogenic RA exposure causes spinal cord defects, mediated by RARγ, but this is not a phenotype of vitamin A deficiency. It is therefore possible to describe three categories of developmental processes: those affected by vitamin A insufficiency, excess, or both. Mechanistically, this might indicate different molecular aspects of retinoid signaling (i.e., transcriptional activation vs cross-coupling to other signaling pathways) or might simply be a consequence of differential sensitivity of each of these tissues to experimental perturbation. Identification of the genes and molecular processes involved in the genetic and experimental phenotypes is clearly a matter of great importance in understanding the normal and pathological details of retinoid signaling. Unfortunately, the specific genes that are regulated in most of these processes are not well characterized at all. This is not to imply that retinoic acid regulated genes are not known, but rather that the downstream genes that are implicated in any specific RA-regulated process are not well understood. This is also to distinguish between a primary transcriptional response to retinoic acid, and secondary and tertiary gene targets whose expression is regulated not directly by retinoic acid but rather by the nature of the responding tissue or cell type, for example in the course of undergoing differentiation. A complete understanding of the biological response to retinoic acid will only be achieved when the molecular details of these primary and secondary signaling processes are elucidated.

Among the first primary target genes of retinoic acid action to be characterized were the RAR genes themselves. The RARβ2 and the RARα2 promoters (and to a lesser extent RARγ2 as well) contain RA response elements in their promoters that are of the DR-5 type (de The et al., 1990; Sucov et al., 1990; Leroy et al., 1991; Lehmann et al., 1992; *see* Fig. 2). As noted earlier, it is not evident that there is a unique functional role for these induced receptor genes. However, autoregulation does result in signal amplification, in that the total receptor level in induced tissues rises, and target genes respond increasingly to an increase in receptor level (up to a saturation level). The RARβ2 promoter is particularly active in the spinal cord and hindbrain (Mendelsohn et al., 1991), indicative of the presence of endogenous retinoids and implicating a role for retinoids in this tissue.

Perhaps the most important known set of target genes for RA activation are the Hox genes. These genes are expressed in the developing hindbrain and spinal cord in a rostral-caudal order, and a variety of evidence points to an essential role for the *Hox* genes in establishing pattern and positional identity in neural development. Most critically, gene knockouts result in defects in those hindbrain or spinal cord derivatives that express the given gene. The *Hox* genes are organized in four clusters (A–D). It was found first in cell lines (Simeone et al., 1990), and then in embryos, that retinoic acid induces the expression of the *Hox* clusters in an interesting pattern: Those genes expressed at the most anterior portion of the embryo are induced in a primary manner by RA, whereas those genes more posterior are induced with delayed kinet-

ics in a secondary manner. Efforts to identify the critical sequences that confer RA responsiveness led to the identification of response elements located at the 3' end of the *Hox* A and B clusters (Langston and Gudas, 1992; Marshall et al., 1994; Ogura and Evans, 1995), near those genes who expression is induced in a primary manner. Interestingly, it has been found that many of the Hox gene promoters contain regulatory sites that are binding sites for Hox proteins. This suggests one model in which a primary RA signal induces the expression of the more anterior Hox genes, which then induce the expression of secondary genes.

Critically, by using markers for specific regions of the developing hindbrain, it was possible to demonstrate that teratogenic RA treatment causes the respecification of cell identity in the hindbrain (Morriss-Kay et al., 1991; Marshall et al., 1992). In general, RA treatment causes a transformation in fate so that rhombomeric segments of the hindbrain take on a more posterior identity. This transformation identified at a molecular level is an exact correlate with what occurs at an embryo-wide level: Many of the defects seen in teratogenic exposed embryos are attributable to defects in the differentiation of the hindbrain. Consequently, the regulation of the *Hox* genes by RA is almost certainly a primary molecular event in the teratogenic effect of RA treatment, and by inference is likely to play an important role in the normal development and differentiation of the unmanipulated embryo.

Primary target genes that encode structural, rather than regulatory, proteins have been identified in many tissues, including neural, and for some, retinoic acid response elements have been found in the gene promoters. Many of these genes, being terminal differentiation markers, are not expressed as a prerequisite of differentiation, but rather as the consequence of differentiation. This illustrates that RA plays a continuing role in adult physiology and homeostasis, modulating the expression of genes in mature differentiated tissues, in addition to its role in differentiation and embryogenesis.

Summary

Animal studies of retinoid deficiency and teratogenesis done over the past 50 yr have served to identify developmental and physiological processes that, by virtue of their sensitivity to experimental perturbation, are likely to utilize retinoids in normal signaling. Established cell lines and primary cell cultures have provided simpler retinoid-dependent model systems that are more amenable to experimental study and manipulation. The critical task in the years ahead will be to define organism- and tissue-level descriptive retinoid teratology by the molecular details of the signal transduction processes that underlie them. A detailed molecular understanding of the mechanism by which the retinoid receptors function has now been elucidated, and provides the intellectual framework for further experimental analysis. The approaches described earlier, of gene knockouts and transgenics, and identification of retinoid responsive genes, will serve to provide these molecular details in the coming years.

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