23. F. P. De Haan *et al.*, *J. Am. Chem. Soc.* **106**, 7038 (1984).
24. P. Giacomello *et al.*, *J. Phys. Org. Chem.* **2**, 467 (1989).
25. V. D. Nefedov, M. A. Toropova, T. P. Simonova, V. E. Zhuravlev, A. M. Vorontsov, *Zh. Org. Khim.* **25**, 156 (1989).
26. S. Fornarini and M. Speranza, *J. Phys. Chem.* **91**, 2154 (1987).
27. ———, *J. Am. Chem. Soc.* **111**, 7402 (1989).
28. F. A. Cotton and G. Wilkinson, *Advanced Inorganic Chemistry* (Wiley, New York, 1980), p. 560 and references therein.
29. E. H. Appelman, *J. Am. Chem. Soc.* **90**, 1900 (1968).
30. V. D. Nefedov, M. A. Toropova, E. N. Sinotova, *Usp. Khim.* **88**, 883 (1989).

Transport and Storage of Vitamin A

RUNE BLOMHOFF, MICHAEL H. GREEN, TROND BERG, KAARE R. NORUM*

The requirement of vitamin A (retinoids) for vision has been recognized for decades. In addition, vitamin A is involved in fetal development and in the regulation of proliferation and differentiation of cells throughout life. This fat-soluble organic compound cannot be synthesized endogenously by humans and thus is an essential nutrient; a well-regulated transport and storage system provides tissues with the correct amounts of retinoids in spite of normal fluctuations in daily vitamin A intake. An overview is presented here of current knowledge and hypotheses about the absorption, transport, storage, and metabolism of vitamin A. Some information is also presented about a group of ligand-dependent transcription factors, the retinoic acid receptors, that apparently mediate many of the extravisual effects of retinoids.

THE TERM "VITAMIN A" IS USED FOR RETINOIDS THAT exhibit the biological activity of retinol (Fig. 1). Humans require only minute amounts of vitamin A in their diets (400 to 1300 μg of retinol equivalents per day, depending on age and sex). This amount can easily be obtained in most countries, but an inadequate intake of vitamin A—especially by children—is a common health problem in some areas of the world. Vitamin A deficiency can result in blindness and is associated with increased risk of severe infection and death (*1*).

Intestinal Absorption of Retinol

The main sources of vitamin A in the diet are provitamin A carotenoids from vegetables and retinyl esters from animal tissues (*2*). Essentially all of the retinyl esters are enzymatically converted to retinol in the intestinal lumen before absorption by intestinal cells (enterocytes); carotenoids are partially converted to retinol in the enterocytes (*2*) (Fig. 2).

In the enterocytes, retinol reacts with long chain fatty acids to form retinyl esters before these esters are incorporated into the chylomicrons, the main intestinal lipoproteins. The two enzymes that seem to be involved in the intestinal esterification of retinol are an acyl coenzyme A:retinol acyltransferase (ARAT) (*3*) and a lecithin:retinol acyltransferase (LRAT) (*4*). Under optimal in vitro conditions, ARAT is far more active than LRAT and is induced by large oral doses of retinol (*3*).

Retinol complexed to an intracellular, retinol-binding protein found in the intestine [CRBP(II)] is the preferred substrate for LRAT (*5*). In contrast, uncomplexed retinol in membranes may be esterified by ARAT (*6*). Thus, it is possible that LRAT esterifies retinol during absorption of a "normal" load of retinol, and ARAT esterifies excess retinol (perhaps for temporary storage) when large doses are absorbed and CRBP(II) becomes saturated.

Tissue Uptake of Chylomicron Remnants

Chylomicrons are exocytosed into the intestinal lymph and then move into the general circulation where several processes, such as triacylglycerol hydrolysis and apolipoprotein exchange, result in the formation of chylomicron remnants (*7*). Chylomicron remnants, which contain almost all of the absorbed retinol in the form of retinyl esters, are primarily cleared by the liver (*8, 9*) (Fig. 2; see below).

Extrahepatic uptake of chylomicron remnants occurs mostly in the bone marrow and the spleen and to a lesser extent in the adipose tissue, skeletal muscle, testes, lungs, and kidneys (*8–10*). Thus, chylomicron remnants may be important in the delivery of retinyl esters to tissues such as the bone marrow and the spleen that may experience periods of intensive cell proliferation and differentiation. It was recently demonstrated that chylomicron remnants effectively deliver retinyl esters to myeloid leukemic cells and thereby inhibit proliferation and induce differentiation of such cells (*11*).

The mechanism for cellular uptake of chylomicron remnants is not fully understood. Both the low-density lipoprotein (LDL) receptor and a recently characterized protein that has strong homology with the LDL receptor (LDL receptor–related protein or LRP) can bind apolipoprotein E on chylomicron remnants and may be involved in uptake (*12*). The quantitative role of these two receptors is unknown and may vary in different cells.

Hepatic uptake of chylomicron remnants. In the liver, parenchymal cells (hepatocytes) are responsible for uptake of chylomicron remnant retinyl esters (*9*). The retinyl esters are probably hydrolyzed at the plasma membrane or in early endosomes by a retinyl ester hydrolase (*13*). Retinol is subsequently found in endosomes with other ligands that are taken up by receptor-mediated endocytosis. In contrast to many other ligands that are transferred to lysosomes after processing in endosomes, retinol is transferred to the endoplasmic

R. Blomhoff, T. Berg, and K. R. Norum are at The Institute for Nutrition Research, School of Medicine, University of Oslo, 0316 Oslo 3, Norway. M. H. Green is at Pennsylvania State University, Nutrition Department, University Park, PA 16802.

*To whom correspondence should be addressed.

reticulum (*14*), where retinol-binding protein (RBP) is found in high concentration. Binding of retinol to RBP apparently initiates a translocation of retinol-RBP to the Golgi complex, followed by secretion of retinol-RBP from the cells (*15*) (Fig. 2).

The Role of Stellate Cells

Paracrine transfer of retinol from parenchymal to stellate cells in the liver. In hepatocytes, most of the retinol that is derived from chylomicron remnant retinyl esters is transferred within 2 to 4 hours to hepatic perisinusoidal stellate cells (*9*). The transfer is quite specific, as other components of chylomicron remnants, such as cholesterol and vitamin D, are not transferred (*9*). Chylomicron remnant–derived retinyl esters must be hydrolyzed before the retinol is transferred to stellate cells (*16, 17*).

The transfer of retinol from hepatocytes to stellate cells in vivo is too rapid to be accounted for by secretion from hepatocytes to the general circulation, followed by uptake into stellate cells (*9*). Since antibodies against RBP effectively reduce the transfer of retinol, it seems likely that RBP mediates the paracrine transfer of retinol from hepatocytes to stellate cells (*16*) (Fig. 2).

Storage of retinyl esters in stellate cells. In mammals, about 50 to 80% of the total body vitamin A is normally stored in liver stellate cells as retinyl esters (*18*). The normal reserve is adequate for several months in adult laboratory rats and in humans. Stellate cells store retinyl esters in large cytoplasmic lipid droplets, the size and number of which depend on amounts of vitamin A present (*19*). Stellate cells are also found in the intestine, kidneys, heart, large blood vessels, ovaries, and testes, and these cells store retinyl esters when large doses of vitamin A are consumed (*19*).

An acute load of retinol does not, however, accumulate in hepatic stellate cells of vitamin A–deficient rats (*9*). Stellate cells in such animals contain much less cellular retinol-binding protein [CRBP(I)] than stellate cells of rats with sufficient vitamin A (*20*). CRBP(I), like intestinal CRBP(II), effectively donates retinol for esterification by LRAT (*4, 21*) and subsequent storage; therefore, reduced levels of CRBP(I) in stellate cells of vitamin A–deficient rats may account for the reduced accumulation of retinyl esters.

Retinol mobilization from stellate cells. Essentially all of the retinol in the plasma that is not associated with chylomicrons and their remnants is bound to RBP and must be mobilized from stellate cells to the plasma retinol-RBP pool. Because cultured hepatocytes synthesize RBP and secrete retinol-RBP (*15*), it was suggested that the hepatocytes were the exclusive site of retinol mobilization from the liver. However, recent studies have shown that stellate cells also contain RBP (*18, 22*) and cultured stellate cells from rat liver secrete retinol bound to RBP. Although it is not clear whether stellate cells can synthesize RBP (*22*), we favor the suggestion that stellate cells synthesize RBP and mobilize retinol-RBP directly into the plasma, without prior transfer of retinol to the hepatocytes (Fig. 2).

Independent support for secretion of retinol into blood by stellate cells was presented by a whole-body multicompartmental model of retinol dynamics in rats (*23*). The existence of extrahepatic retinol-storing stellate cells (*19*) also supports the possibility that liver stellate cells secrete retinol-RBP directly into the blood.

The ability of the stellate cells to control storage and mobilization of retinol ensures that the blood plasma retinol concentration is close to 2 μM in spite of normal fluctuations in daily vitamin A intake (*2*). It is likely that saturation of CRBP(I) and RBP by retinol, along with retinoid-regulated CRBP(I) expression, controls retinol uptake, storage, and mobilization by stellate cells.

Recycling of Retinol Bound to RBP

RBP is one of a family of structurally related proteins that bind small hydrophobic molecules such as bile pigments and odorants (*24*). The three-dimensional structure of RBP predicts that it should contain a hydrophobic pocket able to bind one molecule of retinol (*25*). Most of the retinol-RBP (21 kD) in plasma is reversibly complexed with another protein, transthyretin (TTR) (55 kD), and is therefore less susceptible to filtration by kidney glomeruli (*2*).

Tracer kinetic studies (*26*) indicate that most of the plasma retinol that leaves the general circulation is recycled an average of seven to nine times (*26*) rather than being irreversibly utilized in tissues. Only 20% of the input of retinol-RBP into plasma is therefore predicted to be from the liver. The kidneys contribute 40 to 50% of plasma retinol-RBP, presumably after glomerular filtration of retinol-RBP that is not bound to TTR and subsequent reabsorption of retinol. Several extrahepatic organs, including the kidneys, have been found to contain high amounts of RBP mRNA (*27*).

Cellular Uptake of Retinol

The mechanism for cellular uptake of retinol from plasma is not yet fully elucidated. Since a small amount of free retinol may be in equilibrium with retinol-RBP in plasma, retinol may partition into the plasma membrane of cells without the use of a cell surface receptor (*28*) or may enter cells as a result of fluid-phase endocytosis. Studies of selective cellular uptake (*29*) and saturation kinetics (*30*) indicate that there is a cell surface receptor or transporter for RBP-bound retinol in vivo. Saturable RBP receptors have been demonstrated with both high- (3×10^{-9} M) and low- (9×10^{-8} M) affinity binding components on brush border membranes from human placenta (*31*). The bound RBP has a very high dissociation rate constant; that is, most of the bound RBP is displaced from the putative receptor after 5 min (*31*). Because many uptake assays involve repeated centrifugations to reduce nonspecific association

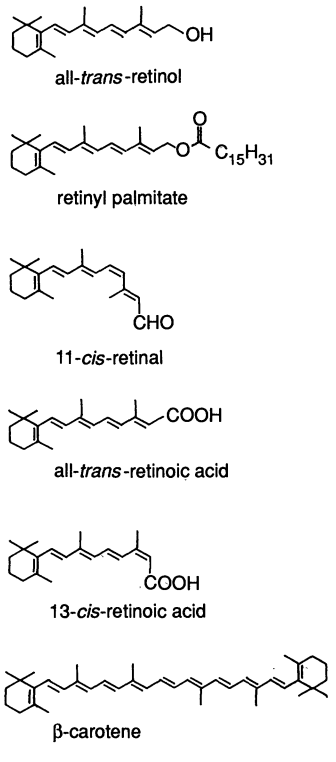

Fig. 1. Structural formulas of β-carotene and some naturally occurring retinoids.

with cells, detection of specific RBP binding may have escaped several previous attempts to identify RBP receptors. At present, it is not clear whether RBP delivers retinol to tissues after receptor-mediated endocytosis (as LDLs deliver cholesterol) or whether the RBP receptor acts as a transporter for retinol (analogous to glucose transporters in adipose tissue).

Cross-linking radiolabeled RBP in retinal pigment epithelial membranes indicated a complex of mass ~90 kD, the formation of which could be blocked by excess unlabeled RBP (*32*), suggestive of an RBP receptor of mass ~70 kD. Similar results were obtained by a ligand-blotting technique (*32*).

In addition to 2 μM retinol-RBP, there is a 5 to 10 nM plasma concentration of retinoic acid, presumably bound to albumin (*1, 2*). Recent data indicate that retinoic acid bound to albumin can be spontaneously transferred to cells and there elicit biological activity (*33*).

The quantitative contribution of the various possible mechanisms of cellular retinoid uptake (specific RBP receptor–mediated retinol uptake, the nonspecific spontaneous transfer of retinol and retinoic acid, fluid-phase endocytosis, and the uptake of chylomicron remnant retinyl esters) to vitamin A metabolism in various tissues is as yet undetermined.

Retinoid-Binding Proteins

RBP is the only retinoid-specific binding protein that has been found in plasma. However, many retinoid-binding proteins that apparently are not found in plasma have been isolated from interstitial fluid (Table 1), and their local function is probably similar to RBP. An interphotoreceptor retinoid-binding protein (IRBP) has

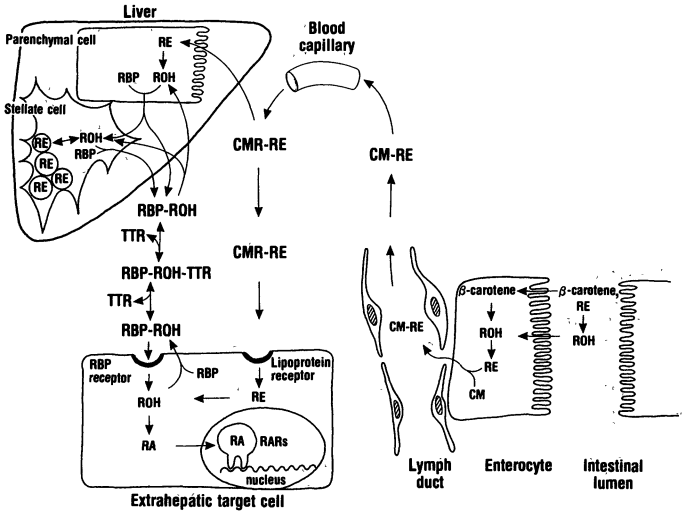

Fig. 2. Major pathways for retinoid transport in the body. Dietary retinyl esters (REs) are hydrolyzed to retinol (ROH) in the intestinal lumen before absorption by enterocytes, and carotenoids are absorbed and then partially converted to retinol in the enterocytes. In the enterocytes, retinol reacts with fatty acids to form esters before incorporation into chylomicrons (CMs). Chylomicrons then reach the general circulation by way of the intestinal lymph, and chylomicron remnants (CMRs) are formed in blood capillaries. Chylomicron remnants, which contain almost all the absorbed retinol, are mainly cleared by the liver parenchymal cells and to some extent also by cells in other organs. In liver parenchymal cells, retinyl esters are rapidly hydrolyzed to retinol, which then binds to RBP. Retinol-RBP is secreted and transported to hepatic stellate cells. Stellate cells may then secrete retinol-RBP directly into plasma. Most retinol-RBP in plasma is reversibly complexed with TTR. The uncomplexed retinol-RBP is presumably taken up in a variety of cells by cell surface receptors specific for RBP. Most of the retinol taken up will then recycle to plasma, either on the "old" RBP or bound to a newly synthesized RBP. (RA, retinoic acid; RAR, retinoic acid receptor.)

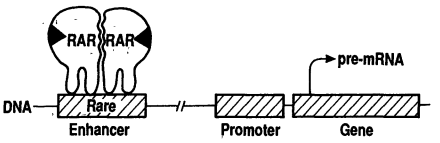

Fig. 3. Suggested mechanism for retinoic acid regulation of gene transcription. After cellular uptake, retinol can be oxidized to retinoic acid and can then diffuse into the nucleus where it can bind to one of its nuclear receptors. All the nuclear RARs are ligand-dependent transcription factors; that is, the retinoic acid–RAR complex regulates gene expression by its interaction with RAREs in the vicinity of target genes.

been identified in the extracellular space between the retinal pigment epithelial cells and the photoreceptor cells (*34*). IRBP binds not only retinol and retinal, but also vitamin E, fatty acids, and cholesterol, and may participate in the intercellular transport of retinoids during the visual cycle (*34*). Several retinol-binding proteins related to but distinct from RBP were found to be secreted by the uterine endometrium of the pig in response to progesterone (*35*). In addition, two interstitial retinoid-binding proteins that bind all-*trans* retinoic acid with high affinity have been isolated from the lumen of rat epididymis (*36*).

Many specific intracellular retinol- and retinoic acid–binding proteins (CRBPs and CRABPs, respectively) have been identified (*37–40*) (Table 1). These proteins show a high degree of homology, and they belong to a protein family that also includes protein P_2, fatty acid–binding protein (Z protein), intestinal fatty acid–binding protein, and mammary-derived growth inhibitor (*37*).

Studies on the distribution of the various types of CRBPs and CRABPs have established that CRBP(I) and CRABP(I) are the predominant intracellular retinoid-binding proteins in most tissues. CRBP(I) is most concentrated in the liver, lung, kidneys, epididymis, and testis, whereas CRABP(I) is most concentrated in the testis, skin, and eyes (*37, 38*). The two proteins are often concentrated in different cell types within a given organ (*38*).

The tissue distribution of CRBP(II), another member of this protein family, is much more restricted. Intestinal absorptive cells in rats and humans contain high concentrations of CRBP(II), but the protein has not been detected in any other cells in the adult rat (*5*). CRBP(II) is, however, present in rat embryonal liver and lung. A third cellular retinol-binding protein, CRBP(III), has recently been isolated from fish eyes (*39*).

Two apparently different CRABPs [both were called CRABP(II)] were recently identified, one in neonatal rats and the other in chick embryos (*40*). Both of these proteins bind all-*trans*-retinoic acid with much higher affinity than 13-*cis*-retinoic acid, whereas CRABP(I) binds these two ligands with similar affinity. Most chick embryonal tissues express CRABP(I), whereas CRABP(II) expression is restricted to skin, muscle, and bone. Chick CRABP(II) seems to be a main CRABP in the developing limb bud of chick embryos, but it is not expressed in any adult tissues examined (*40*).

Another intracellular retinoid-binding protein, called cellular retinal-binding protein (CRALBP), has been detected in the neural retina, in retinal pigment epithelial cells, and in the pineal gland, but not in any other tissues examined (*41*). CRALBP has no homology to other retinoid-binding proteins or other known proteins. It presumably functions in enzymatic reactions of the visual process.

The importance of cellular retinoid-binding proteins for retinoid function is indicated, for example, in some variant cell lines that lack CRABPs and are not responsive to retinoic acid (*42*). Similarly, antisense oligodeoxynucleotides corresponding to CRBP(I) inhibit the induction of alkaline phosphatase and growth response by retinol in malignant keratinocytes (*43*).

Recently, an intriguing function was suggested for CRABP [apparently CRABP(II)] (*44*). In the limb of the chick embryo, the distribution of CRABP(II) is inversely related to that of retinoic

acid, which can function as a morphogen. Thus, CRABP(II) may steepen the gradient of free retinoic acid, which in turn may be the ligand that initiates nuclear action.

In the ventral area of the spinal cord of the developing mouse embryo, the high level of expression of CRBP(I) may serve to concentrate retinol (perhaps by LRAT esterification) from the bloodstream (*45*). The predicted gradient of free retinoic acid along the dorsoventral axis may be further steepened by CRABP(I) in the dorsal but not the ventral area. Such a gradient may be crucial for the differentiation of neurons (*45*).

Many cell types respond to retinoids even though they do not express the corresponding binding proteins (*37, 46*). Hence, although cellular retinoid-binding proteins seem to be necessary for some cells to exhibit retinoid responses, other cells (or other processes in the same cell) seem to function without the binding proteins.

Conversion of Retinol to Retinoic Acid

After cellular uptake, retinol can be oxidized to retinoic acid in target cells. Earlier observations suggested that the oxidations of both retinol and ethanol are catalyzed by the same enzyme. However, recent studies (*47*) demonstrate that retinoic acid is produced from retinol by cytosol in an alcohol dehydrogenase–negative strain of deermouse, perhaps by a pathway that does not involve retinal as a discrete intermediate. Furthermore, retinoic acid may also be synthesized from β-carotene in several tissues, and retinol and retinal are probably not metabolites in this reaction (*47*). Thus, in addition to retinol, β-carotene may be a source of retinoic acid in certain cells. In future experiments, it will be important to determine the involvement of retinoid-binding proteins in these reactions.

The concentration of retinoic acid in various cells may be regulated and mutable. If so, the local intracellular retinoic acid concentration may be determined by the cellular uptake of retinoids, the rate of retinoid synthesis or degradation, or the amount of CRABPs. Alternatively, it is possible that the retinoic acid level is not regulated and most cells have an excess of retinoic acid. Then the availability of protein factors, such as binding proteins and nuclear receptors, would determine the retinoid response.

Nuclear Retinoic Acid Receptors

In 1987, a retinoic acid receptor (RARα) that belongs to the family of steroid-thyroid hormone receptors was cloned (*48*). RARα appears to bind 13-*cis*-retinoic acid, all-*trans*-retinoic acid, and other differentiation-effective retinoids with high affinity, and to bind retinol and retinal with much lower (about 1/100) affinity. However, the concentration of retinol is about 400 times that of retinoic acid in the body fluids (*1, 2*). Therefore, retinol is probably also a ligand for the receptor in vivo. A hepatitis B virus integration site in a human hepatocellular carcinoma was then shown to be a different retinoic acid receptor (RARβ) (*49*). Finally, a third human retinoic acid receptor (RARγ) and the mouse counterparts of all three receptors were cloned (*50*). Comparison of the amino acid sequences of the three human receptors with the mouse receptors showed that the interspecies conservation of a member of the RAR subfamily is much higher than the conservation of all three receptors within a given species, suggesting that RARα, RARβ, and RARγ each have their own specific function (*50*).

The RARα gene is expressed at high levels in specific tissues such as the cerebellum, adrenals, and testes, but otherwise has a low level of expression (*48–50*). RARα is overexpressed in several leukemic cells (*48*). RARβ shows great variation in expression, ranging from undetectable in leukemic cells to relatively abundant in the kidneys, prostate, and cerebral cortex (*48–50*). RARγ is found almost exclusively in the skin, reflecting the effects of retinoids on this organ (*50*). Since almost all cell types in the body contain one or several of the RARs, it is likely that retinoic acid has an even more pleiotropic effect than was previously thought.

Another nuclear retinoic acid receptor (RXRα) has been cloned (*51*) that is substantially different in primary sequence from the previously described RARs. The ligand-binding domains of RARα and RXRα share only 27% homology. There are indications that RXR represents another subfamily. RXRα is more specific for retinoic acid than is RARα, which is activated by several structurally related retinoids. In contrast to the RARs, RXRα mRNA is expressed most abundantly in visceral tissues such as the liver and the kidneys; RXRα may itself be a regulator of vitamin A metabolism (*51*).

Retinoic acid responsive element (RARE). Nuclear receptors are generally ligand-dependent transcription factors and regulate gene expression by binding to short DNA sequences (hormone-responsive elements or enhancers) in the vicinities of target genes (*52*) (Fig. 3). Various RAREs and the genes that are directly regulated by them are being identified. Functional RAREs are located upstream of the genes for RARβ and the B1 subunit of the extracellular matrix protein laminin (*53*), and these RAREs are responsive to RARα, RARβ, and RARγ. Specific binding of RARs to RARE was independent of retinoic acid, suggesting that retinoic acid activates gene expression by binding to RARs already bound to the responsive element (*53*) (Fig. 3).

Table 1. Retinoid-binding proteins and receptors.

Protein	Approximate mass (kD)	Main ligand	Suggested function
RBP	21	Retinol	Blood plasma transport
IRBP	140	Retinol, retinal	Intercellular transport in visual cycle
Four proteins secreted from pig uterus	22	Retinol	Transport to the fetus
Two luminal proteins in rat epididymis	20	Retinoic acid	Intercellular transport
CRBP(I)	16	Retinol	Donor for LRAT reaction, intracellular transport
CRBP(II)	16	Retinol	Donor for LRAT reaction
CRBP(III) from fish eye	15	Retinol	
CRABP(I)	16	Retinoic acid	Intracellular transport, regulate free retinoic acid concentration
CRABP(II) from neonatal rat	15	Retinoic acid	Intracellular transport, regulate free retinoic acid concentration
CRABP(II) from embryonal chick	16	Retinoic acid	Intracellular transport, regulate free retinoic acid concentration
CRALBP	36	Retinal	Enzymatic reactions in the visual cycle
RARα	50	Retinoic acid	Ligand-dependent transcription factor
RARβ	50	Retinoic acid	Ligand-dependent transcription factor
RARγ	50	Retinoic acid	Ligand-dependent transcription factor
RXRα	50	Retinoic acid	Ligand-dependent transcription factor

Other responsive elements. The DNA binding domains of thyroid hormone receptors and RARs are very similar. Indeed, the demonstration that RARα binds to a thyroid hormone responsive element upstream of the growth hormone gene explains the observation that retinoic acid, in synergy with thyroid hormones, can control growth hormone production by pituitary GH1 cells (*54*). Two types of thyroid hormone receptors, TRα and TRβ, both form heterodimers with human RARα (*55*). It seems that the heterodimers bind to a subset of thyroid hormone responsive elements and result in an increase in transcription of some genes, but a decrease for others.

The expression of osteocalcin, an osteoblast-specific bone protein, is regulated by retinoic acid and vitamin D_3 by binding of their respective nuclear receptors to a common responsive element (*56*). This responsive element also contains a consensus AP-1 site, the enhancer element for the *fos-jun* protooncogene family. Binding of the *fos-jun* complex suppresses the induction of osteocalcin by both retinoic acid and vitamin D_3 (*56*). Hence, to understand the effect of vitamin A on gene expression, we must also consider the presence of other nuclear receptors and their ligands, and of proteins that belong to the *fos-jun* family.

Future Challenges

Although advances have been made recently in our knowledge of vitamin A metabolism and function, especially in understanding the function of retinoic acid in morphogenesis and differentiation, many questions remain. For example, the mechanisms for and control of uptake of retinoids by cells, and the in situ synthesis and regulation of retinoic acid must be studied more extensively. Little is known about the quantities of vitamin A used by various body tissues at different stages of development, or about what controls this utilization. The demonstration that retinoic acid is a potent morphogen, the very specific and early embryonal expression of retinoid-binding proteins and nuclear RARs, and the profound effect of retinoid deficiency and excess on fetal development, all point to the importance of retinoids (in particular, retinoic acid) in fetal development. Therefore, transport of appropriate amounts of retinoids to specific cells at specific times and appropriate regulation of cytoplasmic binding proteins and nuclear receptors is crucial for normal development.

Future research may determine whether all extravisual effects of retinoids can be accounted for by the nuclear retinoid receptors. An alternative mechanism may indeed exist: several members of the isoprenoid family (including farnesyl and geranyl derivatives), which also includes retinoids, have been found covalently linked to regulatory proteins such as yeast mating factors, G proteins, and *ras* oncogenes, and have been found to affect the function of these proteins (*57*). The best understood function of vitamin A is the first step in the visual cycle, where 11-*cis*-retinal covalently bound to opsin is isomerized to all-*trans*-retinal in response to light stimulation (*58*). Retinoids may also be covalently linked to regulatory proteins in other cell types. Indeed, several classes of proteins in HL-60 cells covalently bind retinoic acid (*59*). It will be interesting to follow the identification of these proteins and the possible function of their retinoylation.

REFERENCES AND NOTES

1. D. S. Goodman, *N. Engl. J. Med.* **310**, 1023 (1984).
2. G. Wolf, *Physiol. Rev.* **64**, 873 (1984); D. S. Goodman and W. S. Blaner, in *The Retinoids*, M. B. Sporn, A. B. Roberts, D. S. Goodman, Eds. (Academic Press, Orlando, FL, 1984), vol. 2, pp. 1–39.
3. P. Helgerud, L. B. Petersen, K. R. Norum, *J. Lipid Res.* **23**, 609 (1982); *J. Clin. Invest.* **71**, 747 (1983).
4. D. E. Ong, B. Kakkad, P. MacDonald, *J. Biol. Chem.* **262**, 2729 (1987); P. N. MacDonald and D. E. Ong, *ibid.* **263**, 12478 (1988).
5. D. E. Ong, *ibid.* **259**, 1476 (1984); ——— and D. L. Page, *J. Lipid Res.* **28**, 739 (1987).
6. R. W. Yost, E. H. Harrison, A. C. Ross, *J. Biol. Chem.* **263**, 18693 (1988).
7. P. H. R. Green and R. M. Glickman, *J. Lipid Res.* **22**, 1153 (1981).
8. D. S. Goodman, H. S. Huang, T. Shiratori, *ibid.* **6**, 390 (1965).
9. R. Blomhoff, P. Helgerud, M. Rasmussen, T. Berg, K. R. Norum, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7326 (1982); R. Blomhoff, K. Holte, L. Næss, T. Berg, *Exp. Cell Res.* **150**, 186 (1984).
10. M. M. Hussain *et al.*, *J. Biol. Chem.* **264**, 17931 (1989).
11. K. O. Wathne, K. R. Norum, E. Smeland, R. Blomhoff, *ibid.* **263**, 8691 (1988).
12. J. L. Goldstein *et al.*, *Annu. Rev. Cell Biol.* **1**, 1 (1985); J. Herz *et al.*, *EMBO J.* **7**, 4119 (1988); U. Beisiegel *et al.*, *Nature* **341**, 162 (1989); R. C. Kowal *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5810 (1989).
13. E. H. Harrison and M. Z. Gad, *J. Biol. Chem.* **264**, 17142 (1989).
14. R. Blomhoff, W. Eskild, G. M. Kindberg, K. Prydz, T. Berg, *ibid.* **260**, 13566 (1985); H. F. J. Hendriks, E. Elhanany, A. Brouwer, A. M. De Leeuw, D. L. Knook, *Hepatology (Baltimore)* **8**, 276 (1988).
15. L. Rask *et al.*, *Exp. Cell Res.* **143**, 91 (1983); H. Ronne *et al.*, *J. Cell Biol.* **96**, 907 (1983).
16. R. Blomhoff, T. Berg, K. R. Norum, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3455 (1988).
17. W. S. Blaner *et al.*, *Eur. J. Biochem.* **164**, 301 (1987).
18. R. Blomhoff *et al.*, *J. Biol. Chem.* **260**, 13560 (1985); W. S. Blaner *et al.*, *J. Lipid Res.* **26**, 1241 (1985).
19. K. Wake, *Int. Rev. Cytol.* **66**, 303 (1980); R. Blomhoff and K. Wake, *FASEB J.*, in press.
20. M. Kato, K. Kato, D. S. Goodman, *J. Cell Biol.* **98**, 1696 (1984).
21. D. E. Ong, P. N. MacDonald, A. M. Gubitosi, *J. Biol. Chem.* **263**, 5789 (1988); P. N. MacDonald and D. E. Ong, *Biochem. Biophys. Res. Commun.* **156**, 157 (1988).
22. R. Blomhoff *et al.*, unpublished results; M. Yamada *et al.*, *Hepatology* 7, 1224 (1987).
23. M. H. Green, J. B. Green, T. Berg, K. R. Norum, R. Blomhoff, unpublished results.
24. W. S. Blaner, *Endocr. Rev.* **10**, 308 (1989).
25. M. E. Newcomer *et al.*, *EMBO J.* **3**, 1451 (1984).
26. M. H. Green, J. B. Green, K. C. Lewis, *J. Nutr.* **117**, 694 (1987); K. C. Lewis, M. H. Green, B. A. Underwood, *ibid.* **111**, 1135 (1981); M. H. Green, L. Uhl, J. B. Green, *J. Lipid Res.* **26**, 806 (1985).
27. D. R. Soprano, K. J. Soprano, D. S. Goodman, *J. Lipid Res.* **27**, 166 (1986); A. Makover, D. R. Soprano, M. L. Wyatt, D. S. Goodman, *ibid.* **30**, 171 (1989).
28. G. Fex and G. Johannesson, *Biochim. Biophys. Acta* **944**, 249 (1988).
29. D. Bok and J. Heller, *Exp. Eye Res.* **22**, 395 (1976); B. W. McGuire, M. C. Orgebin-Crist, F. Chytil, *Endocrinology* **108**, 658 (1981); T. Gjøen *et al.*, *J. Biol. Chem.* **262**, 10926 (1987); H. Senoo *et al.*, *J. Lipid Res.* **31**, 1215 (1990).
30. J. Heller, *J. Biol. Chem.* **250**, 3613 (1975); ——— and D. Bok, *Am. J. Ophthalmol.* **81**, 93 (1976); L. Rask and P. A. Peterson, *J. Biol. Chem.* **251**, 6360 (1976); U. Eriksson *et al.*, *Cancer Res.* **46**, 717 (1986).
31. A. Sivaprasadarao and J. B. C. Findlay, *Biochem. J.* **255**, 561 (1988).
32. B. C. Laurent *et al.*, *Chem. Scr.* **27**, 185 (1987).
33. M. Botilsrud *et al.*, *Scand. J. Clin. Lab. Invest.* **50**, 309 (1990).
34. Y. L. Lai, B. Wiggert, Y. P. Liu, G. J. Chader, *Nature* **298**, 848 (1982); A. J. Adler and K. J. Martin, *Biochem. Biophys. Res. Commun.* **108**, 1601 (1982); G. I. Liou *et al.*, *Vision Res.* **22**, 1457 (1982); N. G. Bazan *et al.*, *J. Biol. Chem.* **260**, 13677 (1985); S. L. Fong *et al.*, *ibid.* **259**, 6534 (1984).
35. J. Clawitter *et al.*, *J. Biol. Chem.* **265**, 3248 (1990).
36. D. E. Ong and F. Chytil, *Arch. Biochem. Biophys.* **267**, 474 (1988).
37. F. Chytil and D. E. Ong, in *The Retinoids*, M. B. Sporn, A. B. Roberts, D. S. Goodman, Eds. (Academic Press, Orlando, FL, 1984), vol. 2, pp. 90–123.
38. D. E. Ong, J. Crow, F. Chytil, *J. Biol. Chem.* **257**, 13385 (1982); U. Eriksson *et al.*, *ibid.* **259**, 13464 (1984); M. Kato *et al.*, *ibid.* **260**, 4832 (1985).
39. S. Nishiwaki *et al.*, *Biochim. Biophys. Acta* **1037**, 192 (1990).
40. J. S. Bailey and C. H. Siu, *J. Biol. Chem.* **263**, 9326 (1988); T. Kitamoto, M. Momoi, T. Momoi, *Biochemn. Biophys. Res. Commun.* **164**, 531 (1989).
41. J. C. Saari, L. Bredberg, G. G. Garwin, *J. Biol. Chem.* **257**, 13329 (1982); J. W. Crabb, S. Goldflam, S. E. Harris, J. C. Saari, *ibid.* **263**, 18688 (1988).
42. For example, H. Ueda *et al.*, *Cancer Res.* **45**, 3332 (1985); J. Schindler *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1077 (1981); S. Y. Wang and L. J. Gudas, *J. Biol. Chem.* **259**, 5899 (1984).
43. F. O. Cope and J. H. Wille, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5590 (1989).
44. M. Maden, D. E. Ong, D. Summerbell, F. Chytil, *Nature* **335**, 733 (1988).
45. A. V. Perez-Castro, L. E. Toth-Rogler, L. N. Wei, M. C. Nguyen-Huu, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8813 (1989).
46. T. R. Breitman, S. E. Selonick, S. J. Collins, *ibid.* **77**, 2936 (1980).
47. K. C. Posch, W. J. Enright, J. L. Napoli, *Arch. Biochem. Biophys.* **274**, 171 (1989); J. L. Napoli and K. R. Race, *J. Biol. Chem.* **263**, 17372 (1988).
48. M. Petkovich, N. J. Brand, A. Krust, P. Chambon, *Nature* **330**, 444 (1987); V. Giguere, E. S. Ong, P. Segui, R. M. Evans, *ibid.*, p. 624; C. Largman *et al.*, *Blood* **74**, 99 (1989).
49. N. Brand *et al.*, *Nature* **332**, 850 (1988); D. Benbrook, E. Lernhardt, M. Pfahl, *ibid.* **333**, 669 (1988); H. de The, A. Marchio, P. Tiollais, A. Dejean, *EMBO J.* **8**, 429 (1989).
50. A. Krust, P. Kastner, M. Petkovich, A. Zelent, P. Chambon, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5310 (1989); A. Zelent, A. Krust, M. Petkovich, P. Kastner, P. Chambon, *Nature* **339**, 714 (1989).
51. D. J. Mangelsdorf, E. S. Ong, J. A. Dyck, R. M. Evans, *Nature* **345**, 224 (1990).
52. R. M. Evans, *Science* **240**, 889 (1988).
53. H. de The, M. M. Vivanco-Ruiz, P. Tiollais, H. Stunnenberg, A. Dejean, *Nature*

343, 177 (1990); G. W. Vasios, J. D. Gold, M. Petkovich, P. Chambon, L. J. Gudas, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9099 (1989).
54. K. Umesono, V. Giguere, C. K. Glass, M. G. Rosenfeld, R. M. Evans, *Nature* **336**, 262 (1988); G. Bedo, P. Santisteban, A. Aranda, *ibid.* **339**, 231 (1989).
55. C. K. Glass, S. M. Lipkin, O. V. Devary, M. G. Rosenfeld, *Cell* **59**, 697 (1989).
56. R. Schüle *et al.*, *ibid.* **61**, 497 (1990).
57. J. L. Goldstein and M. S. Brown, *Nature* **343**, 425 (1990); J. A. Glomset, M. H. Gelb, C. C. Farnsworth, *Trends Biochem. Sci.* **15**, 139 (1990).
58. L. Stryer, *Sci. Am.* **257**, 32 (July 1987).
59. N. Takahashi and T. R. Breitman, *J. Biol. Chem.* **264**, 5159 (1989).
60. Supported by the Norwegian Research Council for Science and the Humanities, the Norwegian Cancer Society, and the Anders Jahres Foundation. K.R.N. was a Fogarty scholar at the National Institutes of Health, Bethesda, MD, during the preparation of this manuscript.

Research Article

Two Domains of Yeast U6 Small Nuclear RNA Required for Both Steps of Nuclear Precursor Messenger RNA Splicing

PATRIZIA FABRIZIO AND JOHN ABELSON

U6 is one of the five small nuclear RNA's (snRNA's) that are required for splicing of nuclear precursor messenger RNA (pre-mRNA). The size and sequence of U6 RNA are conserved among organisms as diverse as yeast and man, and so it has been proposed that U6 RNA functions as a catalytic element in splicing. A procedure for in vitro reconstitution of functional yeast U6 small nuclear ribonucleoproteins (snRNP's) with synthetic U6 RNA was applied in an attempt to elucidate the function of yeast U6 RNA. Two domains in U6 RNA were identified, each of which is required for in vitro splicing. Single nucleotide substitutions in these two domains block splicing either at the first or the second step. Invariably, U6 RNA mutants that block the first step of splicing do not enter the spliceosome. On the other hand, those that block the second step of splicing form a spliceosome but block cleavage at the 3′ splice site of the intron. In both domains, the positions of base changes that block the second step of splicing correspond exactly to the site of insertion of pre-mRNA–type introns into the U6 gene of two yeast species, providing a possible explanation for the mechanism of how these introns originated and adding further evidence for the proposed catalytic role of U6 RNA.

PRECURSOR MESSENGER RNA (PRE-MRNA) SPLICING IN the nucleus takes place on a large multicomponent particle, termed the spliceosome (*1*). The function of the various components or trans-acting factors in the spliceosome is to fold the intron into a splicing substrate and to catalyze the reaction. The trans-acting factors include a large number of different proteins (perhaps more than 30) and five small nuclear RNA's (snRNA's)—U1, U2, U4, U5, and U6. The snRNA's associate with a set of seven common core proteins, in addition to other proteins occurring only with certain of the snRNA's, to form the small nuclear ribonucleoproteins (snRNP's) (*1, 2*).

Pre-mRNA splicing is a two-step process. In the first step, the 5′ splice site is cleaved and, in a coordinated reaction, the 5′ G residue of the intron is linked in a 2′,5′-phosphodiester linkage to an A residue near the 3′ splice junction. In the second step, the 3′ splice site is cleaved, the exons are joined, and the intron is released as a "lariat." Hydrolysis of adenosine triphosphate (ATP) is required for this process in vitro (*3*).

The group I and group II self-splicing introns are found in mitochondria and chloroplast genes, and group I introns have been extensively studied in a *Tetrahymena* ribosomal RNA gene (*4*). The splicing mechanism for these introns also consists of two phosphotransfer reactions. The group II self-splicing reaction mechanism is, in fact, identical to that for pre-mRNA's, which suggests a common evolutionary origin for the two processes (*5*). Although pre-mRNA splicing and group II self-splicing proceed by the same reaction pathway, the two reactions have distinct biochemical requirements. Group II self-splicing proceeds with no added factors, whereas a myriad of trans-acting factors, as well as hydrolysis of ATP, are required for pre-mRNA splicing. The group II intron itself is the catalyst of the self-splicing reaction and, as such, has a conserved and complex secondary and tertiary structure. By contrast, introns in the nucleus have the bare minimum of information at 5′ and 3′ splice sites required for identification of these sites by the trans-acting factors, and intron sequences appear to have no conserved secondary or tertiary structure (*3*). If these two processes have a common evolutionary origin, then we are led to the hypothesis that pre-mRNA splicing, despite its requirements for protein and ATP, is an RNA-catalyzed reaction. If this is so, then the catalyst for pre-mRNA splicing must be one or more of the snRNA's. In this view, each of the snRNA's can be thought of as a group II intron in pieces, and the spliceosome as a way of reassembling the intron from its parts (*6*).

Unequivocal evidence establishing a catalytic role in splicing for any of the snRNA's is lacking. Functional roles have been established for the U1 and U2 snRNA's. Namely, a sequence at the 5′ end of U1 RNA forms base pairs with the 5′ splice site and a sequence near the 5′ end of U2 RNA forms base pairs with the branchpoint sequence near the 3′ splice site (*1*). These interactions

The authors are at the California Institute of Technology, Division of Biology, Pasadena, CA 91125.