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FINDing the Ligand:
Retinoid Receptor Activation in the CNS

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Cover picture: Close up of the developing brain of a transgenic E11.5 mouse embryo expressing a GAL4-RAR chimeric receptor. Induction of reporter gene expression (blue) can be seen in the lateral ganglionic eminence as well as at the midbrain/hindbrain boundary, suggestive of retinoid synthesis within these regions.
(Modified from Mata de Urquiza et al., *PNAS* 96:13270-13275)

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To my Family

"...For the real amazement, if you wish to be amazed, is this process. You start out as a single cell derived from the coupling of a sperm and an egg; this divides in two, then four, then eight, and so on, and at a certain stage there emerges a single cell which has as all its progeny the human brain. The mere existence of such a cell should be one of the great astonishments of the earth. People ought to be walking around all day, all through their waking hours calling to each other in endless wonderment, talking of nothing except that cell."

LEWIS THOMAS (1979)

"Like the entomologist in search for brightly colored butterflies, my attention hunted, in the garden of the gray matter, cells with delicate and elegant forms, the mysterious butterflies of the soul."

S. RAMON Y CAJAL (1937)

SUMMARY

Nuclear hormone receptors (NRs) comprise a large family of ligand activated transcription factors, found in vertebrates, arthropods and nematodes. Among the members of the NR family are the receptors for small, lipophilic ligands such as the steroid hormones, vitamins A and D, thyroid hormone, oxysterols and bile acids. These lipophilic molecules easily pass through the plasma membrane and enter cells where they encounter and bind their cognate receptor, thereby affecting transcription of target genes.

Retinoids, the biologically active vitamin A metabolites, have been shown to be essential in processes such as organogenesis, neurogenesis, vision and reproduction. The two most important retinoids *in vivo*, namely all-*trans* retinoic acid (atRA) and 9-*cis* retinoic acid (9cRA), exert their functions by binding and activating two classes of NRs, the retinoic acid receptors (RARs) and the retionid X receptors (RXRs). Knockout studies of the genes encoding RARs and RXRs fully recapitulate the phenotypes seen in mice lacking vitamin A, confirming the receptors´ roles in transducing retinoid signals. In addition, RXR also functions as a heterodimer partner for several other NRs. For example, RARs, thyroid hormone receptors (TRs), vitamin D receptor (VDR) and several orphan receptors, are known to mediate their function as heterodimers with RXRs.

An important issue to better understand the roles played by nuclear receptors, is to establish where the receptor is active *in vivo*. We have previously developed a transgenic mouse model, where RAR and RXR activity in mouse embryos was directly reflected by the transcription of a lacZ reporter gene. Here, we present a refined version of the so called effector-reporter system (referred to as the FIND expression system), where ligand-activated receptor not only will induce the expression of the reporter, but also of the receptor itself in an auto-inductive loop. Transgenic mice expressing GAL4-RAR were analyzed, and the resulting reporter expression pattern is consistent with our previous data. Strong expression was detected in the developing spinal cord, forebrain and proximal forelimb buds. In addition, reporter activation was detected at the midbrain/hindbrain boundary, a region not previously reported to contain retinoids at this stage. The FIND expression system can potentially be used with any given NR of interest, and should provide a powerful tool when searching for novel NR ligands.

Expression of the homeobox gene Meis2 is enriched in a ventral structure of the developing forebrain (the lateral ganglionic eminence, LGE) and is induced by retinoic acid in PC12 cells. Thus, we became interested in investigating a potential role for retinoids during the development of the forebrain. By making use of the effector-reporter assay described above, we show that radial glial cells within the LGE are responsible for producing retinoids. Hence, we suggest a model where radial glia not only play a vital role as guiding cells in neuronal migration, but also as direct contributors to differentiation of LGE neurons by local retinoid synthesis.

The importance of retinoids during development is well established, whereas their role in postnatal and adult tissues is less clear. By studying the expression of all known retinoid binding proteins and retinoid receptors, we show that most of these proteins are still expressed after birth. Co-culture experiments show that postnatal striatum, but not hippocampus and cortex, produces retinoids, implicating retinoids as important factors in this structure also after birth.

A surprising result when analyzing different adult brain tissues for RAR and RXR activation, was that most tissues activated RXR stronger than RAR. The fact that 9cRA, the natural ligand for RXR, has been difficult to detect *in vivo*, prompted us to go ahead and purify the RXR ligand(s) present in the adult brain. Biochemical isolation, purification and mass spectrometry thus identified the activity as docosahexaenoic acid (DHA), a polyunsaturated fatty acid highly enriched in postnatal central nervous system. Deficiency studies have shown that DHA is essential for normal growth and development, as deprivation of this fatty acid leads to neurological problems, learning disabilities and growth retardation. Certain RXR mutant mice display similar defects, and both DHA and RXR ligands have beneficial effects on blood cholesterol levels and insulin sensitivity. We therefore suggest that RXR activation by DHA is a novel signaling mechanism, which may in part explain how DHA influences neural processes and maturation of the mammalian brain.

Alexander Mata de Urquiza, 2001

ABBREVIATIONS

aa	amino acid
AF-1, AF-2	activation function 1, 2
AHD2	aldehyde dehydrogenase 2
A-P	anterior-posterior
AR	androgen receptor
CAR	constitutive androstane receptor
CBP	CREB binding protein
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
COUP-TF	chicken upstream ovalbumin promoter transcription factor
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
CTD	carboxy-terminal domain
DA	dopamine
DARPP-32	dopamine- and cAMP-regulated phosphoprotein 32
DBD	DNA binding domain
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
DR	direct repeat
DRIP	vitamin D receptor interacting protein
EcR	ecdysone receptor
ER	estrogen receptor
ER	everted repeat
FA	fatty acid
FIND	feedback-inducible nuclear-receptor-driven
FXR	farnesoid X receptor
GCNF	germ cell nuclear factor
GR	glucocorticoid receptor
GRIP1	glucocorticoid receptor interacting protein 1
GTF	general transcription factor
HAT	histone acetyl transferase
HDAC	histone deacetylase
HNF4	hepatocyte nuclear factor 4
Hox	homeobox
HRE	hormone response element
Hsp	heat-shock protein
IR	inverted repeat
lacZ	β-galactosidase gene
LBD	ligand binding domain
LBP	ligand binding pocket
LGE	lateral ganglionic eminence
LXR	liver X receptor
MGE	medial ganglionic eminence
MR	mineralocorticoid receptor
N-CoR	nuclear receptor corepressor
NGFI-B	nerve growth factor inducible 1
NOR1	neuron derived orphan receptor 1
NR	nuclear hormone receptor
NTD	amino-terminal domain
NURR1	Nur-related factor 1
P450	cytochrome P450 enzyme
p/CAF	CBP associated factor
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
PUFA	polyunsaturated fatty acid
PXR	pregnane X receptor
RA	retinoic acid
atRA	all-*trans* retinoic acid
9cRA	9-*cis* retinoic acid
RAL	retinal
atRAL	all-*trans* retinal
9cRAL	9-*cis* retinal
RALDH	retinal dehydrogenase
RAR	retinoic acid receptor
RARE	retinoic acid response element
RE	response element
RIP160	receptor interacting protein 160
RNA	ribonucleic acid
RNA Pol II	RNA polymerase II
ROL	retinol
atROL	all-*trans* retinol
9cROL	9-*cis* retinol
ROLDH	retinol dehydrogenase
RXR	retinoid X receptor
RXRE	retinoid X response element
SF-1	steroidogenic factor 1
SMRT	silencing mediator for retinoid and thyroid hormone receptor
SRC-1	steroid receptor coactivator 1
TLX	tailX
TR	thyroid hormone receptor
TRAP	TR-associated protein
UAS	upstream activating sequence
USP	ultraspiracle
VAD	vitamin A deficiency
VDR	vitamin D_3 receptor

TABLE OF CONTENTS

SUMMARY 5

ABBREVIATIONS 6

TABLE OF CONTENTS 7

LIST OF ORIGINAL PUBLICATIONS 9

INTRODUCTION 10

The Nuclear Hormone Receptors 11

One big, happy family 11

Nuclear receptors share a common structure 12

DNA binding and dimerization 12

The DNA-binding domain 13

The LBD and ligand-dependent transactivation 15

Coactivators 17

Corepressors 18

Transcriptional initiation – it´s a matter of complexes 20

NRs from an evolutionary perspective 22

Retinoids and Retinoid Receptors 24

La Dolce Vita(min A): A vital vitamin 24

RA synthesis and breakdown 24

Hox genes and pattern formation 26

Retinoids in adult physiology 28

Retinoid binding proteins 28

Retinoid receptors 29

RXR heterodimers and DNA binding 30

Retinoid signaling: permissive vs. non-permissive heterodimers 31

RXR-specific signaling 32

Retinoid receptor expression patterns 33

Retinoid receptor 'knockouts' 34

AIMS OF THE STUDY 36

RESULTS AND DISCUSSION 37

Feedback-inducible nuclear-receptor-driven reporter gene expression in transgenic mice (Paper I) 37

FIND-RAR/lacZ 37

Conclusion (Paper I) 38

Retinoids are produced by glia in the lateral ganglionic eminence and regulate striatal neuron differentiation (Paper II) 39

Retinoids are produced in the LGE 39

Glial cells in the LGE synthesize and release retinoids 40

Retinoids induce striatal neuron differentiation in vitro 40

Conclusion (PaperII) 40

Role of retinoids in the CNS: differential expression of retinoid binding proteins and receptors and evidence for presence of retinoic acid (Paper III) 41

A role for RA signaling in the nigrostriatal dopamine system 41

Conclusion (Paper III) 42

Docosahexaenoic acid, a novel ligand for the retinoid X receptor in mouse brain (Paper IV) 43

DHA – an essential fatty acid 43

Conclusion (Paper IV) 44

FUTURE PROSPECTS 46

ACKNOWLEDGEMENTS 48

REFERENCES 51

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **Alexander Mata de Urquiza***, Ludmila Solomin* and Thomas Perlmann. Feedback-inducible nuclear-receptor-driven gene expression in transgenic mice. (1999) *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 13270-13275

II. Håkan Toresson, **Alexander Mata de Urquiza***, Charlotta Fagerström*, Thomas Perlmann and Kenneth Campbell. Retinoids are produced by glia in the lateral ganglionic eminence and regulate striatal neuron differentiation. (1999) *Development*, **126**, 1317-1326

III. Rolf H. Zetterström, Eva Lindqvist, **Alexander Mata de Urquiza**, Andreas Tomac, Ulf Eriksson, Thomas Perlmann and Lars Olson. Role of retinoids in the CNS: differential expression of retinoid binding proteins and receptors and evidence for presence of retinoic acid. (1999) *Eur. J. Neurosci*., **11**, 407-416

IV. **Alexander Mata de Urquiza**, Suya Liu*, Maria Sjöberg*, Rolf H. Zetterström, William Griffiths, Jan Sjövall and Thomas Perlmann. Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. (2000) *Science*, **290**, 2140-2144

* Equal contributions

INTRODUCTION

This is a tale about how cells communicate with each other by chemical signaling. The controlled release of such signaling molecules, allows cells to regulate the developmental fate of their neighbours. Transcription is the initial step in the process whereby the information that is contained in the DNA of our cells is transformed into the proteins that we need to function properly. This complex and extremely fine tuned sequence of events, starts with the localization of the gene containing the necessary information, followed by the correct assembly of the cellular machinery that reads the DNA code and copies it into RNA. This sets the stage for another cellular machine that has the ability of translating the RNA information into a protein. With the completion of the sequencing of the human genome, we now know that each cell in our body contains around 30 to 40,000 different genes. Thus, it is quite impressive that a cell can keep track of all this stored information, and know when to activate a certain gene. Transcription is initiated when specialized proteins, transcription factors, recognize the gene of interest. These factors then attract other accessory proteins that are needed to copy the gene from DNA into RNA. Ultimately, gene activation leads to changes in cell structure and function.

But how does a cell know when to activate a certain gene? A plethora of different signaling molecules are constantly bombarding the cells of our bodies, each instructing the cell to respond in a certain way, e.g. by activating a certain set of genes. Mediators of one such type of signaling mechanisms include small, fat-soluble molecules, which easily pass the protective plasma membrane of cells. Once inside, they directly bind to the adequate transcription factor (a so-called nuclear receptor), thereby inducing transcription of the correct set of genes. Retinoids (the biologically active metabolites of vitamin A), play fundamental roles in vertebrate development and homeostasis (Giguère, 1994), and function in signaling by entering cells and binding to specific receptors in the nucleus. These receptors are in fact transcription factors, that upon binding the ligand, become activated and induce the expression of specific genes of interest.

The aim of this study has been to gain further insight into the roles played by retinoids *in vivo*, both during embryonal development, and also in the adult organism, thereby unveiling how these tiny molecules, by activating transcription of target genes, can have such widespread effects on processes like organogenesis, neurogenesis, vision and reproduction.

THE NUCLEAR HORMONE RECEPTORS

One big, happy family

Retinoids, including all-*trans* retinoic acid (atRA) and 9-*cis* retinoic acid (9cRA), act as ligands for two types of transcription factors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), both existing in three different subtypes, α, β and γ. RARs and RXRs belong to a large and evolutionary well conserved family of transcription factors known as the nuclear hormone receptors (NRs), found in organisms as diverse as nematodes, flies and mammals (reviewed in Di Croce *et al*., 1999; Mangelsdorf *et al*., 1995a; Perlmann and Evans, 1997). The NR superfamily comprises more than 150 different proteins, most of which are thought to function as ligand-activated transcription factors, exerting widely different biological functions by regulating target gene expression positively and/or negatively. The members of this family are receptor proteins for certain small, lipophilic molecules, including, in addition to retinoids, molecules such as steroid hormones, vitamin D_3, thyroid hormone, cholesterol metabolites and fatty acids. These ligand molecules readily pass through the plasma membrane and interact with their receptors inside the target cell. With the exception of the steroid receptors, some of which encounter their ligands in the cytoplasm prior to entering the nucleus, most other NRs are thought to bind their ligands inside the nucleus. The ligand-activated receptor will recognize specific sequences in the promoters of target genes, thereby inducing transcription of those genes (see Figure 1).

In addition to these classical nuclear receptors that act as ligand-inducible transcription factors, cDNAs encoding members of this family with no known ligands have also been identified. These are usually refered to as "orphan" receptors (reviewed in Giguère, 1999; Kastner *et al*., 1995). During the last few years, ligands have been identified for some of these orphans (Blumberg and Evans, 1998; Chawla *et al*., 2000; Coward *et al*., 2001; Kliewer *et al*., 1999; Tremblay *et al*., 2001), but in most cases, the ligands and functions of nuclear orphan receptors are still to be elucidated.

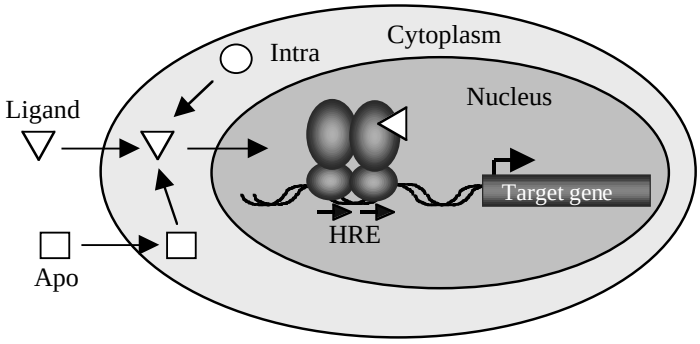

Fig. 1. Ligand activation of nuclear receptors. NR ligands act in at least three different ways: in the classical endocrine fashion, where the ligand (e.g. thyroid hormone) is synthesized by a specialized gland and carried to the target cell by the blood (Ligand); the ligand (e.g. retinoic acid) is synthesized in the cell from an apo-form generated elsewhere (Apo); or the ligand (e.g. fatty acids) is synthesized in the target cell and not secreted (Intra). Most NRs encounter their ligands directly in the nucleus, where they bind specific DNA elements (hormone response elements, HREs), and activate transcription of target genes. (Modified from Mangelsdorf and Evans, 1995)

Nuclear receptors share a common structure

With only a few exceptions, all nuclear receptors share a common structure of functionally separable domains, including an amino-terminal domain (NTD), a central DNA-binding domain (DBD), and a carboxy-terminal (C-terminal) ligand-binding domain (LBD) (Fig. 2A). The highly conserved DBD is responsible for recognizing and binding to specific DNA sequences in the promoters of target genes, and is also important for dimerization between receptors. The LBD, besides binding ligand, plays an essential role in dimerization and ligand-dependent transactivation. A C-terminal region of the LBD harbours a ligand-dependent activation function (AF-2). The NTD is less conserved between different NRs, both in amino acid (aa) composition and length, and contains a ligand-independent activation function (AF-1), shown to play important roles in the basal transcriptional activity of some receptors.

DNA binding and dimerization

The hallmark of the NR family is the well conserved DNA-binding region containing eight cystein residues, tetrahedrally coordinating two zinc ions and forming two zinc finger motifs (Fig. 2B). These recognize and bind specific DNA target sequences, termed hormone response elements (HREs), arranged as one or two half sites of the consensus nucleotide sequence AGGTCA. Two half sites can be arranged as direct- (DR), inverted- (IR), or everted repeats (ER) (Fig. 3). Based on ligand binding, dimerization and DNA-binding, the NRs can be divided

into four classes: (1) the steroid receptors, which are ligand-induced and bind IR HREs as homodimers; (2) ligand-induced receptors, including RAR as well as receptors for thyroid hormone (TR) and vitamin D_3 (VDR), that bind DR elements spaced by one to five nucleotides as heterodimers with the retinoid receptor RXR; (3) non-steroid receptors that bind DRs as homodimers; (4) receptors which bind extended core sites as monomers (see Figure 3). The latter two classes are mostly comprised of the orphan receptors (Mangelsdorf *et al*., 1995a).

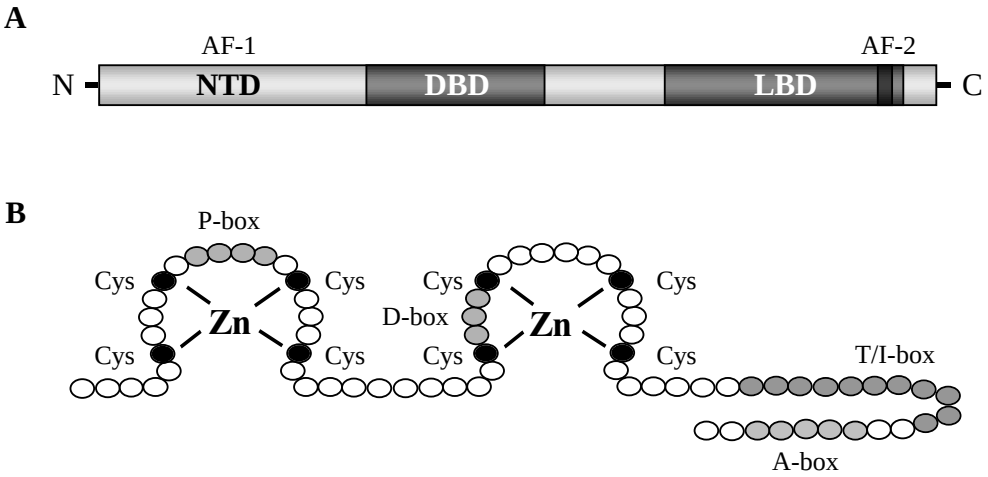

Fig. 2. Nuclear receptor structure. (**A**) NRs consist of defined domains, with variable degrees of conservation within the NR superfamily. The NTD, which is the most variable, has a ligand-independent transactivation function (AF-1) shown to be important in basal transcription by some receptors. The DBD and the LBD are more conserved and are responsible for DNA- and ligand-binding, respectively. A ligand-dependent transactivation function (AF-2) is localized to the LBD (see text for details). NTD, amino-terminal domain; DBD, DNA binding domain; LBD, ligand binding domain; AF-1, activation function 1; AF-2; activation function 2. (**B**) The DBD contains two zinc finger motifs, each consisting of four cystein residues (black) coordinating one zinc atom. Amino acid residues of particular importance for correct DNA-binding and dimerization (gray) have been mapped to subdomains ("boxes") within the DBD (see text for details). Cys, cystein; Zn, zinc.

The DNA-binding domain

Structural and functional analysis of the DBD has identified certain regions of crucial importance for DNA binding and dimerization (Fig. 2B). A strech of amino acids in the C-terminus of the first zinc finger, the P-box, directly contacts the major groove of DNA and determines the sequence specificity of the receptor. Residues in the N-terminus of the second zinc finger, the D-box, are involved in receptor dimerization and correct nucleotide spacing between two half sites (Kurokawa *et al*., 1993; Perlmann *et al*., 1993; Umesono *et al*., 1991). Receptors which are able

to bind DNA as monomers have an additional C-terminal extension, the A-box, which contacts DNA residues at the 5′-end of the core recognition site (Wilson *et al*., 1992). RXR has the ability to bind DNA both as a homodimer and as a heterodimer with several other NRs (discussed below). Critical residues for this function are contained within the T-box in the DBD (Lee *et al*., 1993), but also in a region within the LBD called the I-box (Perlmann *et al*., 1996).

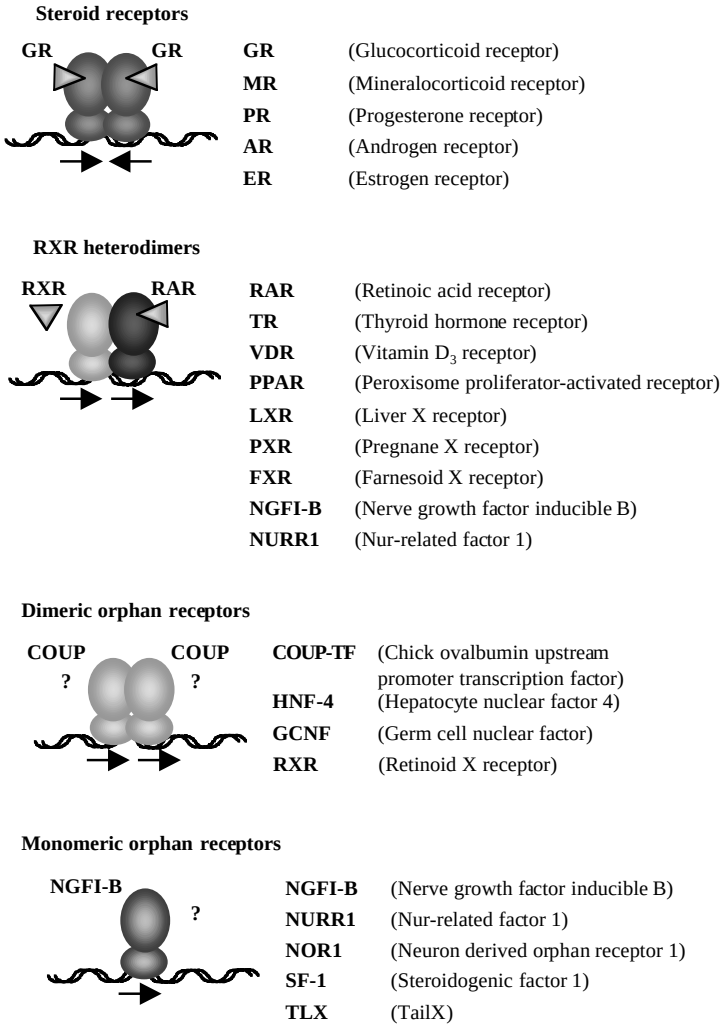

Fig. 3. The different classes of NRs. *Steroid receptors* bind DNA as homodimers, and recognize inverted repeats spaced by three nucleotides. *RXR heterodimers* recognize direct repeat response elements (DRs) spaced by a variable number of nucleotides, ranging from one to five. Depending on the spacing, different heterodimers will be recruited to DNA. *Dimeric orphan receptors* bind DRs as homodimers. The members of this group are currently lacking identified ligands. Note that RXR is found in this group as it has the capability of forming homodimers on DNA. *Monomeric orphan receptors* bind extended DNA half sites, and also lack identified ligands. See text for further details.

The LBD and ligand-dependent transactivation

The crystal structure of the C-terminal ligand-binding domain of several NRs has been solved (reviewed in Egea *et al*., 2000a). The results reveal a common fold, consisting of 12 α-helices (H1 to H12) and one β-turn, arranged in a three-layered antiparallel "sandwich" (Fig. 4A). The center of this sandwich contains the ligand-binding pocket (LBP), lined mostly by hydrophobic and polar residues, some of which are critical for stabilizing the LBD structure (Moras and Gronemeyer, 1998; Wurtz *et al*., 1996). As mentioned, the LBD contains the ligand-dependent activation function AF-2, and residues critical for its function have been mapped to helix 12 (the AF-2 core) (Barettino *et al*., 1994; Danielian *et al*., 1992; Durand *et al*., 1994; Tone *et al*., 1994). Information provided by the three-dimensional structures of various nuclear receptor LBDs, including unliganded (apo-) and liganded (holo-) RXRα (Bourguet *et al*., 1995; Egea *et al*., 2000b), holo-RARγ (Renaud *et al*., 1995), holo-TRα (Wagner *et al*., 1995), and agonist- and antagonist-bound estrogen receptor β (ERβ) (Brzozowski *et al*., 1997; Pike *et al*., 1999; Shiau *et al*., 1998), has provided a model that accounts for the structural transitions involved in ligand-activation of NRs (reviewed in Egea *et al*., 2000a; Moras and Gronemeyer, 1998). In this so called "mouse-trap" model, the initial interaction between ligand and LBD moves helix 11 (H11) out of the ligand cavity, thereby allowing ligand to enter in an induced fit mechanism. In the unliganded receptor, H11 stabilizes the LBD by partially filling the LBP with hydrophobic residues, thereby making the apo-receptor less accessible to ligand. The ligand-induced transition of H11 is followed by a repositioning of helices 3 and 4, which together with H11 now move to form a hydrophobic cleft on the surface of the LBD. In addition, the transition allows the pocket to change shape to better match that of the ligand, and to seal off the cavity.

The most striking conformational change involves H12, harbouring the AF-2 core. In the absence of ligand, H12 protrudes from the LBD and is exposed to solvent, whereas in the holo-receptor, it rotates and folds back towards the LBD, thereby compacting its structure. In its final position, H12 seals the pocket, trapping the ligand inside (compare Fig. 4A and B). In addition, in this new conformation, H12 has a major role in positioning coactivator proteins in the hydrophobic cleft formed by residues on helices 3, 4 and 11, a process important in transcriptional activation (discussed below). Interestingly, the peroxisome proliferator-activated receptors (PPARs) seem to be unique in that the H12 transition observed in other receptors upon ligand binding, is much more subtle. The crystal structures of PPARδ and PPARγ (Nolte *et al*.,

1998; Xu *et al*., 1999a) show that H12 is packed against the LBD both in the apo- and holo-forms of these receptors, and that only minor structural changes occur when ligand is bound. Additionally, the LBP is much larger in the PPARs as compared to other NRs, allowing ligand entry without great difficulty. This property explains the more relaxed ligand specificity found for the PPARs.

In addition to acting as ligand-dependent transcription factors, some NRs also become activated or inactivated in the absence of ligand, e.g. by phosphorylation of the receptor itself or of its coregulatory proteins. This is especially the case for the steroid hormone receptors (Weigel and Zhang, 1998), but also the activity of retinoid receptors can be modulated through phosphorylation, both in the presence and absence of ligand (see e.g. Huggenvik *et al*., 1993; Taneja *et al*., 1997). Ligand-independent modulation of orphan receptors is particularly interesting. Since it is still unknown whether this type of receptors bind ligands *in vivo*, phosphorylation might be their 'true' mode of regulation. Inhibition of transactivation and DNA-binding by the orphan receptor nerve growth factor inducible receptor-B (NGFI-B) is caused by phosphorylation of residues within the DBD both *in vitro* and *in vivo* (Hirata *et al*., 1993; Pekarsky *et al*., 2001), and induces its nuclear export (Katagiri *et al*., 2000). In contrast, phosphorylation of the NTD of steroidogenic receptor-1 (SF-1), increases its transcriptional activity by promoting coactivator recruitment (Hammer *et al*., 1999).

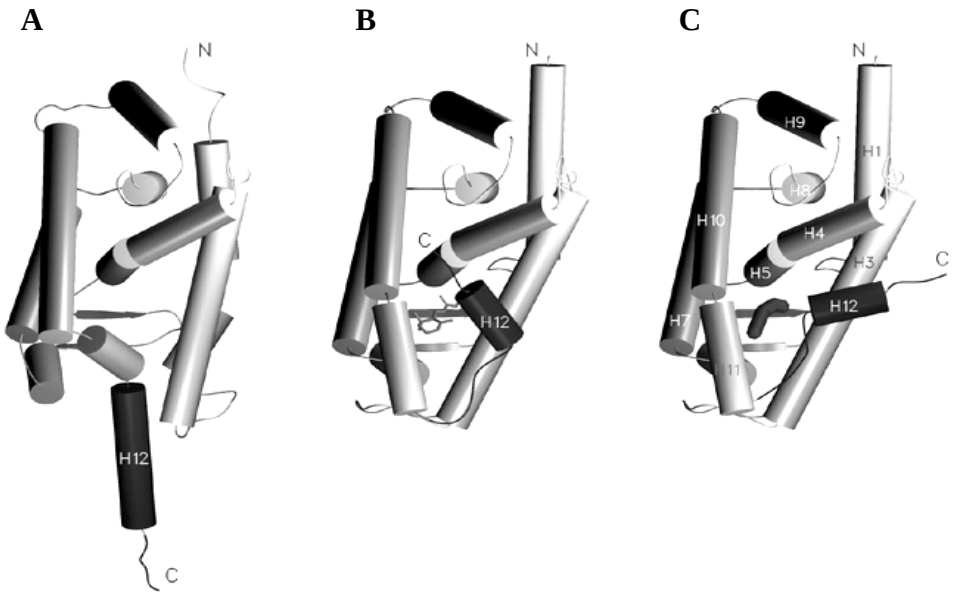

Fig. 4. Schematic drawing of the LBD structure of apo-RARα (**A**), holo-RARγ bound to all-*trans* retinoic acid (atRA) (**B**), and antagonist-bound ERα (**C**). atRA is shown in "stick" form in the center of the LBD in (B), and the ER antagonist, raloxifene, is depicted as a bent cylinder in (C). Note the different positions of helix 12 (shown in black) in each situation. The α-helices of the LBD are represented as numbered rods. (Modified from Moras and Gronemeyer, 1998)

Coactivators

Interactions between nuclear receptors and the basal transcriptional machinery have been described (see e.g. Baniahmad *et al*., 1993; Jacq *et al*., 1994; McEwan and Gustafsson, 1997; Sadovsky *et al*., 1995; Schulman *et al*., 1995). However, these interactions either did not require the nuclear receptor AF-2, or were not ligand-dependent, suggesting that other factors involved in NR signaling were required for the ligand-dependent transactivation (Jacq *et al*., 1994; Sadovsky *et al*., 1995). An early hint that NRs interact with additional factors came from so-called squelching experiments, where *in vitro* overexpression of NR transactivation domains interfered specifically with signaling by other coexpressed NRs (Meyer *et al*., 1989). The first such coactivators to be described interacted with ER in the presence of ligand (Cavailles *et al*., 1994). One such protein, RIP160 (for receptor interacting protein 160), later shown to be identical to SRC-1 (steroid receptor coactivator-1) (Onate *et al*., 1995), interacted with several NRs in a hormone-dependent manner. Since then, an ever-increasing number of coactivators have been cloned and characterized (reviewed in Glass *et al*., 1997; Glass and Rosenfeld, 2000; Xu *et al*., 1999b). Some of these factors are specific for one or a few receptors, whereas others act as general NR coactivators. In addition, certain NR coactivators are common to other signaling pathways, e.g. CBP (CREB binding protein) and its homolog p300, involved in activation via CREB, AP-1, c-myb and Myo D (reviewed in Janknecht and Hunter, 1996).

Coactivators interact with NRs via one or several leucine-rich α-helices, also known as NR boxes, with the consensus sequence LxxLL (where L corresponds to leucine and x is any aa residue) (Ding *et al*., 1998; Heery *et al*., 1997; Le Douarin *et al*., 1996; Torchia *et al*., 1997; Voegel *et al*., 1998). Structural and functional studies of holo-PPARγ bound to an SRC-1 peptide containing two such LxxLL motifs, and of holo-ER and -TR with a peptide containing one LxxLL from the coactivator GRIP-1 (glucocorticoid receptor interacting protein-1) (Darimont *et al*., 1998; Feng *et al*., 1998; Nolte *et al*., 1998; Shiau *et al*., 1998), indicate that the coactivator LxxLL helix is accomodated along the hydrophobic cleft on the surface of the receptor LBD that forms upon ligand-binding (see above). Two strictly conserved aa residues on the receptor, a glutamic acid in the AF-2 core helix and a lysine on helix 3, are critical for correct positioning of the coactivator NR box. These charged amino acids form a "charge clamp" that correctly places the coactivator LxxLL motif on the LBD, leading to transcriptional activation of the receptor. Receptor and ligand-dependent specificity of the coactivator is accomplished both

by the LxxLL motif itself, but also by flanking residues (Darimont *et al*., 1998; Mak *et al*., 1999; McInerney *et al*., 1998; Schulman *et al*., 1998; Shao *et al*., 2000; Zhou *et al*., 1998). In the crystal structures of ER and RAR bound to antagonists, the AF-2 helix of the receptor is not repositioned correctly as in the ligand-bound receptors (compare Fig. 4B and 4C). Instead, it is translocated to overlap the coactivator interaction site, thereby preventing coactivator binding (Bourguet *et al*., 2000; Brzozowski *et al*., 1997; Pike *et al*., 1999; Shiau *et al*., 1998). This in turn would facilitate the recruitment of another group of regulatory factors, corepressors, explaining the molecular mechanism behind antagonistic repression of NRs (see below).

The best characterized coactivators belong to one of three classes: the p160 family, inlcuding SRC-1/N-CoA1, GRIP-1/TIF2, and ACTR/pCIP/RAC3/AIB-1; the homologous CBP and p300 coactivators; and the recently isolated TRAP/DRIP complexes (see Xu *et al*., 1999b for abbreviations and references). Members of the p160 family, together with CBP/p300 and p/CAF (CBP associated factor) show intrinsic histone acetyl transferase (HAT) activity (Bannister and Kouzarides, 1996; Chen *et al*., 1997; Ogryzko *et al*., 1996; Spencer *et al*., 1997; Yang *et al*., 1996), suggesting that coactivators may play direct roles in chromatin remodeling at promoters by acetylation of histone proteins (Montminy, 1997). The TRAP/DRIP complexes, isolated by their ability to interact with ligand-bound TR and VDR, respectively, are large multi-protein complexes that share common subunits (Fondell *et al*., 1996; Rachez *et al*., 1999). Several TRAP/DRIP subunits seem to be homologous to components of the ARC and Mediator complexes, proteins that associate with RNA polymerase II (Di Croce *et al*., 1999), suggesting an important role for this class of coactivators as bridging molecules between DNA-bound NRs and the basal transcription machinery (Freedman, 1999).

Corepressors

Certain NRs, such as RAR and TR, repress basal transcription in the absence of ligand by binding the promoters of target genes, a process known as silencing (Perlmann and Vennström, 1995). The molecular mechanisms behind this phenomenon involves two related corepressor proteins, N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptor), which both interact with RAR and TR in a ligand-independent manner (Chen and Evans, 1995; Hörlein *et al*., 1995; Kurokawa *et al*., 1995). Both proteins share a common structure, with three N-terminal repressor domains (RD1-3) and two C-terminal

receptor interaction domains (ID1 and 2). Initially, it was shown that residues in the N-terminal helix 1 of the NR LBD were required for corepressor interaction (Hörlein *et al*., 1995). However, structural analysis of the LBD has excluded the possibility of a direct interaction, and instead suggests that this region of the receptor plays a more global structural role within the LBD. It has instead been shown that corepressors bind a region on the surface of the receptor LBD that overlaps the coactivator interacting site. The ID motifs of N-CoR and SMRT contain α-helical structures similar in sequence to the LxxLL helix of coactivators, with the consensus LxxxIxxxI/L (where L is leucine, I is isoleucine and x is any aa residue) (Hu and Lazar, 1999; Nagy *et al*., 1999; Perissi *et al*., 1999). Mutagenesis of aa residues within this so-called CoRNR box, suggests that they are critical for corepressor interaction with the NR LBD, with additional residues surrounding the CoRNR box strengthening the interaction (Perissi *et al*., 1999). Thus, due to sequence similarities, corepressors are able to bind the same region on the NR LBD as coactivators. In the absence of ligand, a correct coactivator interaction site cannot be formed since the receptor AF-2 core is positioned away from the LBD. Instead, corepressors bind by virtue of their extended CoRNR motifs, thereby masking the coactivator site and repressing transcription. Structural transitions in the LBD that occur upon ligand binding move the corepressor and allows AF-2 helix repositioning, which further displaces the corepressor. The extended corepressor motif no longer fits in the cavity due to steric hindrance by the AF-2 helix, and instead the coactivator gains entry to the site. Interestingly, the structure of the ER LBD in the presence of antagonist supports this model, and proposes a molecular explanation to antagonist mediated repression. Binding of the antagonist distorts the LBD, thereby preventing a correct coactivator interaction site from forming and instead allowing binding of corepressor (Brzozowski *et al*., 1997).

Analogous to coactivators forming large multi-protein complexes, N-CoR and SMRT also interact with other proteins to repress transcription. Histone deacetylases (HDACs 1 and 2) are bridged to unliganded NRs via N-CoR/SMRT and mSin3 corepressors, thereby mediating silencing (Alland *et al*., 1997; Heinzel *et al*., 1997; Nagy *et al*., 1997). Deacetylation of core histones by HDACs is recognized as a mechanism for keeping chromosome domains transcriptionally silent, and would explain how NRs mediate repression (Pazin and Kadonaga, 1997).

Transcriptional initiation – it´s a matter of complexes

The development of novel techniques for studying protein interactions at promoters of genes, has yielded exciting new insights explaining the steps leading from repression to transcriptional initiation. Such novel techniques include (1) real time imaging in living cells using FRET (fluorescence resonance energy transfer), (2) *in vivo* and *in vitro* chromatin assembly studies, and (3) ChIP (chromatin immunoprecipitation) assays (reviewed in Lee and Lee Kraus, 2001). For example, a recent study using the FRET technique to study GR-DNA interactions, suggests that hormone-bound receptors continuously cycle on and off target promoters, transiently interacting with response elements on DNA, recruiting cofactors to initiate transcription, and subsequently dissociating from DNA again (McNally *et al*., 2000). Using a chromatin-based transcription system *in vitro*, Dilworth and co-workers could show that RAR/RXR-mediated transcription requires the sequential action of both ATP-driven remodeling factors (the SWI/SNF complex) and HAT activity (Dilworth *et al*., 2000). In their model, the SWI/SNF complex acts in an initial step to unwind DNA at the promoter, thus aiding tight binding of the NR, a process that is ligand-independent. Exposure to hormone then leads to recruitment of coactivators with HAT activity, e.g. SRC-1, CBP/p300 and p/CAF. Histones are acetylated, not only at the NR-bound response element, but also further away on the DNA template, to allow better access of proteins to promoter DNA. Some indications exist that also non-histone proteins are acetylated, which may both enhance transcriptional initiation, but also attenuate the signal once transcription is to be turned off (Pazin and Kadonaga, 1997). Finally, binding of TRAP/DRIP protein complexes will attract the basal transcription machinery including RNA Polymerase II (RNA Pol II) to the promoter, leading to transcriptional initiation.

A very accurate assay to study *in vivo* interactions between proteins at active promoters, is the newly developed ChIP assay, where formaldehyde is used to crosslink protein-DNA complexes from cells treated with hormone, followed by immunoprecipitation with specific antibodies (reviewed in Kuo and Allis, 1999). This methodology has been used to study hormone-activated transcription mediated by ERα (Shang *et al*., 2000). The results verify the cyclic mode of assembly and release of the transcriptional machinery at promoters. However, in contrast to the results obtained by Dilworth and colleagues (see above), the recruitment of p160 and TRAP/DRIP coactivators by ligand-activated NRs did not seem sequential. Instead they

appear to bind active receptor in a single step. HAT activities of SRC-1 and p300 modifies DNA through histone acetylation, allowing entry of RNA Pol II via interactions with the TRAP/DRIP complex. The C-terminal domain of RNA Pol II is then phosphorylated, to allow transcriptional initiation. Interestingly, CBP and p/CAF, which also possess HAT activity, were not recruited until after RNA Pol II was bound. This suggests that they might be important actors in the subsequent steps, perhaps by acetylating the p160 coactivators leading to RNA Pol II release. The last step is the release of hormone-bound receptor, followed by CBP and p/CAF, after which the cycle is repeated. The different steps in transcriptional activation by NRs are summarized in Fig. 5.

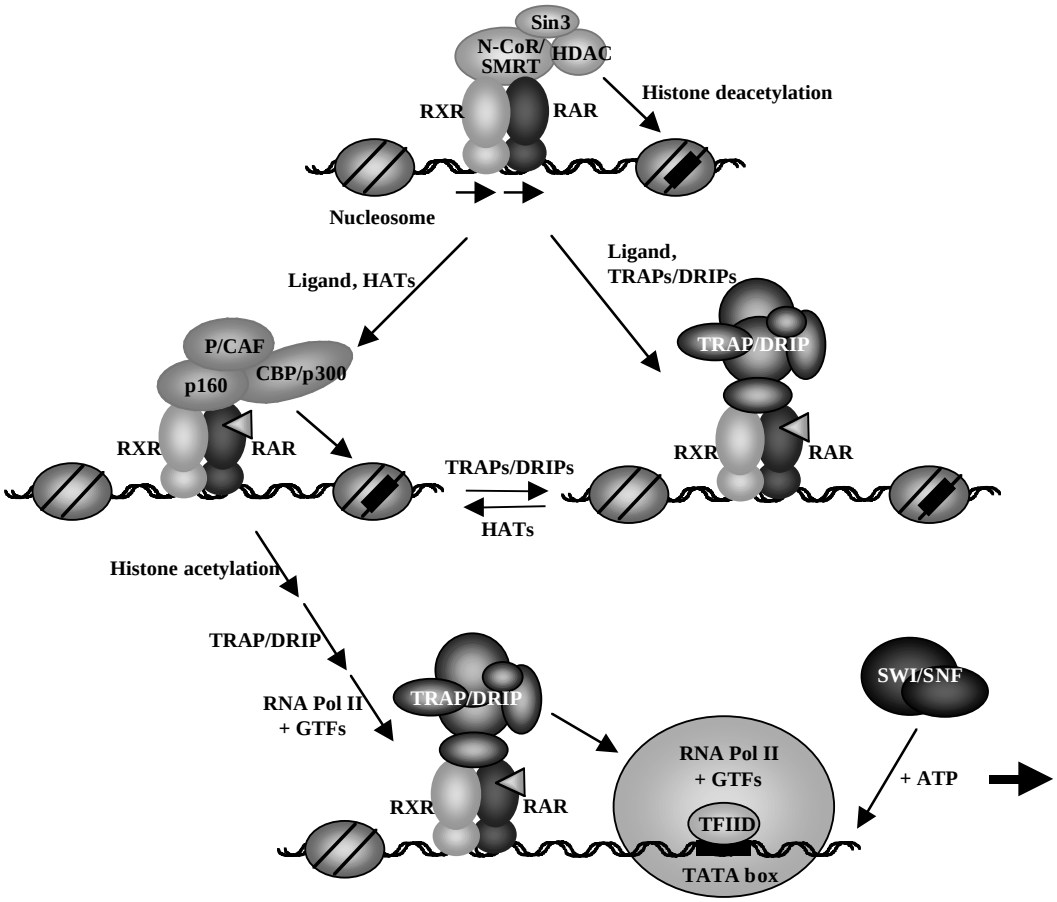

Fig. 5. The different steps and proteins involved in NR transcriptional activation. Unliganded and DNA-bound NRs (here exemplified as an RAR-RXR heterodimer) inhibit transcriptional activation by recruiting corepressors with histone deacetylase (HDAC) activity, keeping promoter DNA packed into histones, i.e. in a silent form. Upon exposure to ligand, corepressors are released and coactivators of the p160 family and CBP/p300 are recruited, either before or together with the TRAP/DRIP complex. The ATP-dependent activity of the SWI/SNF complex, initially acts to unwind DNA at the promoter. Histone acetylation by coactivators allows stronger interaction between the NRs and DNA, ultimately leading to recruitment of RNA Pol II and other accessory factors. These proteins recognize and bind DNA sequences at the TATA box spanning the transcriptional initiation site. Finally, RNA Pol II is released from the promoter and initiates gene transcription. See text for abbreviations and further details. (Modified from Ito and Roeder, 2001)

NRs from an evolutionary perspective

A matter of considerable debate within the field of nuclear receptors has been whether all orphan receptors can be expected to have a natural ligand. As the function of many of these proteins is still unclear, discovering a potential ligand can be helpful in trying to uncover their role *in vivo*. Based upon the homologies within the superfamily, NRs have been divided into six subfamilies (Laudet, 1997). The ability to bind ligand and the identity of the ligand in some cases seem unrelated to which subfamily the respective receptors belong. For example, RAR and TR, which bind two unrelated ligands, are nonetheless more related in sequence than for example RAR and RXR, which both bind retinoic acid isomers, suggesting that ligand-binding ability is independent from the evolutionary origin. The fact that orphan receptors are present in all subfamilies, whereas liganded receptors are not, further suggests that the ancestral receptor did not have a ligand, but was activated by other means, e.g. phosphorylation. Ligand-binding would then have been independently acquired by some receptors during evolution (Escriva *et al*., 1997).

No NR homologs have been found in unicellular organisms such as yeast, making NRs unique to metazoans. In addition, there seems to have been two waves of gene duplications that lead to the present array of receptors: one initial wave prior to the split between invertebrates and vertebrates, giving rise to the various groups of receptors from one ancestral gene; and a second wave unique to vertebrates, resulting in the diversification within each group of modern vertebrate receptors. As a result, most groups in invertebrates such as flies and worms, only have one gene, whereas the corresponding group in higher vertebrates contains several, paralogous genes, e.g. RXRα, β and γ.

The role of RXR during evolution is intriguing. Its function as a heterodimerization partner for other receptors seems to have been conserved, as its fly homolgue, ultraspiracle (USP), heterodimerizes with the ecdysone receptor (EcR). EcR has a ligand, the hormone ecdysone, suggesting that most, if not all, RXR heterodimer partners in vertebrates should have a ligand. Indeed, receptors like RAR, TR, VDR and PPAR all bind ligands. In addition, ligands for the vertebrate homologs of EcR, including farnesoid X receptor (FXR) and liver X receptors α and β (LXRα and β) have recently been discovered (reviewed in Chawla *et al*., 2000). On the other hand, USP does not bind the vertebrate RXR ligand 9cRA, suggesting that this ability was acquired later. In fact, proof of this can be found in the jellyfish *Tripedalia cytosphora*, as its

RXR homologue, jRXR, evolutionary more related to vertebrate RXR than USP, does bind 9cRA (Kostrouch *et al*., 1998).

RETINOIDS AND RETINOID RECEPTORS

La Dolce Vita(min A): A vital vitamin

The first description of beriberi, a disease involving muscular paralysis and weight loss, dates back to about 2,600 B.C. However, it was not until the end of the 19th century that the Japanese naval doctor Takadi discovered that the disease, which was more common among Japanese than English sailors, was caused by dietary deficiencies (Nationalencyklopedin, 1996). The dietary factor missing in the diet of the Japanese sailors was in 1915 found to be an essential component of milk, and later termed vitamin A (also known as retinol; ROL) (McCollum, 1967). Since these initial experiments, much has been learned about the essential roles played by vitamin A and its metabolites, the retinoids, in embryonic development, differentiation and maintenance of homeostasis in the adult organism (Gudas *et al*., 1994; Hofmann and Eichele, 1994). Adult animals suffering from vitamin A-deficiency (VAD) display a number of abnormalities, including impaired vision, fertility, immune response and epithelial differentiation. Furthermore, vitamin A deficient offspring have ocular, cranofacial, limb, heart, respiratory, circulatory and urogenital malformations, further underscoring the importance of vitamin A during gestation (reviewed in Maden, 2000; Morriss-Kay and Sokolova, 1996) (see also Dickman *et al*., 1997; Maden *et al*., 1996; White *et al*., 1998). Similar malformations occur upon exposure of pregnant mothers to excess RA, including craniofacial and CNS defects, as well as limb and genitourinary malformations (Hofmann and Eichele, 1994; Ross *et al*., 2000). These abnormalities are thought to arise due to dysregulation of Hox gene expression, genes which are important in correct patterning of the embryo (see below).

RA synthesis and breakdown

As neither embryonal nor adult cells have the capacity to synthesize vitamin A *de novo*, this substance has to be supplied through the diet. Excess dietary retinol is stored as retinylesters mainly in the liver, which when needed are hydrolyzed to retinol and transported to the target tissue via the bloodstream bound to retinol binding protein (RBP). In the target cell, retinol is converted to retinoic acid in two enzymatically controlled steps (Fig. 6). First, all-*trans* retinol (atROL) is converted to all-*trans* retinal (atRAL), a reaction catalyzed by one of various ROL-dehydrogenases (ROLDH). In the following step, atRAL is converted into atRA by a RAL-

dehydrogenase, e.g. RALDH1, RALDH2 or RALDH3 (reviewed in Duester, 2001; Napoli, 1996). 9cRA can then be formed either by spontaneous conversion from the all-*trans* form, or via a recently suggested alternative route, where atROL is isomerized to 9cROL, subsequently generating 9cRA via 9cRAL in a pathway independent of all-*trans* retinoid synthesis (Romert *et al*., 1998; Tryggvason *et al*., 2001). Although atRA and 9cRA are the two best characterized retinoids, other bioactive forms also exist *in vivo*, e.g. 3,4-didehydroRA and 4-oxo-RA (Pijnappel *et al*., 1993; Thaller and Eichele, 1990). 4-oxo-RA is one of several breakdown products of atRA in catabolic reactions catalyzed by a family of cytochrome P450 enzymes, the CYP26 family, including for example P450RAI-1 (White *et al*., 1997), P450RAI-2 (White *et al*., 2000), and the related $P450_{RA}$ (Fujii *et al*., 1997).

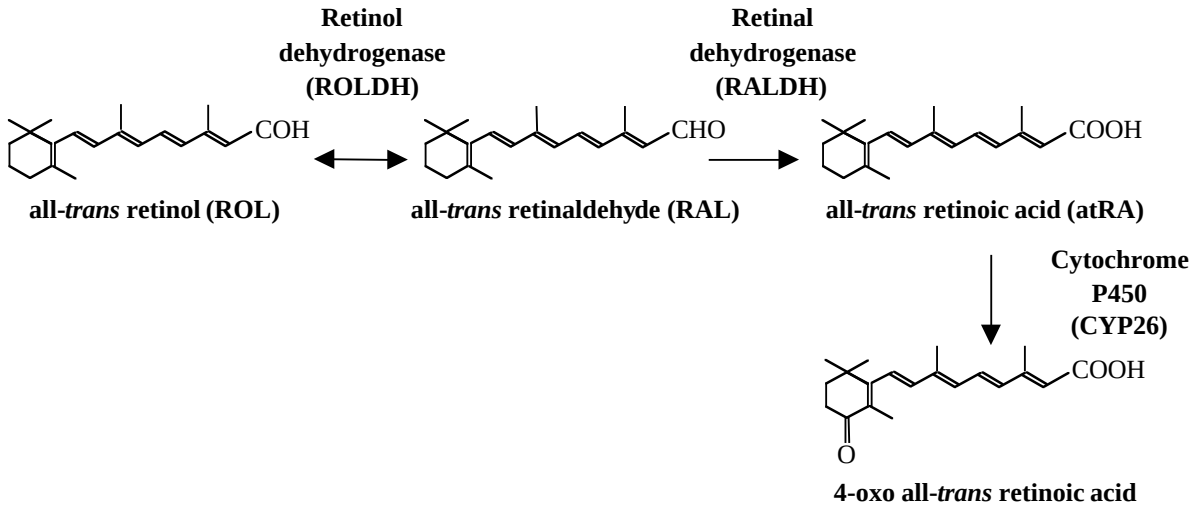

Fig. 6. Metabolic steps leading from retinol to retinoic acid and one of its oxidation products. The initial conversion of retinol to retinal is rate-limiting and the only reversible step in this process. atRA is either isomerized to 9cRA directly, or via an alternative route from 9cROL via 9cRAL (see text for details).

The expression patterns of three enzymes involved in the *in vivo* synthesis of RA, namely RALDH1, -2 and -3, have been studied in detail. As expected, expression is high in embryonal tissues known to be dependent on retinoids. RALDH1 expression is found in the developing eye, thymus and mesonephros (Haselbeck *et al*., 1999), whereas RALDH2 is expressed in the optical vesicles, somites, heart, spinal cord and kidney (Berggren *et al*., 1999; Haselbeck *et al*., 1999; Niederreither *et al*., 1997; Swindell *et al*., 1999; Zhao *et al*., 1996). RALDH3 expression is

confined to the developing ventral retina, otic vesicle and olfactory pit (Mic *et al*., 2000). In addition, adult tissues express RALDH1 in the epithelium of the eye, lung, liver, intestinal and reproductive tissues, while RALDH2 expression is restricted to the reproductive organs (Haselbeck *et al*., 1999).

$P450_{RA}$ seems to be the most abundantly expressed enzyme involved in RA degradation during embryonal development (Fujii *et al*., 1997; Swindell *et al*., 1999). In both mouse and chick, expression is restricted to the anterior- and posterior most structures of the embryo, including the presumptive head and tailbud regions. At later stages in development, expression is detected in neural crest cells, limb buds, otic vesicle and eye. Intriguingly, a comparison of the expression patterns of RALDH2 and $P450_{RA}$ in chick embryos, suggests that both enzymes are present in complementary and non-overlapping domains (Swindell *et al*., 1999). Areas differing in their RA levels can thereby be created via controlled expression of these enzymes, perhaps explaining how RA gradients are established during embryogenesis (see below). Additionally, areas that express RALDH2 (and therefore presumably synthesize RA), resemble those where activation of RA-sensitive reporters can be seen in transgenic mice (e.g. Colbert *et al*., 1993; Rossant *et al*., 1991; Solomin *et al*., 1998).

Hox genes and pattern formation

The formation of an organism from a fertilized egg involves the generation of a large number of cells, which during the course of embryonal development are specified to form different organs and cell types. Cells at a particular position within an embryo or embryonic tissue need to know their exact location in order to correctly form the unique structures or patterns within the animal body. Morphogens, molecules capable of forming gradients in the embryo, are able to dictate such spatial information, and it has been shown that retinoids, in particular retinoic acid, act as morphogens during embryonal development (Brockes, 1989; Eichele, 1989). They do so by regulating the expression of a family of homeobox-containing transcription factors, the *Hox* proteins. These proteins play crucial roles in informing cells of their position along the head-to-tail, or anterio-posterior (AP), axis, thereby establishing a so-called *Hox* code (reviewed in Hofmann and Eichele, 1994; Ross *et al*., 2000). Vertebrate *Hox* genes are arranged in four clusters (*Hox* A to D) of about nine genes each, all sharing the same transcriptional orientation. Expression in the developing hindbrain and spinal cord is both temporally and positionally

controlled in a head-to-tail order. This results in 3' *Hox* genes being expressed earlier and in more anterior areas, whereas 5' *Hox* genes are expressed at later stages and in more posterior regions of the embryo. The overlapping expression domains of these genes along the AP axis, is thought to specify the positional identity of cells along this axis, thereby enabling them to adopt a correct fate. Several "anterior" 3' *Hox* genes contain RA response elements (RAREs) in their promoters, indicating that retinoids are involved in regulating their expression. Indeed, embryos that develop in absence of RA or in presence of excess RA, display altered *Hox* gene expression, leading to so called homeotic transformations (Conlon and Rossant, 1992; Maden *et al*., 1996; Ross *et al*., 2000).

Measurements of endogenous retinoid levels (Chen *et al*., 1992; Maden *et al*., 1998; McCaffery and Drager, 1994b), analysis of expression patterns of RALDH2 and $P450_{RA}$ (Berggren *et al*., 1999; Niederreither *et al*., 1997; Swindell *et al*., 1999) and phenotypes in animals lacking these enzymes (Niederreither *et al*., 1999; Niederreither *et al*., 2000; Sakai *et al*., 2001), suggests the following possible scenario in developing embryos:

(i) During the initial stages of embryo development, RA synthesis is limited to cells in the anterior parts of the embryo, specifically to Hensen´s node and the primitive streak (see Hofmann and Eichele, 1994), with an apparent lack of RA in the anterior- and posterior-most structures. This initial low, anterior RA synthesis results in only 3' *Hox* genes being activated in this region, as they are most RA-sensitive. As the node progresses posteriorly, RA levels increase, leading to 5' *Hox* gene activation in posterior parts of the embryo. RALDH2 expression correlates to the region of RA synthesis, while $P450_{RA}$ is expressed in regions lacking RA, consistent with their roles in RA synthesis and degradation, respectively.

(ii) At later stages of development, head and tail regions still produce low levels of RA, while the posterior parts of the forming hindbrain, and the entire spinal cord excluding the most posterior part, contain high levels of RA. Within the hindbrain, which is particularly sensitive to changes in RA levels during development, RA synthesized by RALDH2 in posterior parts, diffuses anteriorly towards the midbrain. $P450_{RA}$, which is synthesized in fore- and midbrain, will degrade RA, thereby creating a gradient essential for correct *Hox* gene expression within the hindbrain. Disturbances in this gradient leads to posteriorization (excess RA) or anteriorization (shortage of RA) of structures in this region, in part due to misexpression of *Hox* genes.

(iii) In addition, hot spots of retinoid synthesis within the spinal cord are located to brachial and lumbar levels, where e.g. fore- and hindlimbs will develop, suggesting that retinoids are involved in specification of these and other structures. Indeed, limb-innervating motor neurons extending from the spinal cord at these levels show a prominent expression of RALDH2.

Retinoids in adult physiology

Apart from the early realization that retinoids are important in vision (Wald, 1968), little is known about the roles played by retinoids and their receptors in adult physiology. However, the roles played by retinoids in regeneration of auditory cells in the ear have been studied (Lefebvre *et al*., 1993). Additionally, RA has been shown to induce regeneration of lung alveoli in rats (Massaro and Massaro, 1997). Furthermore, locomotion and dopamine signaling is impaired in mice lacking RARβ and either RXRβ or RXRγ (Krezel *et al*., 1998), and compound RARβ/RXRγ -/- mice suffer from impaired memory formation (Chiang *et al*., 1998), an effect that has been corroborated in VAD mice (Misner *et al*., 2001). Recently, proof of localized RA synthesis in the adult brain was presented. A subset of neurons in the brain of adult songbirds were shown to locally synthesizes RA, implicating retinoids as essential for normal song maturation in juvenile birds (Denisenko-Nehrbass *et al*., 2000). Finally, some of the roles played by RXRα in adult physiology have been assessed. As embryonic ablation of RXRα is lethal (see below), various studies have employed conditional knockout strategies to selectively ablate the RXRα gene in adulthood. For example, mice lacking RXRα in skin keratinocytes develop alopecia (hair loss) due to destruction of hair follicles, and display keratinocyte hyperproliferation and aberrant terminal differentiation (Li *et al*., 2001; Li *et al*., 2000). Ablation of RXRα in liver hepatocytes leads to shortened lifespan and compromised regeneration of the liver cells, as well as alterations in pathways controlling fatty acid and cholesterol metabolism (Imai *et al*., 2001; Wan *et al*., 2000).

Retinoid binding proteins

Within cells, cellular retinol binding proteins I and II (CRBP-I and -II) and cellular retinoic acid binding proteins I and II (CRABP-I and -II), act as cytoplasmic carriers for ROL and RA, respectively. Based on expression studies, it was thought that CRBPs function in protecting

retinol from the cellular environment and presenting ROL to RA synthesizing enzymes. In contrast, CRABPs were suggested to be important in sequestering and promoting breakdown of excess RA in embryonic regions sensitive to the teratogenic effects of retinoids (see e.g. Dolle *et al*., 1990; Ruberte *et al*., 1991; Ruberte *et al*., 1993). However, animals carrying null mutations in both CRABP-I and -II are indistinguishable from wild-type littermates, suggesting that CRABPs are dispensable for normal embryonal development and adult physiology (Lampron *et al*., 1995). On the other hand, although not essential for embryonal development, adult mice lacking CRBP-I show decreased liver retinyl ester storage and predisposition to vitamin A deficiency (Ghyselinck *et al*., 1999), suggesting an important role for CBRP-I during vitamin A-limiting conditions. Interestingly, two CRBP-III genes have recently been cloned (Folli *et al*., 2001; Vogel *et al*., 2001), showing partially overlapping patterns of expression, and it will be interesting to see what roles these proteins might play *in vivo*.

Retinoid receptors

The cloning and characterization of the nuclear receptors that bind and transduce the retinoid signal represent landmarks in our understanding of retinoid physiology (Giguère *et al*., 1987; Mangelsdorf *et al*., 1990; Petkovich *et al*., 1987). As already mentioned, two families of retinoid receptors exist, the RARs and the RXRs, each present in three isotypes, α, β and γ (reviewed in Chambon, 1996; Kastner *et al*., 1995; Mangelsdorf *et al*., 1994). In addition, each RAR and RXR isotype exists in several isoforms (e.g. RARα1 and α2) due to differential promoter usage. RAR and RXR function as ligand-activated transcription factors belonging to the NR family. RAR binds both all-*trans* and 9-*cis* retinoic acids, whereas RXR only binds 9-cRA (Heyman *et al*., 1992; Levin *et al*., 1992). The receptors activate transcription by recognizing and binding consensus sequences known as RA response elements (RAREs) in the promoters of target genes. RAR binds DNA as a heterodimer with RXR, while RXR also has the ability to bind DNA as a homodimer. Additionally, RXR forms heterodimers with a number of other NRs, such as TR, VDR, PPAR, LXR and several orphan receptors, including NGFI-B and Nurr77-related receptor 1 (Nurr1) (Kliewer *et al*., 1992; Leid *et al*., 1992; Perlmann and Jansson, 1995; Willy *et al*., 1995; Yu *et al*., 1991), thereby coupling retinoid signaling to a multitude of other cellular signaling pathways.

RXR heterodimers and DNA binding

As already mentioned, most non-steroid and orphan receptors recognize the consensus sequence 5'-AGGTCA-3' in DNA, arranged as one or two copies. RXR and its heterodimer partners bind response elements arranged as two direct repeats, DRs, spaced by one to five nucleotides (the '1 to 5 rule'; Umesono *et al*., 1991) (see Fig. 7). Depending on the spacing between the two repeats, different RXR heterodimers will bind and activate transcription (reviewed in Rastinejad, 2001). In addition, RXR also has the ability to switch its polarity on DR elements, binding either the upstream or the downstream half-site (Kurokawa *et al*., 1994; Kurokawa *et al*., 1995; Perlmann *et al*., 1993). When heterodimerizing with most NRs, e.g. TR or VDR, RXR occupies the 5' upstream half-site. As a heterodimer with RAR, RXR can occupy either of the half-sites, depending on the type of DR motif: (1) on DR1 elements, RXR is downstream of RAR, and the receptor heterodimer acts as a repressor; (2) on DR2 or DR5 elements, RXR is bound upstream of RAR, and the complex functions as an activator of retinoid responsive genes. In addition, RXR forms ligand-activated homodimers on DR1 elements.

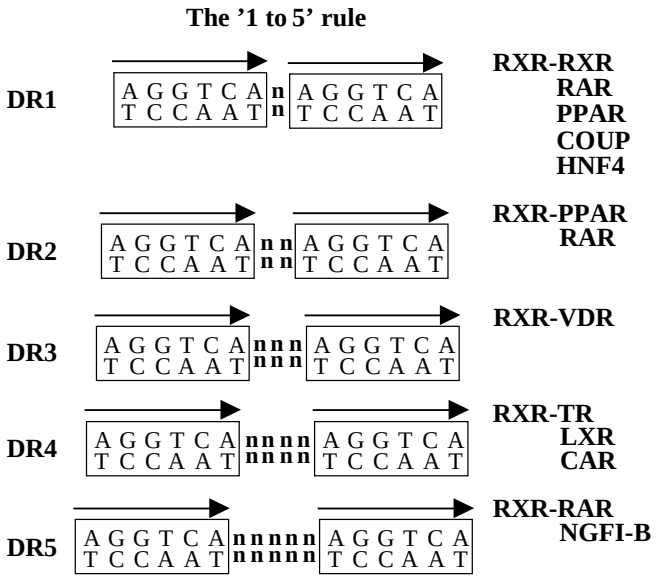

Fig. 7. The 1 to 5 rule of DNA-binding by RXR heterodimers. DNA half sites can be spaced by one to five nucleotides (n), and depending on the spacing, different RXR heterodimers will bind DNA. See text for receptor abbreviations. (Modified from Rastinejad, 2001)

Our understanding of RXR heterodimerization and DNA-binding has been aided by the structural determinations of heterodimers bound to DNA, including RXR homodimers bound to a DR1 motif (Zhao *et al*., 2000), RAR-RXR heterodimers bound to a DR1 element (Rastinejad *et al*., 2000), and RXR-TR heterodimers bound to their cognate DR4 motif (Rastinejad *et al*., 1995). It seems that both heterodimer partners cooperate to ensure correct binding specificity and affinity by making partner-specific protein contacts, that stabilizes the complex only on the correct DR motif. The unique C-terminal extension of each receptor partner only allows a certain nucleotide spacing between the receptors, thereby ensuring correct binding. Additionally, the structure of DNA-bound RXR seems more relaxed than that of its partners, perhaps explaining why RXR can form so many different heterodimeric complexes on DNA. Most likely, it is the partner which modulates binding affinity and selectivity for the various response elements. Importantly, the structures of RXR and RAR-RXR on DNA confirm that structural changes also occur in the DNA, further accommodating the receptor complex.

Retinoid signaling: permissive vs. non-permissive heterodimers

The RXR heterodimer partner not only influences the response element of choice, but also the capability of RXR to become activated by ligand. For example, PPAR-RXR heterodimers can be activated by both PPAR or RXR ligands and are said to be permissive to RXR activation. RAR-RXR heterodimers, on the other hand, are non-permissive in that they require an initial activation by RAR ligands in order to become responsive to RXR ligands (Forman *et al*., 1995; Kurokawa *et al*., 1994). Based on the structures of apo-RXR and holo-RAR (Bourguet *et al*., 1995; Renaud *et al*., 1995), Westin and coworkers have proposed a model to account for this allosteric inhibition of RXR by RAR (Westin *et al*., 1998). In the apo-RAR-RXR-DNA complex, the AF-2 core helix of the RXR LBD is interacting with the coactivator binding site on RAR. This interaction would prevent both closure of the ligand pocket of RXR even in the presence of its ligand, and also inhibit correct formation of a coactivator binding site on RXR. Upon exposure of the complex to RAR-specific ligand, coactivator is recruited and the RXR AF-2 core is displaced from the RAR coactivator binding site. This release induces the RXR AF-2 helix to swing back towards the RXR LBD, thereby relieving allosteric inhibition and allowing RXR to recruit ligand. Binding of ligand to RXR then promotes coactivator interaction with RXR. Importantly, the results also suggest that RAR and PPAR have different affinities for the RXR

AF-2 core, explaining why only RAR is inhibiting RXR activation. Permissive and non-permissive partners of RXR might thus differ in their affinity for the AF-2 helix of RXR, where non-permissive partners like RAR, TR and VDR, probably show a higher affinity than permissive partners like PPAR, LXR and NGFI-B.

However, several groups, including our own, have obtained results which are not easily explained by the 'Westin-model'. For example, we have shown that RAR-RXR heterodimers can become responsive to an RXR ligand even after addition of an RAR antagonist (Botling *et al*., 1997). Under such circumstances, coactivator recruitment by RAR in order to release the RXR AF-2 helix, cannot to be expected to occur since the antagonist represses transcription. Perhaps, any modification of the binding of the RXR AF-2 helix to its non-permissive partner will lead to the necessary changes within the heterodimer that allow RXR to become activated by its own specific ligand. Interestingly, deletion of the RXR AF-2 core helix inhibits activation of RAR, suggesting that unliganded RXR plays an essential role during the initial interaction between the partner and its ligand (Botling *et al*., 1997).

RXR-specific signaling

The signaling status of RXR *in vivo* is still a matter of debate. Nonetheless, several reports show that once the partner has been exposed to its specific ligand, addition of RXR-specific ligands often leads to synergistic effects on gene regulation (see e.g. Botling *et al*., 1997; Lu *et al*., 1997; Roy *et al*., 1995). Therefore, it seems likely that ligand-induced activation of RXR does occur, a conclusion has been corroborated by experiments in transgenic mice (Mascrez *et al*., 1998; Solomin *et al*., 1998). *In vivo*, retinoid signaling is probably also transduced by liganded RXR independently of RAR, as RXR-specific ligands can affect expression of genes that are not induced by RAR-specific ligands (Lu *et al*., 1997; Solomin *et al*., 1998) (see also Paper IV).

In conclusion, retinoid receptor mediated signaling will ultimately depend on many factors. First, cell type specific presence of different retinoid receptors and/or coactivators will decide if a given cell is compentent to become stimulated by retinoids present in its environment. Second, the availability and concentration of ligand, and the interconversion of atRA to 9cRA or vice versa, will decide which genes become activated. In relation to this, it is also possible that the exact nature of the ligand will influence which coactivators are recruited, thereby affecting

the cellular response to a given ligand. Third, chromatin structure at the promoter of target genes plays an important role in transcriptional activation, as it may allow or impede binding of receptor and coregulatory proteins. Fourth, the type of response element in the promoter (permissive vs. non-permissive response elements) will decide to what extent RXR is able to participate actively in transcriptional activation, and depending on the heterodimer partner, also influence what cellular processes are affected. Finally, other NRs may play important modulatory roles, either by sterically hindering retinoid receptor-binding to DNA, or by sequestering important coactivators such as CBP.

Retinoid receptor expression patterns

The expression patterns of RARα, β and γ, have been extensively studied (Dolle *et al*., 1990; Krezel *et al*., 1999; Ruberte *et al*., 1991; Ruberte *et al*., 1993; Smith and Eichele, 1991) (see also Papers II and III). The results show a ubiquitous expression of RARα, both during development and in adult tissues. RARβ and RARγ show a more temporal and spatial restriction, often in a non-overlapping fashion. For example, during early nervous system development, RARβ is expressed after neural tube closure, while RARγ is only expressed in the open neural folds. At later stages of development and in adulthood, RARβ is strongly expressed in epithelial cells of the inner ear, lung, intestine and genital tract, and in the CNS. RARγ expression is confined to all mesenchyme giving rise to bone and facial cartilage, and in the differentiating epithelium of skin (reviewed in Mangelsdorf *et al*., 1994).

RXRs are similarly differentially expressed during development (Dolle *et al*., 1994; Krezel *et al*., 1999; Mangelsdorf *et al*., 1992) (see also Paper III). RXRβ is expressed in a general fashion, perhaps at slightly higher levels in the anterior regions of the spinal cord and hindbrain. RXRα is abundantly expressed in liver, kidney, spleen and intestine. RXRα is also abundant in epidermis, together with RARγ. RXRγ expression is highly restricted, mostly to the developing ear, muscle, retina, pituitary and thyroid gland. In addition, strong expression is found in the developing striatum and spinal motor neurons (where it is coexpressed with RARβ). In adult tissues, expression is strong in muscle and striatum (reviewed in Mangelsdorf *et al*., 1994).

Retinoid receptor 'knockouts'

A tremendous effort has been made during the last few years to understand the specific functions of the different retinoid receptors during development. Despite the unique distributions of the different receptors, genetic ablation studies have revealed a surprising redundancy in function among the different members of each receptor subtype. The results of single and double RAR or RXR mutants, as well as compound RAR/RXR mutants, have been extensively reviewed (Kastner *et al*., 1995; Kastner *et al*., 1997; Lohnes *et al*., 1995; Lohnes *et al*., 1994; Mendelsohn *et al*., 1994). However, a few important points are worth making. RARα, β or γ -/- mice are all viable (reviewed in Kastner *et al*., 1995). The mutant animals display some VAD aspects, e.g. testicular degeneration (RARα -/-), growth deficiency and early postnatal death (α or γ -/-, to some extent β -/-), ocular defects (β -/-), and homeotic transformations of cervical vertebrae, fusion of ribs and irregular trachea (γ -/-). However, many known VAD phenotypes are not apparent in single RAR -/- mice, suggesting that RARs can substitute for each other *in vivo*. In contrast, RAR double mutants die either in utero or shortly after birth, and display most aspects of VAD, including multiple eye and heart malformations, as well as respiratory and urogenital tract abnormalities. In addition, a number of malformations not described in VAD studies are also observed, most likely reflecting the difficulties in achieving complete VAD by dietary deprivation. Taken together, the results suggest that RARs are important for correct regulation of numerous developmental processes, including neural crest cell differentiation, patterning of A-P axis in hindbrain and limbs via *Hox* gene regulation, retinal and heart development, as well as control of apoptosis in retina, fronto-nasal and limb interdigital mesechyme.

Of all retinoid receptor single mutants, RXRα ablation is unique in causing embryonal death (Kastner *et al*., 1994; Sucov *et al*., 1994), probably due to cardiac malfunction. In addition, RXRα -/- mice display ocular defects also found in VAD animals. However, mice lacking either RXRβ or γ are phenotypically normal, except for RXRβ-induced male sterility (Kastner *et al*., 1996) and RXRγ-induced locomotor deficiencies (Krezel *et al*., 1998). On the other hand, compound mutants of RXRα and RARα, β or γ, together reproduce the entire VAD phenotype spectrum, supporting the notion that RAR/RXR heterodimers are the active units for retinoid signaling *in vivo* (summarized in Kastner *et al*., 1997). In contrast, no apparent synergism exists between RARs and RXRβ or γ. In fact, mice carrying a single RXRα allele

(RXRα +/- / RXRβ -/- / RXRγ -/-) are viable and, with the exception for a reduction in size, not more affected than RXRβ -/- mice (Krezel *et al*., 1996), suggesting that RXRα is the most important RXR receptor *in vivo* during embryonal development. Interestingly, transgenic mice have been produced that express a manipulated version of RXRα lacking the AF-2 core important for ligand-dependent transactivation (Mascrez *et al*., 1998). Although less affected than RXRα -/- mice, these animals die during the late fetal period or early after birth, and display phenotypical changes resembling those seen in RXRα -/- mice with variable penetrance. In combination with single targeted RAR isotypes, most defects of vitamin A deficient animals are recapitualted, supporting the conclusion that liganded RXR is important in retinoid signaling. Recently, mice expressing a truncated form of RXRα lacking the ligand-independent AF-1 domain have been engineered (Mascrez *et al*., 2001). AF-1 mutant mice are growth deficient but otherwise apparently normal. Compound mutants that also lack one RAR isotype die in utero and display, with variable penetrance, a majority of the defects previously observed in RXRα/RAR double knockouts. Interestingly, these defects were less severe than those observed in the corresponding mice lacking the AF-2 core of RXRα, suggesting that the functions of the AF-1 domain of RXRα are less important than those of the AF-2 core in RAR-dependent signaling.

AIMS OF THE STUDY

It is well established that retinoids are essential for vertebrate development and homeostasis, but less is known about the exact distribution of retinoids *in vivo*. In particular, the RXR ligand 9cRA has been very difficult to identify in tissue extracts, and its role *in vivo* is still uncertain. We wished to gain further insight into the roles played by retinoids during development, but also to extend our knowledge on the importance of RA and its receptors in the adult central nervous system (CNS).

The specific aims of this study have been:

(i) To develop a transgenic mouse model as well as *in vitro* assays to study the activation patterns of nuclear receptors with focus on retinoid receptor signaling (Papers I, II, III and IV)

(ii) To gain insight into the roles played by retinoids in the development of the forebrain by exploiting the developed activity assays *in vitro* (Paper II)

(iii) To study the distribution and roles of retinoid receptors in the adult brain (Paper III)

(iv) To search for novel nuclear receptor ligands in the adult brain by applying the activity assays (Paper IV)

RESULTS AND DISCUSSION

Feedback-inducible nuclear-receptor-driven reporter gene expression in transgenic mice (Paper I)

In order to better understand the biological function of nuclear receptors, it is necessary to localize the distribution of ligand-activated receptor *in vivo*. We have previously described an assay in transgenic mice, in which the transcription of a *lacZ* reporter gene reflects the presence of active receptor (Solomin *et al*., 1998). Although useful for detecting nuclear receptor activation *in vivo*, we wished to refine and expand the applicability of this so called effector-reporter assay. In the novel version of the effector-reporter system (here referred to as the feedback-inducible nuclear-receptor-driven (FIND) expression system), the effector protein consists of a fusion between the DNA-binding domain of the yeast transcription factor GAL4 and the ligand binding domain of the studied receptor, in this case RAR. The *lacZ* reporter gene is preceded by a minimal promoter from the heat shock protein hsp68 gene and four upstream GAL4-binding sites (UASs). Thus, the presence of ligand will activate the effector fusion protein, subsequently leading to transcription of the reporter. In the original version of the system, the effector fusion protein was expressed under a strong tissue-specific promoter (nestin), thereby restricting detection of ligands to tissues where the effector protein was being expressed. In this modified version of the system, a copy of the same UAS-hsp promoter preceding the reporter construct is driving the expression of the effector (see Paper I for illustration). Since the promoter is ubiquitously expressed, ligand-detection will not become restricted to certain tissues (as was the case in the original version of the system). Instead, auto-regulated gene expression will occur in any tissue where ligands and thus active effector proteins are normally present.

FIND-RAR/lacZ

The new system was tested using a GAL4-RAR chimera as effector gene. Transgenic E11.5 and E12.5 embryos were collected and retinoid-induced reporter gene expression was analyzed. Prominent activation was detected in the developing spinal cord, especially at the levels of the developing limbs. This is consistent with previous reports, showing that the brachial and lumbar

regions of the spinal cord are "hot spots" for retinoid synthesis (Balkan *et al*., 1992; Colbert *et al*., 1993; McCaffery and Drager, 1994b; Mendelsohn *et al*., 1991; Reynolds *et al*., 1991; Rossant *et al*., 1991; Sockanathan and Jessell, 1998; Solomin *et al*., 1998; Wagner *et al*., 1992). Reporter gene expression was additionally detected in the developing forebrain, in proximal forelimb buds, and in the optic cup, also in accordance with previous results (Balkan *et al*., 1992; LaMantia *et al*., 1993; Mendelsohn *et al*., 1991). Interestingly, staining was observed in the ventral aspect of the midbrain/hindbrain boundary, a structure not previously reported to contain retinoids at this stage. Tissue taken from this region was co-cultured with cells transfected with effector and reporter plasmid *in vitro*, and strong reporter activation was observed, indicating that retinoids indeed are produced in this region. Expression of a retinaldehyde dehydrogenase (RALDH1) has recently been detected in an overlapping area, and is perhaps responsible for this localized retinoid synthesis (Haselbeck *et al*., 1999; Mic *et al*., 2000).

Conclusion (Paper I)

The consistency between the results obtained in this and previous studies, suggest that the FIND version of the effector-reporter system functions as expected. However, the *in vivo* results have to be interpreted with caution. As mentioned in the introduction, several nuclear receptors become activated in a ligand-independent manner, which could lead to reporter induction in absence of ligand. In addition, it should be emphasized that the FIND system in its present form might not be applicable to all nuclear receptors. Some receptors might require additional or full-length sequences in order to become activated. Nonetheless, the described assay provides a new tool for studying the activation pattern of potentially any nuclear receptor of choice. Furthermore, the system could prove to be useful in the isolation of novel ligands for nuclear receptors, an important feature since the majority of the known NRs are still lacking identified activators. Additionally, the system can be applied as an *in vivo* model for the characterization of novel drugs, defining the target tissue(s) and pharmacokinetic properties of such compounds. Finally, the use of a green fluorescent protein reporter in this study, shows that the FIND system can be successfully used to study NR activation in living cells.

Retinoids are produced by glia in the lateral ganglionic eminence and regulate striatal neuron differentiation (Paper II)

The early mammalian neural tube develops as a straight structure. Soon, however, three primary vesicles are formed at the anterior end of the tube, the region that will form the brain. These bulges form the forebrain, midbrain and hindbrain. The forebrain then becomes subdivided into the anterior telencephalon and more posterior diencephalon. The telencephalon will form the outermost part of the anterior brain, the two cerebral hemispheres (or cortex), while the diencephalon will form more basal structures, including the thalamus, hypothalamus and certain formations belonging to the so called basal ganglia. Two such structures, the caudate nucleus and the putamen, together form the striatum, which receives neuronal input from areas in the cerebral cortex involved in motor control and emotional behaviour (see Kandel *et al*., 1995). The caudate nucleus and the putamen, are derived from two ventral structures in the developing forebrain, the ganglionic eminences. The lateral ganglionic eminence (LGE) gives rise to most neurons of the striatum and certain populations of cortical neurons, whereas the medial ganglionic eminence (MGE) principally generates neurons of the pallidum and basal forebrain.

Although it is well understood how the ganglionic eminences contribute to the neuronal subtypes of the forebrain, less is known about the signals and molecular mechanisms that control the generation of such neurons. In an attempt to clarify these mechanisms, a subtractive cloning technique was used to isolate genes showing enriched expression in the LGE as compared to the MGE. One of the identified genes, the homeobox gene *Meis2*, has been shown to be induced by retinoic acid in PC12 cells (Oulad-Abdelghani *et al*., 1997), suggesting that retinoids are present in the developing forebrain. Our previous results had demostrated RAR activity in the forebrain of transgenic mice (Paper I), and thus prompted us to investigate the potential role for retinoids in this brain region.

Retinoids are produced in the LGE

The expression patterns of a subset of proteins involved in retinoid synthesis and signalling were examined, including cellular retinol binding protein I (CRBPI), RARα, RARβ and RXRγ. The results show that CRBPI expression is strong in the LGE, suggestive of retinoid production in this structure. To obtain further evidence, we made use of the effector-reporter based assay

described above (Paper I). Cells in culture were transiently co-transfected with plasmids expressing GAL4-RAR effector and a UAS-luciferase reporter gene, and subsequently co-cultured with tissue dissected from embryonal E12.5 LGE, MGE or cortex. The presence of retinoids will then allow RAR to activate *luciferase* expression. Analysis of *luciferase* induction demonstrated that LGE explants gave rise to robust activation of the reporter, whereas MGE and cortex explants only weakly affected *luciferase* expression, suggesting that retinoid synthesis is localized to the LGE.

Glial cells in the LGE synthesize and release retinoids

The morphology of the cells expressing CRBPI in LGE was reminiscent of radial glia, extending from the ventricular zone to the mantel layer of the LGE. In order to determine which cell type(s) in the developing forebrain that are responsible for the retinoid production, we analyzed if these cells were capable of producing retinoids *in vitro*. Glial cultures from E13.5 LGE and MGE were generated, and conditioned medium from LGE glial cell cultures was added to cells transfected with plasmids expressing effector and a reporter genes as above, leading to strong activation of the reporter. In contrast, MGE glial cells were incapabel of doing so. These results further support that retinoids are produced in the LGE, specifically by glial cells.

Retinoids induce striatal neuron differentiation in vitro

To investigate whether retinoids are important for striatal neuron differentiation, we treated LGE *in vitro* cell cultures with RA and studied the expression of the striatal neuron marker DARPP-32. Upon RA treatment, the number of DARPP-32 positive cells rose to approximately 200%, showing that retinoids do indeed play a role in striatal development. RAR- and RXR-specific agonists (TTNPB and SR11237, respectively) were used in similar experiments, and interestingly, both signaling pathways seem to mediate the retinoid-induced increase of DARPP-32 expressing neurons equally efficient, suggesting that both RARs and RXRs are important during striatal differentiation.

Conclusion (PaperII)

In conclusion, our results identify the LGE as a novel site of retinoid production, implicating retinoids in the development of forebrain and striatal structures. Furthermore, we show that radial glial cells within this region are responsible for producing retinoids, thus suggesting that

glial cells play dual roles, namely as guiding cells in neuronal migration, a function which is well established, and also as direct contributors to regional differentiation of neurons by local retinoid synthesis. Interestingly, a novel retinaldehyde dehydrogenase (RALDH3; previously referred to as V1) has recently been shown to be expressed in this region of the developing brain (Mic *et al*., 2000; Ross *et al*., 2000), indicating a key role in glial cell retinoid synthesis within this region.

Role of retinoids in the CNS: differential expression of retinoid binding proteins and receptors and evidence for presence of retinoic acid (Paper III)

As discussed, the importance of retinoids in development has long been known. However, less is known about their role in postnatal and adult tissues. To investigate signaling after birth, we analyzed the expression of all known retinoid binding proteins (CRBP-I and -II, and CRABP-I and -II) and retinoid receptors (RAR and RXRα, β and γ) in newborn and adult CNS, thereby extending a previous study (Zetterström *et al*., 1994). By *in situ* hybridization and imunohistochemistry, most retinoid binding proteins and retinoid receptors could be detected in the postnatal and adult brain, expression generally being stronger in the newborn animals. RXRβ mRNA seems to be ubiquitously expressed, while the other mRNA species have a more restricted and specific pattern of distribution. Of all retinoid binding proteins investigated, only CRBP-II and RARγ showed low or no expression (a more detailed description of the distribution of the different transcripts is listed in Table 2 of Paper III). This is in contrast to the results presented recently by Krezel and collaborators, where the presence of RARγ transcript was detected in for example the olfactory bulb, cortex, hippocampus and striatum (Krezel *et al*., 1999). The reason for this discrepancy is currently not known. Interestingly, strong CRBP-I and CRABP-I expression was detected in the olfactory bulb, a region known to produce retinoids (LaMantia *et al*., 1993). Furthermore, it is tempting to speculate that the presence of CRABP-I in the axons of olfactory dopaminergic neurons indicates that RA bound to CRABP-I can be transferred between neurons at synapses in a transneuronal manner.

A role for RA signaling in the nigrostriatal dopamine system

In Paper II, we show that retinoids are involved in the development of forebrain and striatal structures during embryogenesis. Here, we demonstrate the presence of CRBP-I, CRABP-I and -

II, RARβ and RXRγ in postnatal striatum, nucleus accumbens and olfactory tubercle, areas which are all innervated by midbrain dopaminergic neurons. By using the *in vitro* co-culture assay described above, RAR activation was studied using tissue pieces dissected from newborn striatum, hippocampus and cerebellum. Only tissue derived from striatum gave rise to strong reporter activation, suggestive of retinoid production within this region and in agreement with the findings in Paper II. Additionally, one of the enzymes proposed to oxidize retinal to retinoic acid (RALDH2/AHD2) has been reported in dopaminergic nerve terminals (McCaffery and Drager, 1994a) which are known to innervate the striatum (the nigrostriatal and mesolimbic dopamine neurons). Taken together, these results propose an important role for retinoid-regulated gene expression in postnatal dopamine signaling pathways involved in locomotor control and behavioural responses to drugs of abuse. A retinoid response element has been discovered in the promoter of the dopamine 2 receptor, and mice lacking RARβ and RXRβ or RXRγ exhibit a downregulation of this gene in striatum (Samad *et al*., 1997). In addition, these animals also show impaired locomotion and decreased DA release after cocaine treatment (Krezel *et al*., 1998). Interestingly, mice lacking the nuclear orphan receptor NURR1, a putative RXR heterodimerization partner, exhibit a complete loss of nigrostriatal neurons (Zetterström *et al*., 1997), implicating RXR in additional signaling pathways in the brain as heterodimer partner of other NRs besides RAR.

Conclusion (Paper III)

In conclusion, these results extend our previous knowledge on retinoid action during development, and suggest an important role for retinoids in newborn and adult CNS function. In addition, retinoids could possibly be involved in pathological conditions such as Parkinson´s disease and schizophrenia, which are known to affect dopaminergic signaling pathways.

Docosahexaenoic acid, a novel ligand for the retinoid X receptor in mouse brain (Paper IV)

The function of RXR remains elusive, as its proposed natural ligand, 9-*cis* RA, has proven difficult to identify in mammalian tissue. Nonetheless, the significance of RXR signaling *in vivo* has been suggested from several studies (Botling *et al*., 1997; Lu *et al*., 1997; Mascrez *et al*., 1998; Roy *et al*., 1995; Solomin *et al*., 1998). In a subsequent study we therefore wished to analyze the importance of retinoid receptors in the adult CNS, by searching for endogenous ligands that activate RAR and RXR. Using the previously described effector-reporter assay in transfected cells, we compared the activation patterns of GAL4-RAR and GAL4-RXR in cells co-cultured with serum-free media that had been incubated overnight with various tissues from adult brain. As shown previously (Paper III), little or no RAR activation could be detected. In contrast, a substantial RXR activation was detected. Additional experiments convinced us that the adult brain-conditioned medium directly activated RXR. To characterize the putative RXR ligand(s), biochemical isolation, purification and mass spectrometry of brain conditioned medium was performed. The activity was identified as docosahexaenoic acid (DHA), a long chain polyunsaturated fatty acid (PUFA) that accumulates in the postnatal and adult central nervous system. Interestingly, other PUFAs can also activate RXR, although DHA is the most efficient. DHA is specific for RXR, as it does not activate RAR or the receptors for thyroid hormone or vitamin D_3, and behaves as a bonafide NR ligand in that it promotes the interaction between RXR and the coactivator SRC-1. At the recent EMBO workshop on nuclear receptors in Sicily (EMBO Workshop: "Nuclear receptor structure and function", May 12-15, 2001, Erice, Sicily, Italy), Moras and colleagues presented the crystal structure of RXR LBD bound to DHA, providing a structural mechanism by which DHA binds and activates RXR.

DHA – an essential fatty acid

Fatty acids (FAs) have long been recognized as essential for normal growth and development, as energetic fuels, membrane components and precursors of essential lipid metabolites (Burr and Burr, 1929; Neuringer *et al*., 1988). DHA accumulates in the mammalian CNS during late gestation and early postnatal development, and constitutes as much as 30 to 50% of total FAs in postnatal brain, predominantly associated with membrane phospholipids (reviewed in Neuringer

et al., 1988; Salem Jr *et al*., 1986). Deficiencies in DHA have been shown to cause neurological abnormalities, impaired learning abilities and growth retardation (see for example Gamoh *et al*., 1999; Horrocks and Yeo, 1999; Rose and Connolly, 1999; Sheaff Greiner *et al*., 1999).

Conclusion (Paper IV)

The identification of the fatty acid DHA as a ligand for RXR, provides evidence for an entirely novel pathway for RXR signaling and suggests a mechanism whereby DHA may influence neural processes and maturation of the mammalian brain. DHA is essential for correct CNS development and maturation. Interestingly, the analyses of gene targeted mice lacking one or several genes encoding RXR isotypes have demonstrated overlapping functions with DHA, e.g. in the process of memory formation (Chiang *et al*., 1998), vision (Kastner *et al*., 1994), reproduction (Kastner *et al*., 1996), and growth (Kastner *et al*., 1995). In addition, DHA, a major constituent in nutrients rich in ω3 PUFAs such as fish oils, has beneficial effects on blood cholesterol levels and insulin sensitivity (Horrocks and Yeo, 1999; Storlien *et al*., 1998), and is also used to treat pathological conditions such as cancer (Rose and Connolly, 1999). Interestingly, synthetic RXR ligands have been shown to influence humans and mice in a similar way, providing a possible molecular mechanisms for the effects achieved by dietary ω3 PUFAs (Bischoff *et al*., 1998; Mukherjee *et al*., 1997; Repa *et al*., 2000). Finally, several RXR heterodimerization partners, such as PPARs, LXRs and FXR, contribute to energy and nutritional homeostasis in response to their respective ligands (reviewed in Chawla *et al*., 2000; Willson *et al*., 2000), adding the possibility that DHA is an important modulatory factor in these processes by binding and influencing the RXR subunit of such heterodimers.

Unlike 9cRA which binds and activates RXR with high affinity, DHA is a low affinity ligand for this receptor, reaching half-maximal activation at a concentration of about 50 to 100 μM. On the other hand, while 9cRA has been difficult to identify *in vivo*, DHA constitutes between 30 to 50 % of all membrane-bound fatty acids in the postnatal brain. Therefore, we speculate that a high intracellular concentration of free DHA can exist in neurons. Little is known about the mechanisms of DHA release from its phospholipid stores, although some reports implicate certain phospholipases in this process (see e.g. Garcia and Kim, 1997; Homayoun *et al*., 2000; Spector, 1999). This mobilization could potentially supply free DHA to activate RXR. However, it is important to realize that not all described effects of DHA on

cellular functions are due to RXR activation. For example, DHA is an essential membrane component and plays important roles during structural rearrangements including neurite outgrowth (Ikemoto *et al*., 1997), myelinogenesis (Martinez and Vazquez, 1998), and in synaptic changes ocurring during learning (Yoshida *et al*., 1997). In addition, PPAR, which forms heterodimers with RXR, has been shown to bind and become activated by long chain FAs, including DHA (Forman *et al*., 1997; Nagy *et al*., 1998; Xu *et al*., 1999a). This suggests that some of the effects on cholesterol and lipid metabolism produced by DHA could be mediated by both receptor partners in RXR-PPAR heterodimers.

In conclusion, we show that DHA-mediated activation of RXR can occur both via RXR homodimers and in heterodimers with the orphan receptor NURR1. In addition, DHA could also be important in synergistic activation through non-permissive heterodimers like RAR-RXR and VDR-RXR, and permissive heterodimers like PPAR-RXR, LXR-RXR or FXR-RXR.

FUTURE PROSPECTS

The identification of a novel RXR ligand in the adult mammalian central nervous system (CNS), poses an exciting new twist in RXR signaling. Of outermost importance is to clarify what target genes are involved in the effects of RXR activation, both by retinoid ligands and by fatty acids such as DHA. One approach would be to study the effects of high affinity RXR-specific ligands, so called rexinoids, on gene transcription in the brain, thereby identifying novel target genes. Detailed analysis of expression patterns, induction levels and promoter regions could prove useful in the characterization of such genes. Once a potential target gene has been isolated, DHA or other potential RXR agonists can be used to compare their effect on gene expression. Such studies could either be performed by comparing DNA microarrays from untreated and treated tissues, or by analyzing gene induction in cell lines derived from the CNS. Midbrain dopaminergic and striatal neurons would be two cell types of particular interest, since retinoid receptors are important for their function *in vivo* (see discussion in Paper III).

In addition, more needs to be learned of how DHA is released from membranes, and what the intracellular concentrations are during different conditions. The exact distribution of DHA within the brain is also unknown. "Hot spots" of DHA accumulation might exist, possibly important in DHA function.

DHA levels are known to increase during the final stages of embryonal and early postnatal development. Thus, DHA is probably not essential for the initial differentiation of neuronal subtypes, but could play essential roles in later stages of maturation. Indeed, DHA deficiency leads to impairment of synaptic plasticity and learning abilities, suggesting that DHA is important in cells involved in memory formation. These effects can in principle be studied in DHA-deficient mice, e.g. by recording neuronal activity changes in brain slices derived from deprived animals. Treating such slices with rexinoids would provide valuable insights in the pharmacological importance of RXR activation *in vivo*. In addition, mutagenesis of specific residues in the LBD of RXR to create a DHA binding-deficient receptor, would provide important information about the interplay between DHA and RXR. Analysis of the phenotypes found in mice expressing this type of receptor would be of great interest, as they might provide firm evidence for a role of RXR signaling *in vivo*.

The mechanism behind DHA-activation of RXR signaling *in vivo* is currently unknown. In order to learn more about the effects of PUFAs on RXR mediated transactivation, we have compared the effects of 9cRA and DHA on RXR-mediated transcription of reporter genes carrying two different RA response elements, either DR1 or DR5 response elements. Both response elements have been shown to bind RAR-RXR heterodimers, but by overexpressing RXR in cells, these retinoid receptor binding sites can be saturated with RXR homodimers. Interestingly, when incubated with cells expressing RXR and the DR1 reporter, both DHA and 9cRA gave rise to strong induction of reporter gene expression. In contrast, while 9cRA also gave rise to strong DR5 reporter induction, DHA had no effect. These effects were more clear when natural promoters were used (the apolipoprotein A1 gene promoter for DR1, and the RARβ gene promoter for DR5), but could also be observed on synthetic promoters. The initial experiments were performed in 293T cells (see materials and methods of Paper IV for cell descriptions). When similar experiments were performed in JEG3 cells, none of the reporters were efficiently activated by DHA. This suggests the following: (1) since the effects of the two ligands differ even in the same cell type (DR1 activation by both ligands compared to DR5 activation by 9cRA only), the RXR conformation might be different depending on nature of the ligand; (2) a cell-specific coactivator only expressed in 293T cells is recruited to the RXR-DHA complex and mediates the effects seen on the DR1 reporter (DR1 is activated in 293T cells but not in JEG3 cells); (3) alternatively, a cell-specific corepressor only expressed in JEG3 cells cannot be released when RXR is bound to the DR1 element. Another possible explanation would be differential metabolism of DHA in the two cell types, but since a NURR1/RXR heterodimer is similarly activated by DHA in both cell lines, this is not likely to be the case. These results indicate that different ligands have different effects on RXR-induced transcription, and that DHA modulation of RXR might be specific for a given set of target genes *in vivo*. This could potentially allow modulation of gene responses depending on the RXR ligand of choice, in part influenced by the nature of the heterodimer partner involved.

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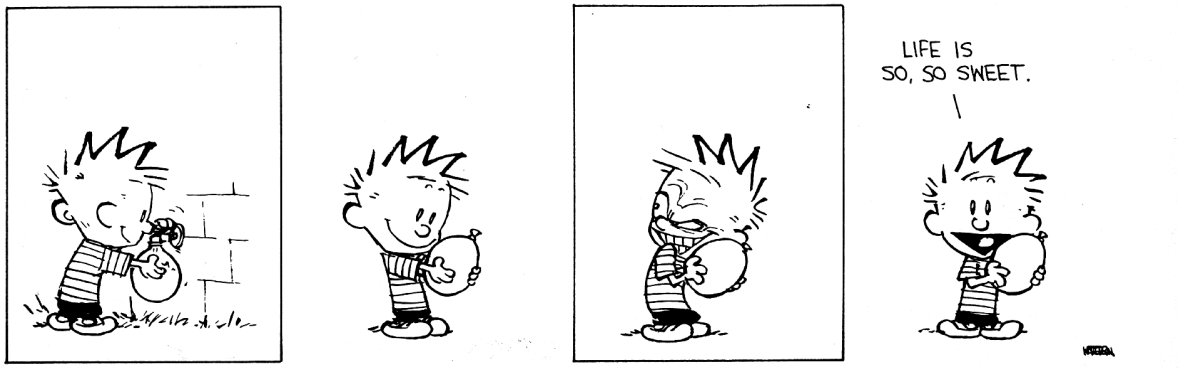
"Between the fifth and tenth days, the lump of stem cells differentiates into the overall building plan of the [mouse] embryo and its organs. It is a bit like a lump of iron turning into the space shuttle. In fact it is the profoundest wonder we can still imagine and accept, and at the same time so usual that we have to force ourselves to wonder about the wondrousness of this wonder."



THE END

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