Review

Recent progress in vitamin A research: nuclear retinoic acid receptors and their interaction with gene elements

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Introduction

Since the initial recognition of vitamin A as a "fat-soluble factor" by McCollum and Davis,[1] and the later discovery of its function in vision by Wald,[2] vitamin A has never occupied a position of such prominence in biology as at the present time. This center-stage position was occasioned by the new developments in molecular biology and in analytical procedures (principally high-pressure liquid chromatography).

The resulting discoveries arrived at the recognition that the mechanism of action of retinoic acid (the active derivative of vitamin A) was closely similar to that of the steroid hormones and thyroxine, involving activation of the expression of specific genes, thus placing retinoids in the category of hormones regulating growth, differentiation, and embryonic development.

Although many other discoveries of considerable importance have been reported recently in the vitamin A field, such as the mechanism of esterification in intestine,[3] the isomerization reaction in the retina,[4] and the organ-to-organ transport and plasma homeostasis,[5] this review is concerned exclusively with the hormone-like action of retinoic acid (RA).

Steroid hormones have specific binding proteins, located in the nucleus for the most part. These binding proteins, when combined with their respective steroid hormone (called "ligand"), have the property of binding to specific regulatory sequences on DNA and of enhancing (or retarding) transcription of specific mRNA and ultimately synthesis of specific proteins. (It should be noted that the steroid-binding proteins are known as steroid "receptors," a terminology used henceforth in this review.)

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The established steroid receptors are: the receptor for glucocorticoids, GR; for mineralocorticoids, MR; for progesterone, PR; for estrogen, ER (all of M_r about 50 kD). They all have a ligand-binding region, and a cysteine-rich DNA-binding region. The DNA-binding region forms two zinc-stabilized DNA-binding "fingers." Here the peptide chain, by means of the cysteine residues, folds around zinc ions, forming finger-like loops that can intercalate into DNA. Similar receptors for thyroid hormone have been found also.

The structures of the genes coding for these receptors now have been established: Because of the similarity of the receptors, they form a gene superfamily which also includes genes coding for two thyroxine receptors (neural and hepatic, $T_3R\alpha$ and $T_3R\beta$), and for a receptor for the active form of vitamin D_3, 1,25-dihydroxyvitamin D_3, VDR. Homology among all the receptor genes is high in the DNA-binding region. This superfamily of genes now has been recognized as consisting of two subfamilies: (1) those coding for glucocorticoid, mineralocorticoid, and progesterone receptors, and (2) coding for thyroid hormone, estrogen, and 1,25-dihydroxyvitamin D_3 receptors. Homology in the subfamilies is 40 to 50% in their DNA-binding regions and about 20% in the ligand-binding regions.[6]

The surprising discovery made in the laboratories of Giguère et al.[7] and Petkovich et al.[8] was the existence of a nuclear receptor for retinoic acid (RA) and the recognition of it as belonging to the superfamily of hormone receptors that bind to DNA. This was achieved by a new methodology. Because of the great similarity of the DNA-binding domains of the different hormone receptors, DNA sequences encoding these domains could be used to scan DNA libraries for new receptor genes bearing ligand domains. cDNA clones were thus obtained with oligonucleotide sequences coding for a new polypeptide with cysteine-rich DNA-binding sequences similar to those found in GR and

T_3R. By exchanging the DNA-binding domain from the DNA coding for the new polypeptide with that of the known GR, a hybrid construct, consisting of a gene coding for the ligand-binding domain of the unknown receptor attached to a gene coding for the DNA-binding domain of the GR receptor, was obtained. To this construct was fused a "reporter" gene coding for the bacterial enzyme chloramphenicol acetyltransferase (CAT). The whole construct was then transfected into cells which were challenged with candidates for binding to the unknown ligand domain. The GR DNA-binding "finger" binds to the promoter region of the GR gene. Attachment of the new ligand to the ligand-binding domain of the new construct would result in activation of the CAT gene and hence expression of the CAT enzyme which could be assayed. RA caused a large increase in CAT activity with an ED_{50} of 6×10^{-10} M. Thus, the unknown receptor was the receptor for RA (retinoic acid receptor, RAR). In the words of the authors: "An expressed chimeric receptor, consisting of the GR DNA-binding domain and the presumptive ligand-binding domain of RA, can act as transcriptional regulator of a glucocorticoid-inducible reporter gene only in response to RA, at a physiological level."[7]

Petkovich et al.[8] also found an RAR in the cell nucleus, with RA as ligand, acting as a transcription regulator. This receptor (or binding-protein, M_r about 50 kD) differed from that of the cellular RA binding-protein (CRABP, M_r about 15 kD). By means of hybridization experiments (Northern blots), Giguère et al.[7] showed the presence of mRNA for the RAR in most tissues, with highest levels in the hippocampus, adrenals, cerebellum, hypothalamus, and testis. However, it was undetectable in liver, an interesting observation in view of the absence of CRABP in that tissue.

An important discovery was the close relationship between the RAR and the thyroid hormone receptor, T_3R. The DNA-binding domain of RAR shows great homology (62% identical amino acid sequences) to the DNA-binding domain of T_3R.[7] On the basis of this homology, Umesono et al.[9] investigated the possibility that the RAR may interact not only with the gene element responsive to RA, but also with that responsive to thyroid hormone (thyroid hormone response element, TRE). By fusing a mouse mammary tumor virus promoter element to a TRE with a CAT reporter sequence, and transfecting this construct into cultured cells, together with the gene element coding for the T_3R, the cells responded, as expected, to thyroid hormone treatment, by a 40-fold increase in CAT activity; if now, instead of co-transfecting the cells with the T_3R gene, they were transfected with the RAR gene and then challenged with RA instead of the thyroid hormone, there would still be a 20-fold stimulation of CAT activity. Indeed, when RAR was synthesized with an ^{35}S-methionine label and the TRE with a biotin label, it was possible to show that the labeled RAR protein had bound to the labeled gene element TRE. It became clear that RA, bound to its nuclear receptor, could activate gene expression (i.e., transcription) not only through the RA-response element, but also through the TRE. Two different receptors (T_3R and RAR) therefore can act through binding to the same nucleotide sequence on DNA. This result suggests that RA and thyroid hormone control overlapping networks of genes.

Recently, Damm et al.[10] found that T_3R can bind to its response element in the absence of ligand: In that case, the receptor acts not by activating transcription, as is usual for receptors with ligands, but by *repressing* transcription. Now, Graupner et al.[11] observed that, in the absence of hormone or ligand, the naked T_3R can also inhibit activation by the RAR of the thyroid hormone response element. The authors suggest that the naked T_3R specifically interferes with the binding of the RAR to TRE.

Thus, receptors in the nucleus of cells exist that can modify rates of gene transcription, up or down, when after interaction with their ligands they bind to specific sequences on the cell's DNA. In that way, RA and thyroid hormone can regulate growth and metabolism.

A further extension of this concept was made by Glass et al.[12] Most of the hormone receptors bind to DNA as dimers, because the hormone response elements in DNA are mostly dyadic palindromic (i.e., two inverted repeating units of the relevant oligosaccharide sequence). Glass et al.[12] now demonstrate that a monomer each of the T_3R and the RAR interact with each other and the resulting hetero-dimer can activate specific TREs. There exist such thyroid response elements in the rat growth hormone gene and in the α-myosin heavy chain gene, with each of which the hetero-dimer reacts, but produces different effects. The interaction between the two receptor molecules was shown to be through their carboxyl termini (*Figure 1*). With the TRE from the growth hormone gene, the two receptors stimulated transcription cooperatively. With the TRE from the myosin gene, in presence of low levels of receptors, cooperation of the two receptors led to a decrease in transcription, though the T_3R alone led to an increase in transcription. Thus, the authors state, "The observation that thyroid hormone and RA receptors can functionally interact raised the possibility that they may also interact with other members of the steroid receptor gene superfamily, resulting in novel patterns of gene expression."[12] The interaction between receptors would, therefore, represent new means of hormonal regulation of gene expression.

The next question that presented itself was: Which specific genes are activated by the RAR binding and, hence, which specific protein would therefore increase in its synthesis? Although the T_3R and the RAR bind to a TRE of two different well-defined genes (the growth hormone and the α-myosin heavy chain gene), as yet no specific protein has been identified as being produced.

On the other hand, a recent report by Bedo et al.[13] shows that RA together with T_3 synergistically increased the output of growth hormone by a culture of

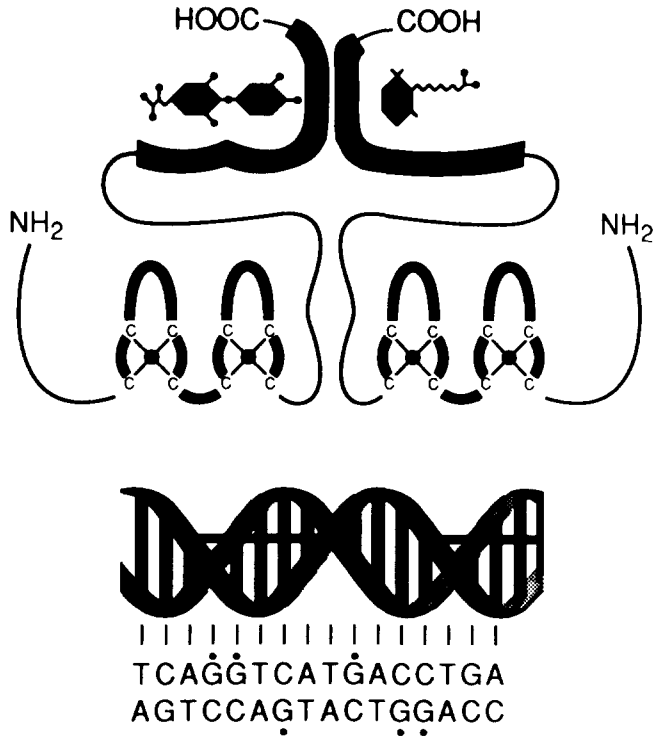

Figure 1 Model of the interaction between the thyroid hormone and retinoic acid receptors on promoters containing TRE-PAL and TRE-MHC. Proposed arrangement of a thyroid hormone-retinoic acid receptor heterodimer. The receptors are shown contacting each other in a region that overlaps with their ligand-binding domains. The DNA-binding domains of the two receptors, shown forming two fingers coordinated by zinc ions (closed circles), are each shown in contact with DNA. The N-terminal regions of the two receptors do not appear to be involved in DNA binding or cooperative interaction. (From: Glass et al., ref. 12, with permission.)

pituitary cells. The rate of growth hormone mRNA was increased. A plasmid containing 1800 base pairs of the 5′-flanking (i.e., promoter) region of the rat growth hormone gene was linked to the CAT reporter gene, and transfected into cultured pituitary cells. The cells, which contained the RAR and T_3R, could be stimulated to give increased CAT activity with RA or T_3 alone, but showed a very large, synergistic increase with RA and T_3 together. Most probably, the T_3R and the RAR combine cooperatively with the regulatory region of the growth hormone gene, as discussed above.

A protein also affected by RA is laminin. This glycoprotein, together with collagen type IV and heparin sulfate, makes up the basement membrane, underlying the epithelial cells. When a certain strain of mouse embryonic cells (F9 teratocarcinoma stem cells) are treated with RA, they differentiate into extraembryonic endoderm.[14] At the same time, the rate of synthesis of laminin-mRNA increases. One subunit of laminin is coded for by the laminin B1 gene, which has been cloned. Three different RA receptors now have been recognized and their genes cloned, RAR-α, RAR-β, and RAR-γ,[15] all of which are present in the F9 cells.[16] Vasios et al.[16] constructed a plasmid of 3900 base pairs upstream of the laminin B1 exon (i.e., the structural gene of laminin B1 attached to its promoter), and fused it to the CAT gene. This gene construct, when co-transfected into F9 cells with RAR-α or RAR-β genes, and the cells treated with RA, led to a significant increase in CAT activity. By means of a number of deletion mutants in the promoter region, the binding of the three RARs was located ultimately as residing in a stretch of 77 base pairs, between base pairs 410 and 486 upstream from the laminin B1 exon. The laminin promoter itself was not affected by the RAR and RA. Thus, all three RARs can bind to a small specific segment of the promoter region of the laminin gene and thereby, in presence of RA, induce its transcription into mRNA and finally the laminin protein (*Figure 2*).

In summary, two proteins (known to be directly involved in the function of vitamin A: growth hormone and laminin; the first in the regulation of growth [together with thyroid hormone], the second in the earliest stages of development of the embryo) are regulated by the binding of the RARs to the promoter regions of their respective genes.

Another recent finding approaches the action of RA on embryonic development from a different angle. It had been known for some time that RA has the remarkable property of controlling chick embryonic development of limbs from limb buds. Local application of RA to the limb buds caused duplication of the pattern of limbs. Embryologists had long postulated the existence of signals between cells which would regulate development by influencing gene expression. These signals showed the properties of a concentration gradient of a substance which was termed "morphogen." Thaller and Eichele,[17] in a truly heroic series of experiments, extracted over 5000 chick embryo limb buds and found that RA was present and formed a concentration gradient, thereby specifying the anterior–posterior digit pattern of the developing chick wings. Thus, RA is the first morphogen to be discovered. Admittedly, the gradient found was extremely shallow and represented small differences of merely picogram quantities of RA across microscopic parts of embryonic limbs. Nonetheless, RA must be interacting with some part of the limb bud's DNA to induce differentiation to take place. Indeed, a considerable number of experiments with embryonic cells in culture have shown RA to induce cell differentiation.[14]

An important experiment to show that RA is indeed involved in the action of RA on limb development was done by Giguère et al.[18] Limbs of newts were amputated and their regeneration was followed 7 days post-amputation, after injection of RA, by means of a constructed anti-sense mRNA corresponding to the RA-binding domain of the RAR. It was found that mRNA in mesenchymal cells under the wound hybridized with anti-sense RAR–mRNA more intensely than in normal control limbs. It is known that RA af-

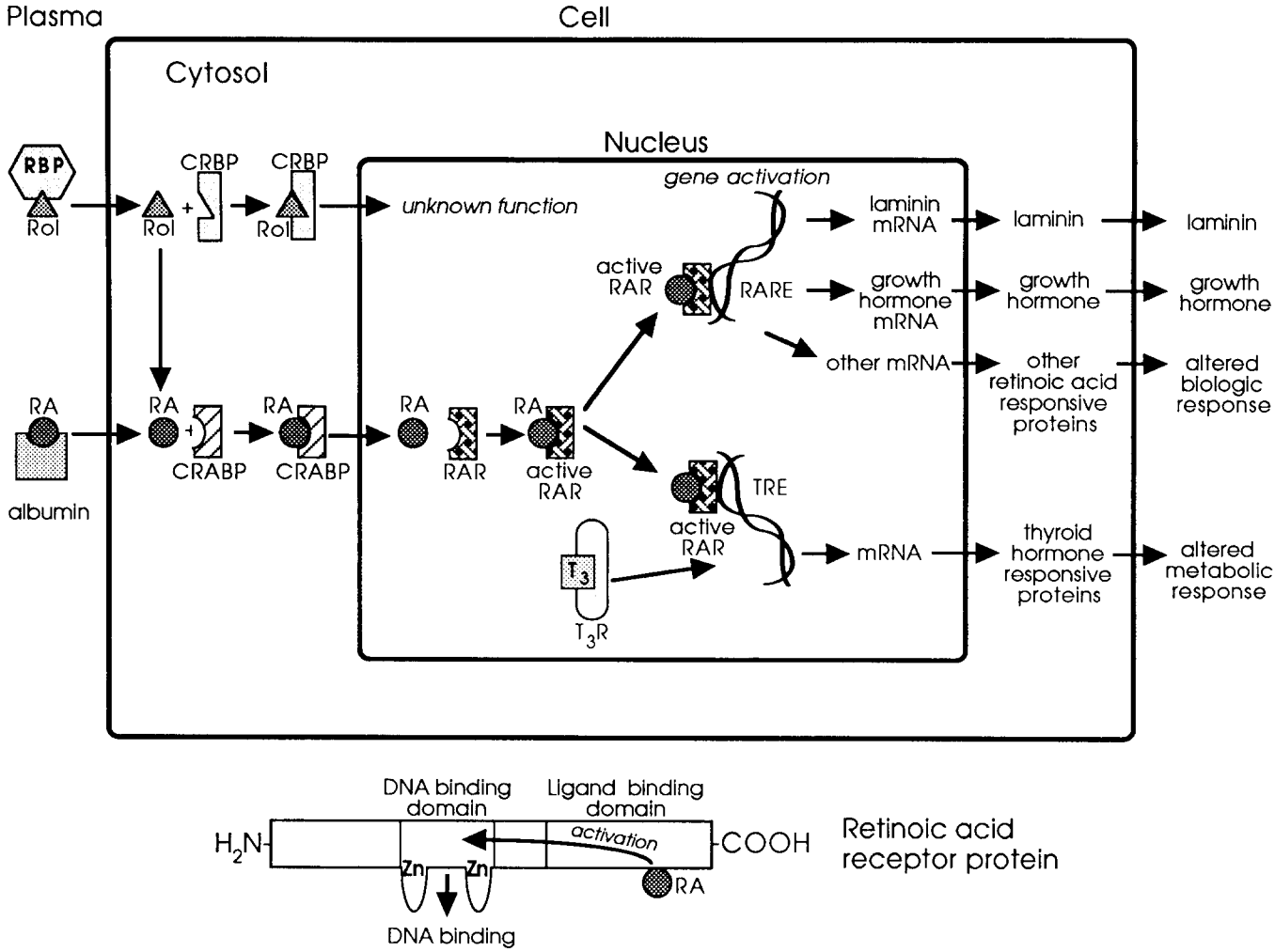

Figure 2 Schematic diagram of the genomic action of retinoic acid. Abbreviations: RBP, serum retinol-binding protein; Rol, retinol; RA, retinoic acid; CRBP, cellular retinol-binding protein; CRABP, cellular retinoic acid-binding protein; RAR, retinoic acid receptor; RARE, retinoic acid-responsive element on gene (part of the promoter region of the gene; enhancement of transcription upon binding of RA-RAR); TRE, thyroid hormone responsive element; T_3, thyroxine; T_3R, thyroxine receptor; mRNA, messenger RNA.

fects mesenchymal cells during limb regeneration. According to the authors, "RAR–mRNA expression is selectively activated in the correct cell type and at the correct point (i.e., time) during regeneration for it to mediate the effect of RA as a morphogen."[18] However, as distinct from the results of Thaller and Eichele[17] who found a gradient of RA in developing chick embryo limb buds, Giguère et al.[18] detected no gradient in the expression of the RAR–mRNA, either in the anterior–posterior or the proximal–distal axes of the developing newt limb. It seemed, therefore, that the action of RA is regulated not by availability of RAR (which is uniform), but by the availability of RA (which forms a gradient). What is the mechanism for this gradient formation?

Chytil and Ong,[19] and later many others, have established the existence of intracellular retinol-binding (CRBP) and retinoic acid-binding (CRABP) proteins (M_r about 15 kD). These bind to the respective retinoids and carry them to the nucleus, where the retinoids (but not the binding proteins) interact with the nuclear RARs (M_r about 50 kD). Maden et al.[20] argue that, in the developing chick embryo limb bud the concentration of RA (as determined by Thaller and Eichele[17]) is such that it would saturate the RARs, as calculated from their binding coefficients. The authors[20] concluded that the RA gradient therefore must be generated by another factor, and that this would be CRABP. By means of CRABP-antiserum and immunohistology, they determined that, during development of the limb, CRABP is present in the limb bud; but its differential distribution is reciprocal to that of the RA concentration gradient across the anterior–posterior axis, being highest at the anterior margin, whereas RA is highest on the posterior side. Therefore, it appears that CRABP binds the RA where it is not needed and releases it where it can interact with RAR. Thus, the authors argue[20] that CRABP, by its reciprocal gradient to that of RA, functions to steepen the free RA gradient and thereby to amplify it and

make it more effective in determining limb development through interaction with RAR. An essentially similar but more detailed analysis of the mRNAs expressed during mouse embryo limb development was made by Dollé et al.[21] They used *in situ* hybridization with labeled anti-sense mRNA of RAR-α, β, γ, and CRABP. At 10 days post-coitum, limb buds appeared non-differentiated. Appearance of precartilaginous blastemas was at 12.5 days, and beginning ossification and digit separation was seen at 14.5 days. At 10 days post-coitum, forelimb bud sections expressed mRNAs of RAR-α and RAR-γ homogeneously distributed through the limb bud. CRABP-mRNA was present abundantly with a proximal-to-distal increasing gradient. At day 12.5, RAR-transcripts were found in the central precartilaginous blastemas of forelimb and hindlimb, with CRABP-mRNA there excluded. At that stage, RAR-α mRNA was distributed widely. At day 14.5, RAR-γ-mRNA was in the fetal skin and cartilage, RAR-α-mRNA in muscle, skin, and perichondrium. RAR-β-mRNA was found only in mesenchymal cells in the interdigital regions. The authors concluded[21] that every RAR has its own specific function; localization of RAR-γ-mRNA in cartilaginous and skin cells may effect the action of RA on chondrogenesis and skin differentiation; RAR-β-mRNA expression may program cell death, since it occurred exclusively in the mesenchyme between digits at the time of digit separation. In all cases, CRABP- mRNA was not transcribed in locations expressing the RAR-mRNAs. The authors could not find an anterio–posterior gradient of CRABP-mRNA reciprocal to that of RA, though they believe there may be a proximo-distal gradient, maximal distally, giving rise to a gradient of free RA across the limb bud.

Conclusion

In summary, it is clear that the retinoic acid receptors, by activating gene transcription, can regulate embryonic development, an activity in which they are aided by the intracellular retinoic acid-binding protein in the control of available free RA.

What is the link between these phenomena and the known functions of vitamin A? Vitamin A can be regarded as a hormone, similar in its mechanism of action to the steroid hormones, thyroxine and 1,25-dihydroxyvitamin D_3, except that it is not produced by an endocrine gland (or by the skin, as for vitamin D), but must be ingested in the diet. The liver, where it is stored, can then be regarded as the "endocrine organ." The functionally active form is retinoic acid.[22] It can affect growth—lack of growth is the most striking effect observed in vitamin A deficiency—by activating the gene for the production of growth hormone.[13] Its action on metabolism, with the many metabolic functions observed,[23] can be rationalized by its activation (or deactivation) of a number of genes, including the thyroid-hormone responsive element and possibly steroid-response elements, thus controlling an overlapping network of genes.

The molecular mechanism of its well-known action on embryonic development[22] is taking shape in the discovery of at least three different RA receptors, distributed differently in developing embryo tissues and thereby activating specific genes and resulting in specific developmental phenomena. Thus, many of the multiple functions of vitamin A can be explained on a molecular basis through the nuclear retinoic acid receptors.

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