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Biological activity of a novel retinoic acid metabolite, *S*-4-oxo-9-*cis*-13,14-dihydro-retinoic acid

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In Dankbarkeit meinen Eltern

Summary

Vitamin A and its analogues (retinoids) regulate a broad range of physiological processes such as differentiation and proliferation. In contrast some retinoids are shown to be biologically inactive degradation products. All-*trans*-retinoic acid (at-RA) is considered as the most active endogenous occurring retinoid in mammalians which mediates its function via Retinoid acid receptors (RAR). Recently, a novel major retinoic acid metabolite was identified and characterised as *S*-4-oxo-9-*cis*-13,14-dihydro-RA (*S*-4o-9c-dh-RA). The present work describes the recognition of *S*-4o-9c-dh-RA as a biological active RA metabolite *in vivo* and *in vitro* investigating its potential to mimic the action of at-RA.

Using cell based model systems, it has been demonstrated that *S*-4o-9c-dh-RA induce RAR-dependent transcriptional activity from transfected luciferase reporter plasmids in different cell lines. *S*-4o-9c-dh-RA was shown to have a positive and dose-dependent effect on RARE (RAR responsive element) regulated genes, both from a simple 2xDR5 element, but also from a more complex promoter region derived from the natural retinoid target gene, RA receptor beta 2 *(RARβ2)*, in P19, HC11, Hela, Hepa-1, and CV1 cells. The potential of *S*-4o-9c-dh-RA was about factor 200 lower compared to at-RA. *S*-4o-9c-dh-RA was able to mediate the transcriptional activity of RARE regulated genes via both RAR subtypes -α or -β in partnership with retinoid X receptor-β (RXR-β). On the other hand, *S*-4o-9c-dh-RA was not capable to activate the transcription from the RXR-element, DR1, in combination with RXRα or RXRβ. Using quantitative real-time PCR (qRT-PCR) it has been found out, that treatment of P19-cells with *S*-4o-9c-dh-RA induced the expression of the direct at-RA target gene *RARβ2* endogenously. The effect was dose-dependent and increased with treatment time. Compared to the untreated controls, *S*-4o-9c-dh-RA induced the relative expression of *RARβ2* mRNA transcripts significant ($P < 0.05$) already after 1 hour of treatment (2-fold at 1 µM and 4-fold at 10 µM). After 24 hours of treatment the relative expression levels were significantly increased to a 3-fold induction at 1 µM and 32-fold induction at 10 µM, respectively. Compared to at-RA, *S*-4o-9c-dh-RA was 200-fold less active at inducing *RARβ2* gene induction.

Mechanistically, *S*-4o-9c-dh-RA induced changes in the protein conformation of RARα and -β in the same manner as at-RA. This effect was observed in digestion experiments of labelled RA receptors incubated with the new metabolite. *S*-4o-9c-dh-RA provoked the resistance of receptor fragments to Trypsin-proteolysis, resulting in accumulation of a 30-kDa resistant proteolytic fragment. The proved effect is a direct result of a ligand binding reaction. Taken together, the data from the different *in vitro* and biochemical experiments strongly suggests that the new RA-metabolite is a novel endogenous ligand for the RAR subtypes -α and -β, thus can regulate gene transcription *in vitro*.

S-4o-9c-dh-RA causes morphological effects in the developing chick wing and hence has a biological activity also *in vivo*. *S*-4o-9c-dh-RA induced digit pattern duplications with additional digits in a dose-dependent fashion after local application to the wing bud in form of beads soaked in a solution containing the retinoid. Wing patterns with additional digit 2 became most prevalent at soaking concentrations of 0.2 and 0.5 mg/ml *S*-4o-9c-dh-RA, whereas patterns with additional digit 3 and 4 were seen at soaking concentrations equal or greater that 1 mg/ml. Using qRT-PCR analysis, it was shown that *S*-4o-9c-dh-RA can control the expression of RA-target genes in the limb buds. *S*-4o-9c-dh-RA induced the expression of genes which are involved in limb morphogenesis (Sonic hedgehog *shh;* Homeobox gene-8, *hoxb8;* and Bone morphogenetic protein-2, *bmp2*), as well as direct at-RA regulated genes (*RARβ2*; Cytochrome P450, *Cyp26;* and *hoxb8*) which are known to contain a evolutionary conserved RARE in their promoter region. This work has clearly shown that *S*-4o-9c-dh-RA is a biologically active retinoid metabolite *in vitro* and *in vivo*.

Keywords: RAR-ligand, gene expression, vitamin A

Zusammenfassung

Vitamin A und dessen Derivate (Retinoide) sind an der Regulation einer Vielzahl physiologischer Prozesse beteiligt z.B. Differenzierung und Proliferation. Einige Retinoidmetaboliten scheinen aber inaktive Abbauprodukte zu sein. In Säugetieren gilt all-*trans*-Retinsäure (at-RA) allgemein als der Metabolit mit der höchsten biologischen Aktivität. At-RA vermittelt seine Wirkung über Retinsäurerezeptoren (RAR). Vor einiger Zeit wurde ein neuer endogen vorkommender Retinsäuremetabolit in Mäusen und Ratten entdeckt, der als *S*-4-oxo-9-*cis*-13,14-dihydro-RA (*S*-4o-9c-dh-RA) charakterisiert wurde. Die vorliegende Arbeit beschreibt die biologische Aktivität von *S*-4o-9c-dh-RA *in vivo* und *in vitro* durch die Anwendung unterschiedlicher Techniken zur Untersuchung des Potenzials von *S*-4o-9c-dh-RA die gleichen Effekte wie at-RA zu induzieren.

Durch die Verwendung zellbasierter Modelsysteme wurde gezeigt, dass *S*-4o-9c-dh-RA eine RAR-abhängige Transkriptionsaktivität von Luziferasereporterplasmiden in verschiedenen Zelllinien aktiviert. *S*-4o-9c-dh-RA induzierte die Transkription von Luziferase-gekoppelten Genen in transfizierten P19, HC11, Hela, Hepa-1 und CV1 Zellen. Die Gene wurden durch regulatorische RAR-Sequenzen (RAR responsive Elemente*,* RAREs) gesteuert. Die Aktivität von *S*-4o-9c-dh-RA in diesen Modelsystemen war verglichen mit at-RA um den Faktor 200 geringer. *S*-4o-9c-dh-RA konnte die transkriptionale Aktivität von RARE regulierten Genen durch zwei RAR subtypen (-α oder -β) in Verbindung mit dem Retinoid X Rezeptor-β (RXR-β) regulieren. *S*-4o-9c-dh-RA zeigte keine transkriptionelle Aktivität bei RXRE-(RXR-responsives Element, DR1) regulierten Genen in Kombination mit RXRα- oder RXRβ. *S*-4o-9c-dh-RA induzierte die endogene Expression des at-RA-Zielgenes *RARβ2* in P19 Zellen. Die Expression war bereits nach 1 Stunde Behandlung signifikant ($P < 0.05$) induziert (2-fach bei 1 µM bzw. 4-fach bei 10 µM) gegenüber der unbehandelten Kontrolle. Die relativen Expressionsraten (RER) stiegen nach 24 Stunden Behandlung auf bis zu 32-fache Induktion bei 10 µM bzw. 3-fach bei 1 µM an. Im Vergleich zu at-RA war *S*-4o-9c-dh-RA ebenfalls etwa 200-fach geringer aktiv.

S-4o-9c-dh-RA induzierte allosterische Konformationsänderungen an RARα und -β-Proteinen in der gleichen Weise wie at-RA. Dieser Effekt wurde in proteolytischen Verdauungsexperimenten festgestellt, wo markierte RAR Proteine mit *S*-4o-9c-dh-RA inkubiert und anschließend in Proteolysereaktionen mittels Trypsin verdaut wurden. *S*-4o-9c-dh-RA induzierte die Resistenz eines 30-kDa Fragmentes, welches in unbehandelten Kontrollproben nicht detektiert werden konnte. Der nachgewiesene Effekt ist die direkte Folge einer Ligandenbindungsreaktion. Die Daten der verschiedenen *in vitro* und biochemischen Experimente zeigen, dass *S*-4o-9c-dh-RA ein neuer endogener Ligand für die RAR-Subtypen -α und -β ist und dadurch die Transkription von Genen regulieren kann.

Der neue Metabolit zeigte auch *in vivo* eine biologische Aktivität. Ionenaustauschkügelchen wurden in einer Lösung mit *S*-4o-9c-dh-RA getränkt und in anteriore Regionen sich entwickelnder Hühnchenflügel appliziert. Dort induzierte *S*-4o-9c-dh-RA dosisabhängig die Duplikation des Fingerstrahlenmusters. Flügelmuster mit einem zusätzlichen Finger 2 (Muster 2234, von anterior nach posterior) wurden bei Konzentrationen von 0,2 und 0,5 mg/ml Tränkungslösung festgestellt, während Muster mit einem zusätzlichen Finger 3 bzw. 4 (32234 bzw. 432234) bei Konzentrationen ab 1 mg/ml vorherrschten. Mittels qRT-PCR wurde festgestellt, dass *S*-4o-9c-dh-RA die Expression von Genen kontrolliert, die zum einen an der Flügelknospenmorphogenese beteiligt sind (Sonic hedgehog, *shh;* Homeobox Gen-8, *hoxb8;* Bone morphogenetic protein-2, *bmp2*) und zum anderen direkte at-RA Zielgene sind, die ein RARE in ihrer Promotorregion beinhalten (*RARβ2*; Cytochrome P450, *Cyp26; Hoxb8*). Die vorliegende Arbeit hat eindeutig gezeigt, dass *S*-4o-9c-dh-RA *in vitro* und *in vivo* ein biologisch aktiver Retinoidmetabolit ist.

Keywords: RAR-ligand, Genexpression, Vitamin A

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LIST OF ABBRIVIATIONS

4o-9,11dc-dh-RA	4-oxo-9,11-di-*cis*-13,14-dihydro-retinoic acid
4o-9c-dh-RA	4-oxo-9-*cis*-13,14-dihydro-retinoic acid
4o-11c-dh-RA	4-oxo-11-*cis*-13,14-dihydro-retinoic acid
4o-at-dh-RA	4-oxo-all-*trans*-13,14-dihydro-retinoic acid
4-OH-9-*cis*-RA	4-hydroxy-9-*cis*-retinoic acid
4-OH-all-*trans*-RA	4-hydroxy-all-*trans*-retinoic acid
4-oxo-9-*cis*-13,14-dihydro-RA	4-oxo-9-*cis*-13,14-dihydro-retinoic acid
4-oxo-9-*cis*-RA	4-oxo-9-*cis*-retinoic acid
4-oxo-13-*cis*-RA	4-oxo-13-*cis*-retinoic acid
4-oxo-all-*trans*-RA	4-oxo-all-*trans*-retinoic acid
9,11-di-*cis*-RA	9,11-di-*cis*-retinoic acid
9,11-di-*cis*-4-oxo-dh-RA	9,11-di-*cis*-4-oxo-13,14-dihydro-retinoic acid
9,13-di-*cis*-RA	9,13-di-*cis*-retinoic acid
9-*cis*-RA	9-*cis*-retinoic acid
9*c*-dh-RA	9-*cis*-13,14-dihydro-retinoic acid
11-*cis*-4-oxo-dh-RA	11-*cis*-4-oxo-13,14-dihydro-retinoic acid
11-*cis*-RA	11-*cis*-retinoic acid
13,14-di-OH-retinol	13,14-di-hydroxy-retinol
13-*cis*-RA	13-*cis*-retinoic acid
14-OH-4,14-retro-retinol	14-hydroxy-4,14-retro-retinol
18-all-*trans*-OH-RA	18-all-*trans*-hydroxy-retinoic acid
ADH	alcohol dehydrogenases
AER	apical ectodermal ridge
all-*trans*-3,4-didehydro-RA	all-*trans*-3,4-didehydro-retinoic acid
all-*trans*-3,4-didehydro-retinol	all-*trans*-3,4-didehydro-retinol
all-*trans*-dh-RA	all-trans-13,14-dihydro-RA
all-*trans*-4-oxo-dh-RA	all-*trans*-4-oxo-13,14-dihydro-retinoic acid
all-*trans*-5,6-epoxy-RA	all-*trans*-5,6-epoxy-retinoic acid
APo	anterior-posterior
APS	ammonium persulfate solution
ARAT	acyl-CoA:retinol acyltransferase
ATP	adenosine 5'-triphosphate
at-RA	all-*trans*-retinoic acid
BFB	bromphenol blue
Bmp	bone morphogen protein

BSA	bovine serum albumin
bw	bodyweight
$CaCl_2$	calcium chloride
CD	circular dichroism
Cf.	confer
CNS	central nervous system
CM	chylomicrons
C_{max}	maximal concentration
CM-RE	chylomicron remnants
CRBP	cellular retinol binding protein
CRABP	cellular retinoic acid binding protein
Ct	comparative threshold
ctrl	control
CYP	cytochrome P450
CYP26	cytochrome P450RAI (retinoic acid inducible)
Cyp26	cytochrome P450RAI gene
d	day
DBD	DNA-binding domain
ddH_2O	double distilled water
DEPC	diethylpyrocarbonat
d.l.	detection limit
DMEM	dulbecco`s modified eagle medium
dNTP	nucleotide
DR	direct repeat
DTT	dithiothreitol
E	qRT-PCR efficiency
EDTA	ethylene-diamine-tetraacetic acid
EGF	epidermal growth factor
FBS	fetal bovine serum
FCS	fetal calf serum
FGF	fibroblast growth factors
G418	Geneticin®
h	hour
Hac	acetic acid
HH	Hamburger-Hamilton
Hox	Homeobox gene
HPLC	high-performance liquid chromatography

Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hz	hertz
IL	interleukin
IR	infrared
IS	internal standard
IU	international units
kDa	kilo Dalton
Kac	potassium acetate
KCL	potassium chloride
l	litre
LB	lysogeny broth
LBD	ligand-binding domain
LRAT	lecithin:ROL acyltransferase
luc	luciferase
M	molar
MDR	medium-chain dehydrogenase/reductase
$MgCl_2$	magnesium chloride
mM	mini molar
MS	mass spectral
NaCl	sodium chloride
nM	nanomolar
NEA	non essential amino acids
NMR	nuclear magnetic resonance
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEST	Penicillin/Streptomycin solution
PIPES	Piperazine-N-N'-bis-2-ethane sulfonic acid
PRV	percentage respecification values
PZ	progress zone
qRT-PCR	quantitative real-time-polymerase chain reaction
R-4o-9,11dc-dh-RA,	4-oxo-9,11-di-*cis*-13,14-dihydro-retinoic acid, *R*-type
R-4o-9c-dh-RA,	4-oxo-9-*cis*-13,14-dihydro-retinoic acid, *R*-type
R-4o-11c-dh-RA	4-oxo-11-*cis*-13,14-dihydro-retinoic acid, *R*-type
R-4o-at-dh-RA	4-oxo-all-*trans*-13,14-dihydro-retinoic acid, *R*-type
RAL	retinal
RALDH	retinal dehydrogenases
RAR	retinoic acid receptor

RARE	retinoic acid responsive elements
RBP	retinol binding protein
RBP	retinol binding protein
RDH	retinol dehydrogenases
RE	retinyl esters
REH	retinyl ester hydrolase
RER	relative expression ratio
RetSat	All-*trans*-retinol:13,14-dihydroretinol Saturase
ROL	retinol
RT	reverse transcriptase reaction
RT-PCR	real-time-polymerase chain reaction
RXR	retinoid X receptor
RXRE	retinoid X responsive element
^{35}S	labeled sulphur
S-4o-9c-dh-RA	4-oxo-9-*cis*-13,14-dihydro-retinoic acid, *S*-type
S-4o-9,11dc-dh-RA,	4-oxo-9,11-di-*cis*-13,14-dihydro-retinoic acid, *S*-type
S-4o-11c-dh-RA,	4-oxo-11-*cis*-13,14-dihydro-retinoic acid, *S*-type
S-4o-at-dh-RA	4-oxo-all-*trans*-13,14-dihydro-retinoic acid, *S*-type
SD	standard deviation
SDR	short-chain dehydrogenase/reductase
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
S/N	signal-to-noise ratio
PAGE	polyacrylamide gel electrophoresis
shh	sonic hedgehog
TCDD 2	2,3,7,8-tetrachlorodibenzo-p-dioxin
TFA	trifluoroacetic acid
TGF	transforming growth factor
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
TTNPB	4-(E-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl) benzoic acid
TTR	transthyretin
U	units
UV-Vis	ultraviolet visible
v	volume
WCEB	whole cell extraction buffer
ZPA	zone of polarizing activity

1. Introduction

1.1. Vitamin A and retinoids

1.1.1. Nomenclature and structure

Vitamin A (CAS-Nr. 68-26-8) is a generic term that summarises all lipophilic compounds which possess the same biological activity of all-*trans*- retinol and its esters (BLOMHOFF *et al.*, 1992; SPORN & ROBERTS, 1985), whereas the term retinoids describes the class of natural and synthetic compounds that are chemically related to all-*trans*-retinol but are not necessarily active in biological systems. The parent compound all-*trans*-retinol is an unsaturated isoprenoid alcohol with five conjugated all-*trans* double bounds (fig.1.1) and the molecular weight of 286. The most important naturally occurring retinoids include all-*trans*-retinol, all-*trans*-retinal, all-*trans*-retinoic acid (at-RA) and retinyl esters (conjugates of all-*trans*-retinol with fatty acids, such as palmitine-, stearine-, and linolic acid). In the following the abbreviation RA refers to the term retinoic acid. The predominant retinoid in the tissue of most animals is retinyl palmitate beside retinyl oleate and retinyl stearate. The structure of retinoids can be generally classified into a hydrophobic β-inone ring, a conjugated tetraen side-chain and a polar end group. Most of these metabolites occur in the all-*trans* configuration, although several stereo-isomers of RA, such as 9-*cis*-RA, 11-*cis*-RA, 13-*cis*-RA and 9,13-di-*cis*-RA exist beside the all-*trans*-form. The 11-*cis* aldehyde form, 11-*cis*-retinal, is present in retina of eyes.

Retinol and its derivatives are highly unstable compounds which isomerise easily in presence of oxygen, acid and light. All natural vitamin A is ultimately derived from the provitamin A carotenoids which belongs to a class of compounds that generally contain eight isoprenoid units and is synthesised by plant and microorganisms. The most abundant provitamin A carotenoid is β-carotene which has the greatest potential in vitamin A activity.

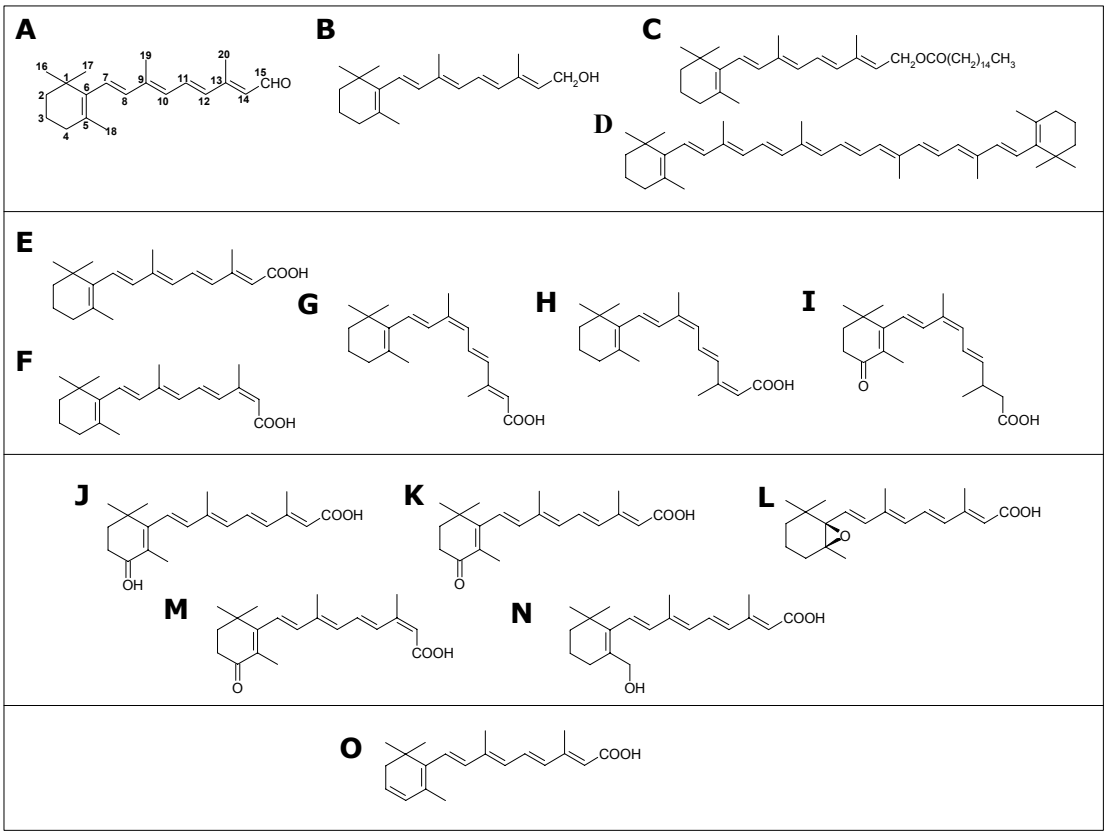

Fig.1.1: Molecular structures of important retinoids.

A) All-*trans*-retinol (most abundant natural occuring retinoid), **B)** all-*trans*-retinal (intermediate in the „activation" of all-*trans*-retinol to at-RA & active principle in the visual cycle), **C)** all-*trans*-retinyl palmitate (most abundant storage form of retinol), D) b-carotene (provitamin A, precursor of retinol); E-I) Main RA isomers, **E)** at-RA, **F)** 13-*cis*-RA, **G)** 9-*cis*-RA, **H)** 9,13-di-*cis*-RA, **I)** 4-oxo-9-*cis*-13,14-dihydro-RA; J-N) Most important polar metabolites, **J)** 4-OH-all-*trans*-RA, **K)** 4-oxo-all-*trans*-RA, **L)** all-*trans*-5,6-epoxy-RA, **M)** 4-oxo-13-cis-RA, **N)** 18-OH-all-*trans*-RA; **O)** all-*trans*-3,4-didehydro-RA (active retinoid principle in chick limb buds).

1.1.2. Absorption and transport

The main dietary sources of Vitamin A are provitamin A carotenoids from plant sources and preformed vitamin A, mostly retinyl esters, from animal tissues. Retinyl esters are enzymatically hydrolised to retinol in the intestinal lumen prior to absorption by enterocytes solubilised in mixed micelles (ONG, 1993) (schematic illustration of major retinoid pathways see fig.1.2). These reactions are catabolised by lecithin:retinol acyltransferase (LRAT) and acyl-CoA:retinol acyltransferase (ARAT) (HELGERUD *et al.*, 1982; HELGERUD *et al.*, 1983; MACDONALD & ONG, 1988; ONG *et al.*, 1987). Carotenoids are absorbed unchanged by a passive mechanism (ONG, 1993). Within the enterocytes, provitamin A carotenoids are partially

converted to retinol which is esterified to long-chain fatty acids. Most of the retinyl esters are packaged in nascent chylomicrons (HUANG & GOODMAN, 1965). Chylomicrons are released into lymph and subsequently blood stream where they are converted to chylomicron remnants. Approximately 75% of the chylomicron retinoid is finally taken up as part of the chylomicron remnants by the liver (BLOMHOFF *et al.*, 1991). The liver is the major storage site for vitamin A containing 50-80% of the total body retinol stores in mammals (BLOMHOFF *et al.*, 1990).

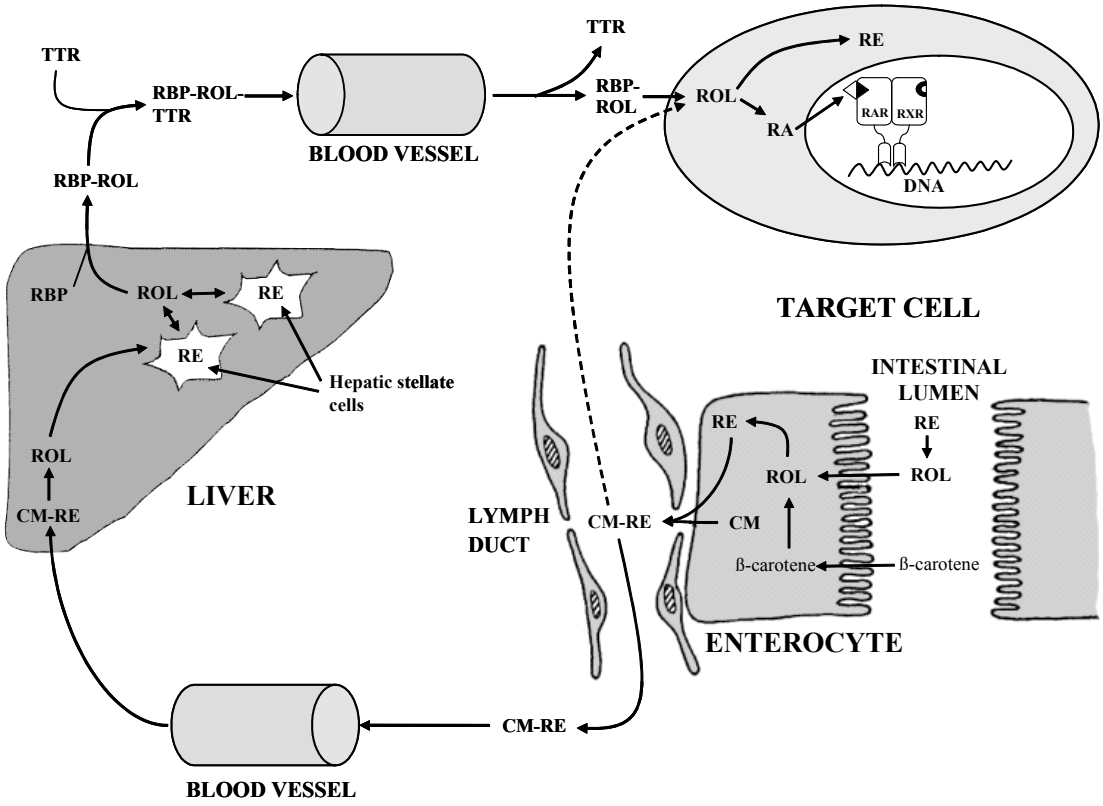

Fig.1.2: Major pathways for retinoids in the body.

See text for details. Abbreviations (see also abbreviation list): CM, chylomicrons; CM-RE, chylomicron remnants; RBP, retinol binding protein; RE, retinyl esters; ROL, retinol; TTR, transthyretin.

Within the hepatocytes retinol is re-esterified with long-chain fatty acids catabolised by LRAT and ARAT (MACDONALD & ONG, 1988; ROSS, 1982). Vitamin A is stored in form of retinyl esters in hepatocytes, primarily in stellate cells (ROSS, 1982). Although stellate, or also called Ito cells, comprise about 7% of the total

number of cells in livers, which is only about 1% of tissue by mass, these cells contain 90-95% of total retinoids present in livers (BLANER *et al.*, 1985; BLOMHOFF *et al.*, 1985). Prior to the mobilisation, stored retinyl esters are hydrolysed by carboxyl esterases to retinol which is secreted from hepatocytes bound to serum retinol binding protein (RBP) (reviewed in HARRISON, 2000). The transport form of the hydrophobic retinol molecule in the hydrophil bloodstream is a complex of RBP-bound retinol and transthyrethin which transfers retinol to the extrahepatic target tissues and prevents a glomerular filtration in the kidneys (VAN BENNEKUM *et al.*, 2001). In target tissue retinol is taken up by cells where retinol can either be stored as retinyl ester, released back into the circulation, enter the RA synthesis pathway, or metabolised to catabolic forms. The exact mechanism responsible for the regulation of homeostasis is not fully understood.

1.1.3. Metabolism

Retinoic acid biosynthesis

All-*trans*-retinol is the main precursor metabolite for at-RA which is the active vitamin A metabolite in most biological systems. Bioactivation of all-*trans*-retinol to at-RA is divided into two steps. First all-*trans*-retinol is oxidised to all-*trans*-retinal in a reversible rate-limiting step, followed by an irreversible oxidation step of all-*trans*-retinal to at-RA (reviewed in BLANER *et al.*, 1999 and BLANER & OLSON, 1994). The all-*trans*-retinol oxidation is catalysed by retinol dehydrogenases (RDH1 and RDH2) as well as several alcohol dehydrogenases (ADH1-4), whereas all-*trans*-retinal is metabolised to RA by retinal dehydrogenases (RALDH1-4). These enzymes belong to several distinct families of cytosolic and membrane-bound dehydrogenases.

Retinoid binding proteins

Retinoid binding proteins play an important role in regulation of retinoid metabolism (reviewed in NAPOLI, 1999a and ONG *et al.*, 1994). Specific cellular retinoid binding proteins are widely expressed in different tissues and are associated with a wide range of functions in the retinoid metabolism, such as protecting retinoid molecules

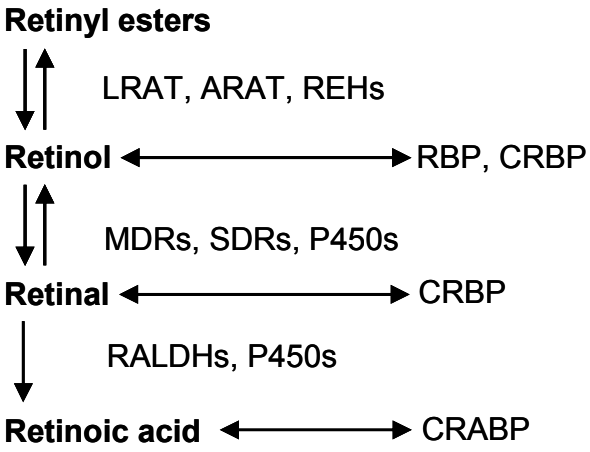

Fig.1.3: RA biosynthesis process and involved enzymes.

Retinoid metabolising enzymes regulate the synthesis of the bioactive RA in a fine tuned interplay together with retinoid binding proteins. For details see text. Abbreviations (see also abbreviation list): CRBP, cellular retinol binding protein; CRABP, cellular RA binding protein; MDR, medium-chain dehydrogenase/reductase; REH, retinyl ester hydrolase; RAL, retinal; ROL, retinol; SDR, short-chain dehydrogenase/ reductase.

from reactive cellular nucleophiles, electrophiles and oxidants, protecting cells from membranolytic effects, directing retinoids to specific metabolising enzymes, and as enhancers of transcriptional activity by delivering the retinoids to a transcription factor (NAPOLI, 1999b; NOY, 2000). Intracellular occurring RA is bound to specific enzymes, the cellular retinoic acid binding proteins, CRABPI and CRABPII, whereas the predominant intracellular fraction of retinol and retinal is bound to the cellular retinol binding proteins, CRPBI and CRPBII (ONG, 1994). The metabolism of RA bound to CRABP is about 7-times more efficient compared to free RA (REGAZZI *et al.*, 1997). Every cell type has the essential enzymes and binding proteins that are necessary to regulate the specific need of RA for several cellular processes.

Retinoic acid metabolism and catabolism

Several metabolites of at-RA have been reported that are generated *in vivo,* including 13-*cis*-RA, 9-*cis*-RA, retinoyl-glucuronide, all-*trans*-5,6-epoxy-RA, 4-OH-all-*trans*-RA, 4-oxo-all-*trans*-RA, and all-*trans*-3,4-didehydro-RA (BLANER & OLSON, 1994), whereas mammalian plasma and tissues additionally contains retinoids such as 9,11-di-*cis*-RA, 4-oxo-13-*cis*-RA (NAU & ELMAZAR, 1999) and the new me-

tabolite 4-oxo-9-*cis*-13,14-dihydro-RA (SCHMIDT *et al.*, 2003a). All-*trans*-3,4-didehydro-RA does not occur in most mammalian species, however, this metabolite was detected in chicken (THALLER & EICHELE, 1990), especially in developing limb buds of embryos (SCOTT, JR. *et al.*, 1994). Moreover, the likely precursor of all-*trans*-3,4-didehydro-retinol has also been detected in chick embryos (THALLER & EICHELE, 1990). Information about the formation of *cis*-configurated RA metabolites is very rare, with exception of the isomerisation of all-*trans* retinoids to 11-*cis*-isomers catalysed by specific enzymes. The formation of the stereoisomer 9-*cis*-RA in cells has not been clearly established yet. Various pathways of 9-*cis*-RA formation have been discussed including isomerisation of at-RA, probably through non-enzymatic processes or enzymatic oxidation of 9-*cis*-retinol to 9-*cis*-retinal and then to 9-*cis*-RA (LABRECQUE *et al.*, 1995; MERTZ *et al.*, 1997; ROMERT *et al.*, 1998; URBACH & RANDO, 1994a; URBACH & RANDO, 1994b). In several investigations the enzymes RDH, RALDH1 and RALDH2 have been shown to oxidise 9-*cis*-retinol and 9-*cis*-retinal to form 9-*cis*-RA *in vitro* (EL AKAWI & NAPOLI, 1994; LABRECQUE *et al.*, 1995; MERTZ *et al.*, 1997; PAIK *et al.*, 2000). These findings were supported by the detection of 9-*cis*-retinol and 9-*cis*- retinyl esters in livers of mice, albeit in much lower levels compared to all-*trans*-retinol (PAIK *et al.*, 2000). PIJNAPPEL *et al.* (1998) reported the identification of 4-oxo-9-*cis*-RA as an *in vivo* retinoid metabolite in *Xenopus* embryos. Some other 9-*cis* isomers of at-RA were detected in mice by TZIMAS *et al.* (1994) after administration of 9-*cis*-RA. 9,13-di-*cis*-RA was found as a major metabolite in plasma, whereas a number of polar metabolites including β-glucuronides of 9-*cis*-RA and 4-oxo-9-*cis*-RA were also detected. SHIRLEY *et al.* (1996) reported the occurrence of 13-*cis*-RA, 9,13-di-*cis*-RA and at-RA, as well as 4-OH-9-*cis*-RA, 4-oxo-9-*cis*-RA, and 9-*cis*-13,14-dihydro-RA in a minor degree after the administration of 9-*cis*-RA to rats.

Oxidative metabolism of retinoic acid

After processing their physiological actions at-RA and its isomers are catabolised by specific enzymes and excreted. In this phase-I-metabolism polar metabolites such as all-*trans*-5,6-epoxy-RA, 4-OH-all-*trans*-RA, and 4-oxo-all-*trans*-RA are formed

(BLANER & OLSON, 1994). It must be pointed out that the metabolism of at-RA may on the one hand form catabolic metabolites in terms of protecting cells of teratogenic levels of at-RA, but can on the other hand also lead to biologically active metabolites. Although some oxidised RA metabolites have been suggested to be biologically active as well (cf. chapter 1.2.7) (IDRES *et al.*, 2002; PIJNAPPEL *et al.*, 1993). Oxidation is generally viewed to be the first step in the elimination pathway of RA *in vivo*.

Role of cytochrome P450 system in the retinoic acid oxidation

It is likely that members of the microsomal cytochrome P450 mono-oxygenase superfamily play a key role in the oxidative inactivation pathways of RA. Several research groups have confirmed that various cytochrome P450 enzymes – including members of the CYP1A, CYP2B, CYP2C and CYP3A families – can oxidise RA to polar metabolites (AHMAD *et al.*, 2000; MARILL *et al.*, 2000; MARTINI & MURRAY, 1993; ROBERTS *et al.*, 1992). Other studies showed that the oxidative RA metabolism is inhibited by P450-inhibitors (PIGNATELLO *et al.*, 2002; STOPPIE *et al.*, 2000) and that acute administration of at-RA induces CYP26 expression in the early mouse embryos (RAY *et al.*, 1997). Initially RA is converted by a hydroxylation on the β-inone ring at the C4- or C18-position (ROBERTS & FROLIK, 1979; VAN WAUWE *et al.*, 1992; WILLIAMS & NAPOLI, 1985). The liver is the principle organ for RA inactivation, because it receives the bulk of retinoids circulating in blood and due to a tailored machinery of retinoid-metabolising enzymes. Therefore the liver plays a key role in homeostasis of retinoid metabolism. MCSORLEY & DALY (2000) reported that CYP2C8 and CYP3A4 are the major at-RA 4-hydroxylating cytochrome P450 enyzmes in human liver microsomes. Additionally MARILL *et al.* (2002) demonstrated that these enzymes also metabolise 9-*cis*- and 13-*cis*-RA to 4-OH- and 4-oxo-metabolites. The CYP26 subfamily, also called P450RAI (retinoic acid inducible) is connected to C4-hydroxylation, too. *In vitro* studies showed that CYP26 catalysed the hydroxylation of at-RA as well as 9-*cis*- and 13-*cis*-RA to form 4-OH- and 4-oxo-metabolites and 18-OH-RA (ABU-ABED *et al.*, 1998; FUJII *et al.*, 1997; NADIN & MURRAY, 1996; WHITE *et al.*, 1996).

CYP26 transcripts have been detected in many human tissues. Highest levels were found in fetal and adult liver, heart, pituitary gland, adrenal gland, placenta and regions of the brain (TROFIMOVA-GRIFFIN & JUCHAU, 1998). A possible major role of CYP26 in embryos could be the protection of specific tissues from excess RA levels during development (TROFIMOVA-GRIFFIN & JUCHAU, 1998). Many questions about the exact role of this enzyme family in the RA metabolism and catabolism remain to be unknown.

Other metabolism of retinoic acid

Another metabolic pathway of retinoids beside the phase-I oxidative metabolism is the glucuronidation of the carboxyl group to form retinyl β-glucuronide (the glucuronide form of all-*trans*-retinol) and retinoyl β-glucuronide (the glucuronidated form of RA) (GENCHI *et al.*, 1996; MELOCHE & BESNER, 1986; TUKEY & STRASSBURG, 2000; ZILE *et al.*, 1982).

1.1.4. Endogenous levels of retinoids

All-*trans*-retinol is by far the most predominant retinoid in most tissues such as plasma, liver and kidney (BRINKMANN *et al.*, 1995; NAU & ELMAZAR, 1999; SCHMIDT *et al.*, 2003a). BRINKMANN *et al.* (1995) detected also a low proportion of other retinol isomers (9-*cis*-, 13-*cis*-, 9,13-di-*cis*-retinol) in liver samples. The endogenous levels of at-RA are very low compared to all-*trans*-retinol. Tab.1.1 summarises the levels of several RA metabolites detected in serum and liver of mice and humans from diverse studies. It has been shown by several researchers that the increase of levels of RA and its isomers is not identical in certain tissues after vitamin A supplementation (ARNHOLD *et al.*, 1996; ECKHOFF & NAU, 1990; SCHMIDT *et al.*, 2002; SCHMIDT *et al.*, 2003a). Feeding experiments with mice and vitamin A supplementation studies with humans revealed that the increase of at-RA levels in tissues such as liver and kidney was relative low (ARNHOLD *et al.*, 1996; ECKHOFF & NAU, 1990; SCHMIDT *et al.*, 2003a). In contrast, the increase of levels of other RA metabolites such as 13-*cis*- and 4-oxo-13-*cis*-RA was more pronounced in several tissues of mice and human followed by vitamin A supplementation or liver

consumption (ARNHOLD *et al.*, 1996; ECKHOFF & NAU, 1990; SCHMIDT *et al.*, 2003a). The levels of at-RA are very stringently regulated and therefore do not fluctuate that obviously, which is clearly reflected in the serum levels. Metabolites such as 13-*cis*- and 4-oxo-13-*cis*-RA seem to be the prominent plasma metabolites, especially in human (ECKHOFF *et al.*, 1991; ECKHOFF & NAU, 1990; NAU, 1990). The endogenous occurrence of 9-*cis*-RA in mammals is still debated. HEYMAN *et al.* (1992) reported the occurrence of relative high 9-*cis*-RA levels in liver and kidney of untreated wild type mice. Such high levels were not detected in any other study again. Other investigators reported the occurrence of 9-*cis*-RA and 9,13-di-*cis*-RA in human plasma only after consumption of liver or vitamin A supplementation (ARNHOLD *et al.*, 1996), whereas these compounds were under the detection limit in "normal" plasma. It was the first time that these RA metabolites were identified in humans. However, the plasma levels of 9-*cis*-RA after liver consumption decreased within a few hours to levels at or below the analytical detection limit of 0.2 ng/ml. It is still unclear, if 9-*cis*-RA is occuring endogenously in mammalian blood or tissue, including the embryo. If at all, the concentrations appear to be very low. Regarding to these facts the role of 9-*cis*-RA in retinoid signaling pathways as a putative RXR ligand is difficult to evaluate (NAU & ELMAZAR, 1999; WERNER & DELUCA, 2001).

1.1.5. The retinoic acid signaling pathway

Molecular mechanism of action of retinoic acid

With exception of the visual process, where retinal is the active principle, the major effects of retinoids are linked to RAs, whereas at-RA is viewed as the most active naturally occurring retinoid. The activity of at-RA on the cellular level is mediated through two families of nuclear receptors, the retinoic acid receptors (RARs), and the retinoid X receptors (RXRs) (reviewed in BASTIEN & ROCHETTE-EGLY, 2004).

Tab.1.1: Levels of important RA metabolites in mouse and human.
(Mean levels are marked in bold)

		at-RA	9-*cis*-RA	13-*cis*-RA	9,13-di-*cis*-RA	4-oxo-13-*cis*-RA	*S*-4o-9c-dh-RA	Reference
Human					**c[ng/ml]**			
Plasma	Mean	**3.5**	-	-	-	-	-	(DE LEENHEER *et al.*, 1982)
	Range	2.7-4.2	-	-	-	-	-	
	Mean	**4.9**	-	-	-	-	-	(NAPOLI *et al.*, 1985)
	Range	2.8-6.6	-	-	-	-	-	
	Mean	**0.8**	n.d.	**1.1**	n.d.	**2.4**	-	(ARNHOLD *et al.*, 1996)
	C_{max} (a)	2.0	2.7	21.5	17.1	32.1	-	
	Mean	**1.3**	n.d.	**1.6**	n.d.	**3.7**	-	(ECKHOFF & NAU, 1990)
	C_{max} (b)	3.9	-	9.8	-	7.6	-	
Serum	Mean	**1.4**	-	**1.4**	-	-	-	(TANG & RUSSELL, 1990)
	Range	1.1-1.9	-	1.0-2.2	-	-	-	
	Mean	1.4	-	1.8	-	2.4	< d.l.	(SCHMIDT *et al.*, 2003a)
Liver					**c[ng/g]**			
	Mean	**15.8**	**0.6**	**1.1**		**2.1**&**0.6** (c)	**10.3** (d)	(SCHMIDT *et al.*, 2003a)
Mouse					**c[ng/ml]**			
Serum	Mean (e)	**1.1**	**0.3**	-	-	-	**0.6**	(SCHMIDT *et al.*, 2002)
	Mean (f)	0.5	0.4	-	-	-	6.6	
					c[ng/g]			
Liver	Mean (e)	**5.6**	-	**1.2**	-	-	**11.4**	(SCHMIDT *et al.*, 2002)
	Mean (f)	7.6	-	1.5	-	-	117	
		-	< d.l.	-	-	< d.l.	-	(SCHMIDT *et al.*, 2003a)
		-	4.0	-	-	-	-	(HEYMAN *et al.*, 1992)

(a) After liver consumption
(b) max. concentration after a diet of 833 IU vitamin A/kg bw, max. 6 h after dosing
(c) only detected in two samples
(d) only detected in one sample
(e) fed with a diet of 15,000 IU vitamin A (retinyl palmitate)/kg bw
(f) fed with a diet of 150,000 IU vitamin A (retinyl palmitate)/kg bw
< d.l. = under detection limit

RARs and RXRs are members of the superfamily of nuclear receptors, which are ligand-dependent transcription factors that regulate the expression of large gene networks (GRONEMEYER & LAUDET, 1995; PERLMANN & EVANS, 1997). These receptors consist of six domains (A-F, see fig.1.4), the A/B region is responsible for ligand independent transactivation, the C domain containing two zinc fingers is responsible for DNA-binding, the E domain for ligand binding and ligand-dependent transactivation (CHAMBON, 1994; MANGELSDORF & EVANS, 1995).

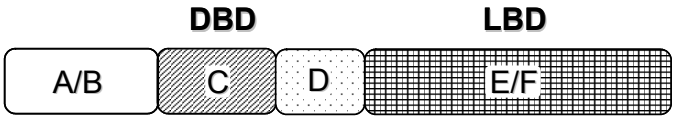

Fig.1.4: Schematic domain structure of retinoid nuclear receptors.
For details see text, Abbr.: DBD, DNA-binding domain; LBD, ligand binding domain.

Within the retinoid receptor subfamily there are different receptor subtypes with multiple isoforms. The RAR exists in the three subtypes α, β or γ (BENBROOK *et al.*, 1988; GIGUERE *et al.*, 1987; KRUST *et al.*, 1989; PETKOVICH *et al.*, 1987) as well as RXR (HAMADA *et al.*, 1989; LEID *et al.*, 1992; MANGELSDORF *et al.*, 1990; YU *et al.*, 1991). Each subtype is encoded by a single gene. Every RAR and RXR subtype is expressed in a tissue and developmental specific manner, suggesting that each receptor subtype may have a specific role in regulating gene activity in a certain developmental stage of tissue (JAVIER PIEDRAFITA & PFAHL, 1999). All cells which have been studied so far express one or several retinoid receptors. Therefore it is likely that retinoids are involved in cell regulatory mechanisms of every single cell in the organisms.

At-RA binds to the ligand-binding domain (LBD) of RAR (fig.1.5A), which functions as a heterodimer together with RXR (fig.1.5B). The ligand-receptor-heterodimer complexes act as transcriptional regulators of a number of retinoid regulated genes, while the DNA-binding domain (DBD) of the heterodimer-complex binds to specific RA responsive elements (RARE) (fig.1.5C) in the promoter region of target genes and thus initiates the transcription (see fig.1.5). RAREs generally consist of 6-base pair repeated motifs that are either a direct or invert repeats of these sequence (5'AGGTCA) separated by two (DR2) or five (DR5) base pairs (CHAMBON, 1994; MANGELSDORF & EVANS, 1995). The DR5 type is most frequent on RA-regulated genes, whereas the DR2 type is very rare (BALMER & BLOMHOFF, 2005). Gene transcriptional activation by RAR/RXR heterodimers is mainly activated by RAR-selective ligands (FORMAN *et al.*, 1995).

The RXRs can, beside being a heterodimerisation partner for the RARs, also form RXR/RXR-homodimers, and regulate transcription of certain genes via a retinoid X responsive element (RXRE), characterised by a DR1 (reviewed in BASTIEN & ROCHETTE-EGLY, 2004). RXRs are viewed as a "silent-partner" since they can also function as heterodimer partners for several other receptors of the nuclear receptor superfamily. Via the mentioned pathways retinoid receptors regulate the expression of a multitude of target genes involved in development such as growth factors, growth factor receptors, cell adhesion molecules, intercellular matrix molecules, other transcription factors such as *hox* genes, some hormones and cytokines, as well as other receptors of the hormone receptor superfamily. In addition, the retinoid pathways themselves are affected through the control of expression of retinoid binding proteins, metabolising enzymes and autoregulation of retinoid receptors (CHAMBON, 1996; DOLLE *et al.*, 1990; KASTNER *et al.*, 1995; MORRISS-KAY & SOKOLOVA, 1996; NAU & ELMAZAR, 1999; SMITH *et al.*, 1998; VAN DER SAAG, 1996). Regarding to this diverse receptor-mediated effects, at-RA is an important regulator of cell growth and differentiation in both embryonic development and adult organism (CHAMBON, 1996; COLLINS & MAO, 1999).

Several researchers observed synergistic effects between RAR and RXR-selective ligands. Co-administration of RAR- and RXR-selective agonists to developing mice revealed strong synergistic responses in regard to a number of teratogenic effects such as spina bifida, craniofacial and urogenital malformations, whereas single administration of RAR-specific ligands had a weaker effect and RXR ligands had no effect, respectively (ELMAZAR *et al.*, 1997). Similarly, LU *et al.* (1997b) observed, that the expression of certain genes such as *RARβ* and *Hoxb*-1 was more effectively activated by a combination of RAR- and RXR-selective ligands in the chick wing bud. Taken together these results suggest that the activation of certain genes profits from the presence of ligand-bound RAR and ligand-bound RXR and therefore results in a synergistic teratogenic response.

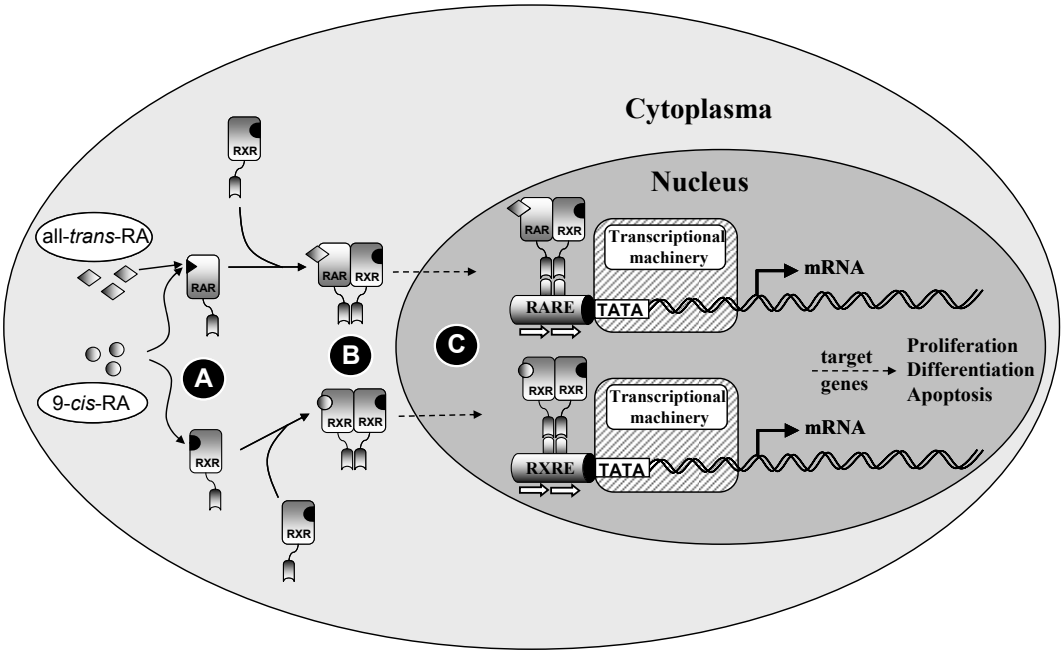

Fig.1.5: Molecular mechanism of action of RA.

A) The initial step in the retinoid regulated expression of target genes is the binding reaction of the ligand to the LBD of the corresponding receptor, whereas at-RA binds to RARs and 9-*cis*-RA binds to both RAR or RXR. In response of the ligand binding, the receptor changes its allosteric conformation in the LBD, which allows the interaction with co-activators (not shown). **B)** These coactivators can activate the formation of RAR/RXR heterodimers or RXR/RXR homodimers. **C)** The ligand-receptor-dimer complex binds to the specific response elements (RARE or RXRE, for details see text) in the promoter regions of target genes and activates the transcription machinery. Abbreviations are listed in the abbreviation list.

1.1.6. Physiological functions

Retinoids control numerous processes which are critical for reproduction and development such as differentiation of epithelial tissues, proliferation, apoptosis and morphogenesis, while another major task of retinoids, apart from the gene regulatory pathway of retinoids, is the function of 11-*cis*-retinal as a chromophore in the visual signal transduction cascade (for reviews see NAU & BLANER, 1999 and SPORN *et al.*, 1994). Effects of retinoids are extensive and comprehensive. This chapter presents only a brief overview.

Proliferation and differentiation of epithelial tissues

Vitamin A is one of the critical factors regulating processes such as differentiation, proliferation and cell death (apoptosis). While providing a balance between those processes, retinoids contribute to the maintenance of tissue homeostasis in adult tissues and directs normal development during embryonic morphogenesis (HARVAT & JETTEN, 1999). The role of retinoids in promoting proliferation, differentiation or apoptosis has been extensively described both *in vivo* (reviewed in LU *et al.*, 1999; MADEN, 1999; PACKER & WOLGEMUTH, 1999 and ZILE, 1999) and *in vitro* (reviewed in AGADIR & CHOMIENNE, 1999; HARVAT & JETTEN, 1999; NASON-BURCHENAL & DIMITROVSKY, 1999 and VAKIANI & BUCK, 1999). Proliferation processes are controlled through retinoids by modulating the action of negative and positive growth factors, including EGF, TGFα, TGFβ, insulin, IL1α, IL6, interferon γ, estrogen and vitamin D_3 (for reviews see BLUTT *et al.*, 1997; KOLLA *et al.*, 1996; MATIKAINEN *et al.*, 1996), whereas the regulation can occur at the level of expression of growth factors or cytokines, their corresponding receptors, binding proteins or downstream genes in the signaling pathway. In leukemia cells retinoids provoked the inhibition of growth, proliferation and the induction of differentiation, as well as in both normal and malignant cells (AGADIR & CHOMIENNE, 1999). The regulating function of retinoids on growth and differentiation is also vital for the maintenance of epithelial cell integrity in most superficial linings (e.g. mucous membranes) of the body (DE LUCA, 1991). Vitamin A deficiency causes *squamous metaplasia* in epithelial tissues, primarily in mucous secretory tissues, provoked by an overall increase in Keratin synthesis (DE LUCA *et al.*, 1985; DE LUCA, 1991). This regulating function of retinoids in epithelial cells is inevitable at all phases of life from conception, to growth of the embryo and maintenance of the adult organism (DE LUCA, 1991).

Reproduction

Vitamin A is essential in both male and female reproduction. In male all-*trans*-retinol is required by the testis to maintain spermatogenesis, whereas in the female all-*trans*-retinol is essential for oogenesis as well as for the placental and embryonic develop-

ment to avoid fetal resorption (reviewed in ESKILD & HANSSON, 1994 and PACKER & WOLGEMUTH, 1999).

Optic process

11-*cis*-retinal has an important role in phototransduction process of visual cycle of vertebrates, where it functions as a light-sensitive molecule in the photoreceptor cells of the retina. 11-*cis*-retinal is a cofactor of the visual pigment Rhodopsin together with the protein Opsin, where it is covalently bound to. In presence of light 11-*cis*-retinal isomerises and changes its configuration which results in a nerve impulse in the ocular system of the central nervous system (reviewed in SAARI, 1994 and SAARI, 1999).

Immunfunction

Retinoids are involved in interactions of immune cells and soluble factors and therefore act as important regulators in the immune system (HAYES *et al.*, 1999). Both clinical and experimental work has shown that vitamin A deficiency is associated with decreased resistance to infection (SEMBA *et al.*, 1993; WOLBACH & HOWE, 1978). Furthermore, retinoids are associated with a functioning immune system while maintaining epithelial barriers which are the first defence lines of immune systems and normally counteract environmental pathogens (reviewed in ROSS & HÄMMERLING, 1994).

Vitamin A deficiency and excess

It is known for a long time that a correct balance of vitamin A is required for development of the CNS, eye, face, dention, ear, limb, urogenital system, cardiovascular system, thyroid, thymus, vertebral column, skin, and lungs, and any disturbance of that balance either in excess vitamin A (COBERLY *et al.*, 1996; LAMMER *et al.*, 1985; NAU, 1993; reviewed in NAU *et al.*, 1994) or in deficiency (WILSON *et al.*, 1953; reviewed in ZILE, 1999 and ZILE, 2001) will result in a disturbed development.

Vitamin A is required when the primitive heart and the cardiovascular system forms up and the hindbrain begins to specify (ZILE, 1999). The absence of sufficient vitamin A at this critical time results in abnormalities, such as failed embryo segmentation, growth, and vascularisation, and can eventually cause early embryonic death (THOMPSON *et al.*, 1969; WELLIK & DELUCA, 1995). Beside heart and CNS, major target tissues, which are influenced by vitamin A deficiency, include structures derived from these organs such as the circulatory, urogenital and respiratory system, and other organs which are dependent on these systems like the skull, skeleton and limbs (ZILE, 1999). The spectrum of congenital malformations in vitamin A deficient embryos resembles that of embryos exposed to excess vitamin A (reviewed in HOFMANN & EICHELE, 1994; LU *et al.*, 1999 and MADEN, 1994).

Vitamin A and retinoids are classical teratogenes in various species. Retinoid treatment of fetuses during early organogenesis resulted in abnormalities of central nervous and cardiovascular systems, defects of the genitourinary tract, and the palate (NAU *et al.*, 1994). Limb malformations, including missing, fused and misshaped elements, are typically observed in the mouse following excessive systemic at-RA exposure at fetal stages (KOCHHAR, 1973; KWASIGROCH & KOCHHAR, 1980). Nearly all tested retinoids induced the same spectrum of malformations, which were depended on dose and developmental stage of embryos (NAU *et al.*, 1994; NAU & BLANER, 1999).

In adult organisms vitamin A deficiency and excess severely changes the differentiation state of cells from epithelial tissues. Epithelial cell from vitamin A deficient adult organisms cannot differentiate normally and due to a loss of the ability to secrete glycoproteins they change their structure and become stratified and cornified (reviewed in DE LUCA *et al.*, 1985 and DE LUCA, 1991). On the other hand, excess vitamin A is membranolytic and hepatotoxic in adult organisms (NAU *et al.*, 1994). The intake of high vitamin A quantities, via supplementation or liver consumption, over a prolonged time causes a hypervitaminosis A with its characteristic appearances. Chronic symptoms emerge after a daily vitamin A dose of 20.000-50.000 IU

over a longer period (HATHCOCK *et al.*, 1990). An example for negative effects of vitamin A excess are disturbed bone development and function (FORSYTH *et al.*, 1989; HOUGH *et al.*, 1988; MELLANBY, 1941). MELHUS *et al.* (1998) reported a decreased bone mineral density and an increased risk for hip fractures in humans already after a daily dietary vitamin A intake of 5.000 IU.

Retinoids in the embryonic development

Retinoids are critical signaling molecules for cell growth and differentiation during embryogenesis (for reviews see DE LUCA, 1991; GUDAS *et al.*, 1994; GUDAS, 1994; MENDELSOHN *et al.*, 1992; REICHEL & JACOB, 1993; SUMMERBELL & MADEN, 1990; and TABIN, 1992). The effects of vitamin A deficiency in embryonic development are already described (see above). The action of retinoids in normal and teratogenic development cannot be explained by a single mechanism (ROSS *et al.*, 2000). Rather at-RA is part of a cascade of signaling molecules regulating morphogenetic events within the embryo. Beginning with the first cell division, several combined processes regulate the organisation and septation of different tissues, which leads to an embryo and thereafter to a fetus. These events, comprising the determination of axial polarity and cell differentiation, are directed by signaling substances, whose concentrations vary locally within the embryo. Cells are exposed to different concentrations of at-RA depending on their position. At-RA, in partnership with its different receptors (RARs and RXRs), creates a specific gene expression pattern which is in the end translated into a specific phenotype with discrete structures (ROSS *et al.*, 2000). The RARs and RXRs play an important role in the morphogenesis. Mice mutants from retinoid receptor knockouts, lacking complete genes or certain isoforms of RARs and RXRs, display discriminative abnormalities (LOHNES *et al.*, 1993; LUFKIN *et al.*, 1993), which resemble those observed in the offspring of vitamin A deficient wild type mice (ROSS *et al.*, 2000). At-RA (THALLER & EICHELE, 1987) as well as the certain RARs and RXRs (MICHAILLE *et al.*, 1994; SMITH *et al.*, 1995; SMITH & EICHELE, 1991; VIALLET & DHOUAILLY, 1994) exhibit a specific spatial and temporal distribution pattern within developing embryos. RARα and RXRβ are expressed ubiquitously,

whereas the expression of certain subtypes and isoforms of RARs and RXRs is limited to specific tissues and specific stages of the embryonic development. The mechanisms responsible for the regulation of the spatial and temporal differences in at-RA concentrations during the embryogenesis are widely unknown. It is likely that the RA binding proteins (CRBP-I and CRABP-I) are involved in these regulation processes, which are also expressed in a spatiotemporally confined manner within the developing embryo (MORRISS-KAY & SOKOLOVA, 1996; ROSS *et al.*, 2000).

Retinoids in the limb development

The developing limb has been extensively used to study morphogenesis (reviewed in EICHELE, 1989; HOFMANN & EICHELE, 1994 and LU *et al.*, 1999). The undifferentiated mesenchymal cells of limb buds differentiate into muscle, cartilage, and bone cells of the later wing. The digits of the wing, oriented along the anterior-posterior (APo) axis of the wing bud, are ordered by the zone of polarising activity (ZPA) in the posterior region of the bud (KOUSSOULAKOS, 2004) (cf. fig.1.6). The fate of mesenchymal cells along the APo axis is dependent on the distance to the ZPA. Cells next to ZPA form a digit 4; cells farther away become a digit 3 or 2, respectively.

1.1.7. Chick limb bud model

In developing chick limbs it was discovered that local application of at-RA released from a carrier bead placed to the anterior margin of a chick limb bud evokes digit pattern duplications in a dose-dependent fashion (see also methods and SUMMERBELL, 1983; TICKLE *et al.*, 1982; TICKLE *et al.*, 1985; reviewed in HOFMANN & EICHELE, 1994). The normal digits pattern of 234 is invariable changed into a six digit mirror-image duplication pattern with a full set of additional digits (432234, 43234 pattern) Reducing the concentration of at-RA produced fewer additional digits with an additional digit 2 and 3 (2234, 32234 pattern). The effect is thus dependent on the dose of applied at-RA but also on the position of implantation *(application of at-RA to the posterior side of the limb bud had no effect)* (HOFMANN & EICHELE,

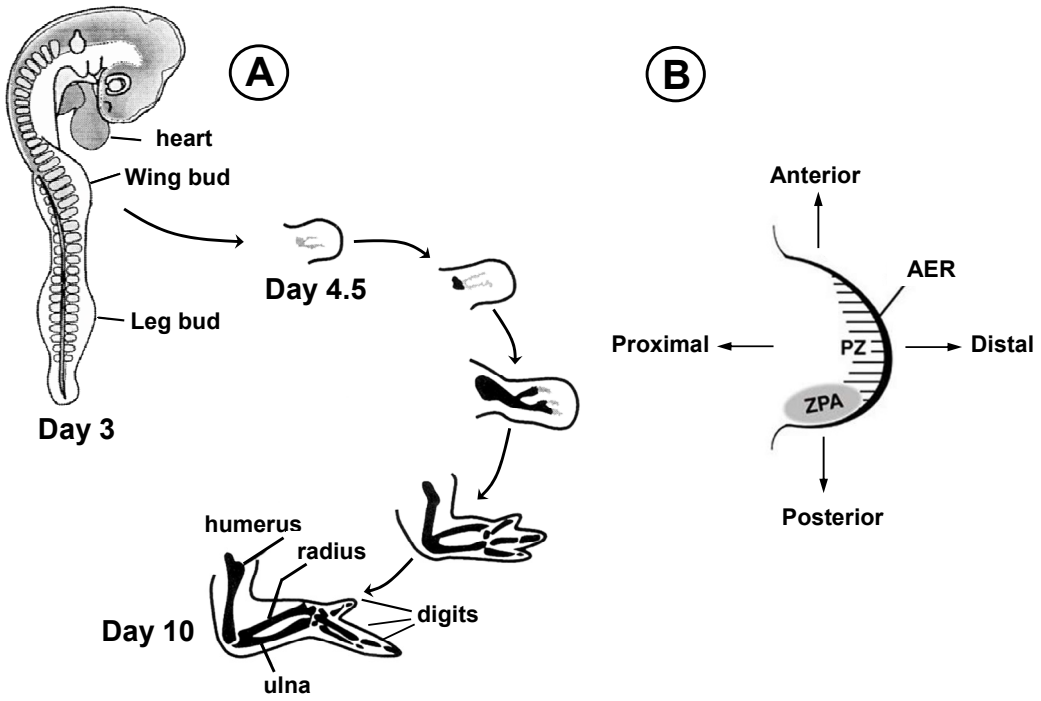

Fig.1.6: Embryology and signaling regions in the chick limb bud.

A) At day 3 (HH-stage 20) cells from the wing field begin to grow distally and form the limb bud which is at that time about 1 mm wide, by day 10 (stage 36) the development of the wing with the skeletal elements humerus, radius, ulna and the three digits is completed. **B)** There are two major signaling regions that mediate patterning and growth of developing limb buds. The apical ectodermal ridge (AER) promotes the distal outgrowth of the limb. The ZPA (positioned posteriorly) acts as an organiser for the anterioposterior axial pattern. The cells from the progress zone (PZ) at the distal tip receive growth signals from the AER and ZPA. Removing any of theses zones results in a truncated limb.

1994). At-RA has to be implanted into Hamburger-Hamilton (HH-)stage 22 (HAMBURGER & HAMILTON, 1951) old chick embryos, whereas the minimum period of approximately 10-12 h treatment time is required to induce duplications (EICHELE *et al.*, 1985).

Implanted beads release constantly at-RA which diffuses into the mesenchymal tissue and forms a concentration gradient across the APo axis (EICHELE & THALLER, 1987). This asymmetrical spatial distribution is responsible for the induction of duplications (EICHELE & THALLER, 1987). It has been proposed that at-RA mimics the ZPA (TICKLE *et al.*, 1975), by initiating a cascade of signaling

molecules [HoxD gene products, SHH (gene product of *shh*), Fibroblast growth factors (FGFs) and presumably other molecules] that, when expressed together, bring about the formation of additional digits (HELMS *et al.*, 1994). In other words at-RA induces effector genes that regulate the limb development. Examples of such genes are Bone morphogenetic protein-2 (*bmp-2*) (FRANCIS *et al.*, 1994), various Homeobox genes including *Hoxb-8* (CHARITE *et al.*, 1994; HAYAMIZU & BRYANT, 1994; HELMS *et al.*, 1994; IZPISUA-BELMONTE & DUBOULE, 1992; LU *et al.*, 1997a; STRATFORD *et al.*, 1997) and Sonic hedgehog (*shh*) (HELMS *et al.*, 1994; RIDDLE *et al.*, 1993). Cytochrome P450 (*Cyp26*; (MARTINEZ-CEBALLOS & BURDSAL, 2001; SWINDELL *et al.*, 1999) and RA receptor beta 2 (*RARb2*; (HAYAMIZU & BRYANT, 1994; LU *et al.*, 1997a) are also locally induced in the limb bud by exogenously applied at-RA although their role in normal limb development is not fully understood. Nonetheless they are likely mediators of the well-known teratogenic effects of retinoids on limb development. The local application of RA in from small ion-exchange beads implanted into embryos is a suitable tool for studying the action of vitamin A compounds in vertebrate development by several reasons: The chick limb bud is very easily assessable for experimental manipulations, and the local placement of the retinoids provides the advantage to mimic the action of retinoids in a special region (WEDDEN *et al.*, 1990).

1.1.8. "Biological active" retinoid metabolites

Several endogenous occurring RA metabolites have shown to be biologically active *in vivo* and *in vitro*, including those which are viewed as catabolic products. 4-oxo-all-*trans*-RA is a highly active modulator in embryonic development and influenced the development of the APo body axis of *Xenopus* embryos in a way similar to at-RA (PIJNAPPEL *et al.*, 1993). Additionally, 4-oxo-all-*trans*-RA was able to induce cell differentiation in F9 mouse teratocarcinoma cells equipotent to at-RA (NIKAWA *et al.*, 1995). VAN DER LEEDE *et al.* (1997) showed that 4-oxo- and 4-OH-all-*trans*-RA could inhibit the proliferation of RA-sensitive breast cancer cell lines in a concentration-dependent fashion. 4-OH-all-*trans*-RA, 4-oxo-all-*trans*-RA as well as all-*trans*-5,6-epoxy-RA showed significant biological activity in human keratinocytes,

mouse melanoma cells and in mouse skin (REYNOLDS *et al.*, 1993). IDRES *et al.* (2001) have shown induced maturation (inhibited cell growth, blocked cell cycle progression and induced differentiation) of NB4 promyelocytic leukemia cells by the four at-RA metabolites 4-oxo-all-*trans*-, 4-OH-all-*trans*-, 18-OH-all-*trans*-, and all-*trans*-5,6-epoxy-RA. 9,13-di-*cis*-RA induced the expression of RARα, RARβ and the production of a plasminogen activator in human liver stellate cells (IMAI *et al.*, 1997). As already mentioned, at-RA is viewed as the retinoid with the highest biological activity in mammals. However, it seems likely that this is not the case in several other vertebrate classes, since THALLER & EICHELE (1990) demonstrated that all-*trans*-3,4-didehydro-RA is the principle active retinoid in the developing limb bud of chick embryos. All-*trans*-3,4-didehydro-RA was equipotent at inducing digit duplications in chick limbs compared to at-RA, but it was detected in 4-6-fold higher concentrations (SCOTT, JR. *et al.*, 1994; THALLER & EICHELE, 1990). Even though 9-*cis*-RA was not identified in chick wings, it was shown to be morphogenetically active in this system (THALLER *et al.*, 1993). 9-*cis*-RA evokes digit duplications with a greater potency compared to at-RA. Findings from BLUMBERG *et al.* (1996) in early *Xenopus* embryos showed that at- and 9-*cis*-RA isomers were not detectable, rather all-*trans*-4-oxo-retinal and -retinol have been identified and characterised as the major bioactive retinoids in these embryos.

The natural retinoids which are known to act as retinoid receptor ligands *in vitro* can be divided into two groups: Ligands that activate only RAR-RXR heterodimers via RARs and those which activate both RARs and RXRs. The first group of ligands involves beside at-RA (reviewed in CHAMBON, 1996) other metabolites such as 4-oxo-all-*trans*-retinol and 4-oxo-all-*trans*-retinal (ACHKAR *et al.*, 1996; BLUMBERG *et al.*, 1996), 9,13-di-*cis*-RA (IMAI *et al.*, 1997; OKUNO *et al.*, 1999), 4-oxo-all-*trans*-RA (IDRES *et al.*, 2002; PIJNAPPEL *et al.*, 1993), all-*trans*-5,6-epoxy-RA, 13-*cis*-RA, 18-all-*trans*-OH-RA, 4-OH-all-*trans*-RA (IDRES *et al.*, 2002), all-*trans*-3,4-didehydro-RA (ALLENBY *et al.*, 1993; SANI *et al.*, 1997; THALLER & EICHELE, 1990) and all-*trans*-3,4-didehydro-retinol (COSTARIDIS *et al.*, 1996). The second group of RXR and RAR ligands and transactivators in-

cludes 9-*cis*-RA (ALLENBY *et al.*, 1993; DURAND *et al.*, 1992; HEYMAN *et al.*, 1992; IDRES *et al.*, 2002; LEVIN *et al.*, 1992; NAPOLI, 1996), 9-*cis*-3,4-didehydro-RA (ALLENBY *et al.*, 1993) and 4-oxo-9-*cis*-RA (PIJNAPPEL *et al.*, 1998). The mentioned retinoids were shown to bind and transactivate RAR- or RXRα, -β or -γ, respectively, in several *in vitro* systems, predominantly binding and transactivation assays. 9-*cis*-RA has been shown to activate RAR-RXR heterodimers more efficiently than natural RAR ligands (MINUCCI *et al.*, 1997). Other retinoids, such as 14-OH-4,14-retro-retinol, anhydro-retinol (BUCK *et al.*, 1991; BUCK *et al.*, 1993) and 13,14-di-OH-retinol (DERGUINI *et al.*, 1995; EPPINGER *et al.*, 1993) which are essential for the proliferation of lymphocytes, mediate their function independently from the retinoid receptors.

1.1.9. Identification and characterisation of the new metabolite

Recently, a novel major endogenous retinoid metabolite, occurring primarily in the liver of mice and rats in comparatively high levels has been isolated and characterised by SCHMIDT *et al.* (2002). It was found that the levels of the new metabolite increase dose-dependently in serum, kidney, and especially in liver of mice supplemented with retinyl palmitate (SCHMIDT *et al.*, 2002). These findings were surprisingly because this increase was complementary to a dose-dependent decrease of at-RA levels in serum, kidney, and brain in the samples from the same animals. The metabolism of the new metabolite seems to be sensitive for exposure to environmental pollutants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). TCDD exposure had a profound effect on the hepatic levels of the new metabolite in mice, with a decrease of approximately 90% (HOEGBERG *et al.*, 2005). Altered retinoid homeostasis has been known for a long time as one of the most sensitive markers of exposure to environmental pollutants such as TCDD (reviewed in NILSSON & HAKANSSON, 2002). Recent studies analysing the tissue levels of the new metabolite after dioxin exposure show that it is an extremely sensitive marker in both mice (HOEGBERG *et al.*, 2005) and rats (SCHMIDT *et al.*, 2003b).

The newly discovered metabolite was isolated from mouse livers and characterised by mass spectral- (MS), ultraviolet- (UV-Vis), and nuclear magnetic resonance analyses (NMR) as 9-*cis*-4-oxo-13,14-dihydro-RA (4o-9*c*-dh-RA) (SCHMIDT *et al.*, 2002). To obtain sufficient material to record an 1H-NMR spectrum and to perform further studies, it was necessary to isolate the new metabolite preparatively extent with several pooled livers (described in SCHMIDT *et al.*, 2002). The chemical structure of the isolated new RA-metabolite (fig.1.7) was finally assigned by its proton-nuclear magnetic resonance (1H-NMR) spectrum. 4o-9*c*-dh-RA is characterised by a chiral carbon at C13 (fig.1.7).

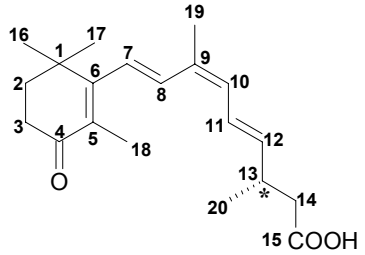

Fig.1.7: Molecular structure of the new RA-metabolite, *S*-4o-9*c*-dh-RA.

To investigate the biological activity of the new metabolite, it was necessary to develop a synthetic pathway for *S*-4o-9*c*-dh-RA to generate sufficient quantities of the compound. This work has been recently accomplished by M. Stefan and is described in a dissertation thesis (STEFAN, 2006). More precisely the thesis describes the development of an enantio-selective synthesis pathway of the new metabolite *S*-4o-9*c*-dh-RA, and other diastereomers of 4-oxo-13,14-dihydro-RA (4-o-dh-RA) such as all-*trans*-, 11-*cis*-, 9,11-di-*cis*-4-oxo-dh-RA. The diastereomers were separated and collected using column chromatography. Collected fractions were characterised by standard spectroscopic techniques (NMR, MS, IR and UV-Vis) and circular dichroism (CD) spectroscopy, and fractions were assigned by their NMR spectra. The chiral identity of the natural occurring new metabolite was evaluated by comparing the retention times of the new RA-metabolite isolated from mice liver (described in SCHMIDT *et al.*, 2002) with the synthesis product, which was a racemic mixture of 4o-9*c*-dh-RA isomers. Identical retention time of authentic isolated metabolite and synthetic *S*-4o-9*c*-dh-RA on different HPLC columns, reversed and normal phase conditions, together with all spectroscopic results, confirmed the assigned structure. Chromatogram comparison of synthetic and isolated metabolite on a Chiracel OJ column showed that the new

metabolite derived from mice livers is the pure *S*-enantiomer (see fig.1.8). A minor content in the sample of the *S*-4o-9*c*-dh-RA sample isolated from mice livers appears to be the *S*-enantiomer of the 4-oxo-all-*trans*-isomer (4o-at-dh-RA), which is most likely a contamination due to isomerisation processes of *S*-4o-9*c*-dh-RA during the separation and purification procedure, where the retinoids were exposed to a slightly acidic phase.

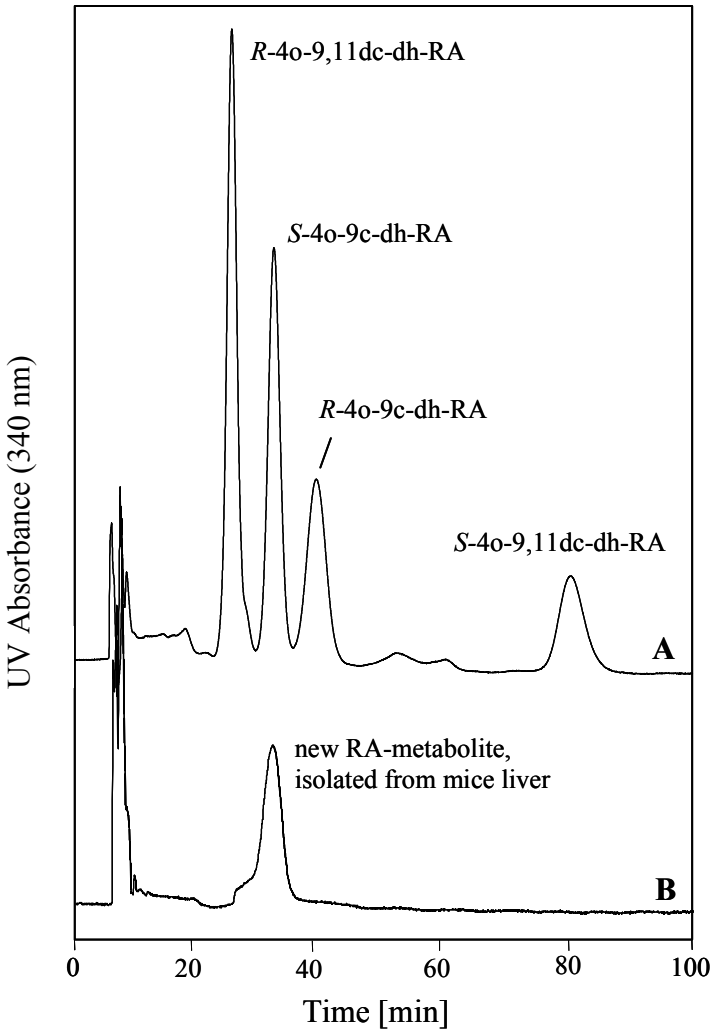

Fig.1.8: Comparison of the new RA-metabolite with synthetic S-4o-9c-dh-RA separated by chiral phase HPLC (modified from STEFAN *et al.*, 2005).

A) Racemic standard mixture of synthetic 4o-9,11dc-dh-RA and 4o-9c-dh-RA containing both enantiomers (*R*- & *S*-type). **B)** New RA-metabolite isolated from liver samples of NRMI mice. Note that isolated new RA-metabolite is the pure *S*-enantiomer of 4o-9c-dh-RA. (Chiracel OJ-H column, 4.6 x 250 mm, 10 µm particle size).

2. Aim of the thesis

The new metabolite *S*-4o-9c-dh-RA is occurring endogenously in considerably high concentrations in the liver of mice and rats (SCHMIDT *et al.*, 2002; SCHMIDT *et al.*, 2003a). It has been found out, that the metabolic formation of *S*-4o-9*c*-dh-RA within the liver is highly dependent on vitamin A intake (SCHMIDT *et al.*, 2002), with dramatically increasing levels after vitamin A supplementation. Based on this facts it seems likely that this new metabolite has a significant role the metabolism of retinoids. To contribute to the understanding of the physiological role of *S*-4o-9c-dh-RA in the multiple pathways of retinoids in the body, this work aims to clarify the question if this metabolite exhibits a biological activity in terms of mimicking the well-known effects of at-RA. Different *in vivo* and *in vitro* techniques have been used to answer this question. By using the classical chicken embryo limb bud model, it was investigated whether this new retinoid metabolite is morphogenetically active *in vivo*. Further, it was examined if *S*-4o-9c-dh-RA mediates its potential function via the same pathway such as at-RA and was able to regulate cellular signaling pathways by activating the nuclear retinoid receptors. Similar to at-RA, other RA metabolites mediate their biological effects also via binding and activating RARs or RXRs. To get knowledge about a possible physiologic role of the new metabolite, it is interesting to reveal if this metabolite has the ability to bind and activate these retinoid receptors, and thus is capable to regulate gene transcription. Using reporter cell systems at this early stage was the most efficient way to analyse the question whether *S*-4o-9c-dh-RA could be a novel ligand for retinoid receptors.

To analyze the possible transcriptional activity of *S*-4o-9c-dh-RA, several cell lines were transiently transfected with different luciferase reporter plasmids under the regulation of a minimal RARE (2xDR5 element), or a more complex regulated sequence, a part of the promoter region of a direct target gene for retinoids, the RARβ2 gene. Both the 2xDR5 element and the natural RARE sequence are exclusively recognized by ligand activated RAR heterodimers. Since it was found out that the natural occurring 4o-9c-dh-RA enantiomer, derived from mice liver, is

the *S*-type, it was interesting to observe, whether both the *R*- and the *S*-enantiomer could transactivate RAR/RXR heterodimers and if there are differences between both types in the potency. Equally, it was interesting to examine, whether the two enantiomers of 4o-at-dh-RA exhibit a potential to induce transcriptional activity on RARE-regulated genes. It has been reported by MOISE et al. (2005) that 4o-at-dh-RA is occurring *in vivo*, albeit in transgenic mice gavaged with all-trans-13,14-dihydro-retinol and in comparatively low concentrations. However, it is likely that both isomers, *S*-4o-9c-dh-RA and *S*-4o-at-dh-RA, exist endogenously under certain circumstances which are not known so far. Hence, the function of all four compounds to transactivate RAR/RXR heterodimers has been tested in the described reporter cell systems. The possibility that *S*-4o-9c-dh-RA could have antagonistic or synergistic effects against at-RA, regarding ligand properties towards RAR/RXR heterodimers, was analysed in comparative experiments. By testing the potency of *S*-4o-9c-dh-RA to induce transcriptional activation of RXRE regulated genes through RXR activation, it was evaluated, whether *S*-4o-9c-dh-RA as well as *S*-4o-at-dh-RA could be novel endogenous ligands for some RXR isoforms rather than being RAR ligands.

Using quantitative real-time polymerase chain reaction analysis (qRT-PCR), the potency of *S*-4o-9c-dh-RA to induce the endogenous expression of the at-RA regulated gene *RARβ2* was investigated in a mouse embryonic carcinoma cell line (P19). Mechanistically, the effect of *S*-4o-9c-dh-RA on ligand-induced conformational changes of retinoid receptors has been studied in limited proteolytic digestion experiments. Hormone binding to a nuclear receptor induces conformational changes within the receptor, which renders the ligand binding domain to become resistant to protease digestion. Using a chicken embryo model, the potency of exogenously applied *S*-4o-9c-dh-RA to mimic patterning activities of at-RA has been investigated in the limb bud. Additionally, using qRT-PCR, the effect of *S*-4o-9c-dh-RA to regulate the expression of at-RA target genes in the chick wing bud has been examined.

3. Material and Methods

3.1. Material

3.1.1. Retinoids

At-RA, 13-*cis*-RA and 9-*cis*-RA were purchased from Sigma-Aldrich (Steinheim, Germany). 4-oxo-13-*cis*-RA, 4-oxo-all-*trans*-RA, all-*trans*-Acitretin (RO101670) and all-*trans*-3,4didehydro-RA were used in this study were kindly provided by F. Hoffmann-La Roche (Basel, Switzerland or Nutley, NJ, USA). All retinoids were diluted in ethanol. The 8 diastereomers (isomers and enantiomers) of 4o-dh-RAs, including the new metabolite *S*-4o-9c-dh-RA, were synthesised and characterised (cf. 1.1.10.) by our collaborator M. Stefan under the supervision of H. Hopf and is described in STEFAN (2005). The synthesis of such compounds has been performed both as racemic as well as eantio-selectiv for configuration assignment (cf. chapter 1.1.10). A stereo-specific synthesis was not investigated because during synthesis, due to light, heat or acid influence, isomerisation occurs, which makes an HPLC separation steps anyway obligatory. Even small amounts of isomerisation products can influence the biological activity. To avoid such misleading results, HPLC purification was always performed prior to biological tests (described in chapter 3.2.1.2.). Additionally, the stock solutions were also regularly checked for purity using reversed-phase high performance liquid chromatography (HPLC) analysis (described in 3.2.2.) and did not show contamination (cf. 3.2.1.1.).

3.1.2. Cell culture reagents, additives and media

Product	**Manufacturer & sources of supply**
Ampicillin	Sigma-Chemical Co., St. Louis, USA
Dulbecco`s modified eagle medium (DMEM)	Gibco™ Invitrogen, Karlsruhe, Germany
Epidermal growth factor (EGF)	Sigma-Chemical Co., St. Louis, USA
Fetal bovine serum (FBS)	Gibco™ Invitrogen, Karlsruhe, Germany
Gelatin, G-6650, Bovine skin	Sigma-Chemical Co., St. Louis, USA
Geneticin® (G418)	Gibco™ Invitrogen, Karlsruhe, Germany
Gentamycin	Gibco™ Invitrogen, Karlsruhe, Germany
Insulin	Gibco™ Invitrogen, Karlsruhe, Germany

LB medium	Sigma-Chemical Co., St. Louis, USA
L-Glutamine	Gibco™ Invitrogen, Karlsruhe, Germany
Low glucose Dulbecco`s modified eagle medium (DMEM)	Gibco™ Invitrogen, Karlsruhe, Germany
Non essential amino acids	Gibco™ Invitrogen, Karlsruhe, Germany
Phosphate-buffered saline (PBS)	Biochemica-MikroSelect, Fluka, Munich, Germany
Penicillin/Streptomycin solution (PEST)	Gibco™ Invitrogen, Karlsruhe, Germany
Pyruvate	Gibco™ Invitrogen, Karlsruhe, Germany
RPMI 1640+ medium	Gibco™ Invitrogen, Karlsruhe, Germany
Trypsin-EDTA (10x)	Gibco™ Invitrogen, Karlsruhe, Germany

3.1.3. Other reagents and sources of supply

Product	**Manufacturer & sources of supply**
β-Mercaptoethanol	Sigma-Chemical Co., St. Louis, USA
Acetic acid	Merck, Darmstadt, Germany
Acrylamid (Liqui-Gel 40%, N,N'Methylene-bis-acrylamide)	ICN Biomedicals Inc., Aurora, OH, USA
Alcian Blue dye	Sigma-Aldrich, Steinheim, Germany
Ammonium acetate	Sigma-Aldrich, Steinheim, Germany
Amplify, NAMP 100 V	Amersham Pharmacia Biotech AB, Uppsala, Schweden
Calcium chloride	Merck, Darmstadt, Germany
Chloroform for sample preparation, Uvasol grade	Merck, Darmstadt, Germany
Chloroform (as HPLC eluent), ROTISOLV HPLC	Carl Carl Roth GmbH, Karlsruhe, Germany
Dithiothreitol (DTT), 1 M	Carl Roth GmbH, Karlsruhe, Germany
Diethylpyrocarbonat (DEPC)	Carl Roth GmbH, Karlsruhe, Germany
Ethanol, Uvasol grade	Merck, Darmstadt, Germany
Ethylene-diamine-tetraacetic acid (EDTA)	Sigma-Aldrich, Steinheim, Germany
iQ SYBRGreen Supermix	Bio-Rad Laboratories, Munich, Germany
Helium 5.0	Linde, Wiesbaden, Germany
Hydrogen peroxide (30 w/w-% sol. in water)	Sigma-Aldrich, Steinheim, Germany
Hydrochloride (HCL)	Merck, Darmstadt, Germany
Isoamylalcohol zur Analyse	Merck, Darmstadt, Germany
Isopropanol, Uvasol grade	Carl Roth GmbH, Karlsruhe, Germany
Isopropyl alcohol	Merck, Darmstadt, Germany
[^{35}S]methionine	Amersham Pharmacia Biotech AB, Uppsala, Schweden
Methanol, HPLC gradient grade	Mallinckrodt-Baker, Greisheim, Germany
Methyl salicylate	Sigma-Aldrich, Steinheim, Germany

Phenol (Aqua PhenolTM water saturated for DNA and RNA Extractions)	Qbiogene, Carlsbad, CA, USA
Phenol red	Sigma-Aldrich, Steinheim, Germany
Potassium acetate	Merck, Darmstadt, Germany
Protein-Standard, Precision Plus ProteinTM, all Blue Standards	Bio-Rad Laboratories, Munich, Germany
Sodium-dodecylsulfate (SDS)	Sigma-Chemical Co., St. Louis, USA
Sodium hydroxide	Sigma-Aldrich, Steinheim, Germany
Tetramethylethylendiamin (TEMED)	Carl Roth GmbH, Karlsruhe, Germany
Trichloroacetic acid	Carl Roth GmbH, Karlsruhe, Germany
Tris	Riedel-de Haen, Seelze, Germany

3.1.4. Consumables

Product	**Manufacturer & sources of supply**
6-well culture plate	Falcon Labware, Cockeysville, MD, USA
96-well-PCR-Plates	iCycler™, Bio-Rad Laboratories, Munich, Germany
96-well-PCR-Plate-Tape	iCycler™, Bio-Rad Laboratories, Munich, Germany
AG1-X2 ion-exchange beads	Bio-Rad, Richmond, CA, USA
Aminopropyl phase cartridge Bakerbond, C18 ODS cartridge	Phenomenex, Aschaffenburg, Germany
Cell culture plates	Nunc™, Roskilde, Denmark
Cling film	Melitta, Minden, Germany
Cryo tubes, CryoPlus 1 ml	Sarstedt AB, Landskrona, Sweden
Dissection needle	Carl Roth, Karlsruhe, Germany
Falcon tubes	Falcon Labware, Cockeysville, MD, USA
Glass vials, Wheaton dispossible scintillation vials	Obtained from Carl Roth, Karlsruhe, Germany
Micro centrifuge polypropylene tube	Eppendorf, Hamburg, Germany
Milli-Q system	Millipore, Eschborn, Germany
Multidish 12- and 24-well plates	Nunc™, Roskilde, Denmark
Nail scissors	Carl Roth, Karlsruhe, Germany
Parafilm ® M	America National Can™, Neehnah, WI, USA
Pipettes for cell culture	Sterilin, Bibby Sterilin Ltd., UK
Polypropylene Round-Botton Tube	Falcon Labware, Cockeysville, MD, USA
RP_{18} column, Spherisorb (ODS 2 mm, 150 x 2.1 mm, 3 µm particle size)	Waters, Eschborn, Germany
SPE NH2, 500 mg, 3 ml	Mallinckrodt-Baker, Griesheim, Germany

Tungsten wire (0.3 mm diameter)	Bio-Rad, Richmond, CA, USA
Watchmaker's -forceps (type 5)	Carl Roth, Karlsruhe, Germany
X-ray-film (Curix, Ultra UV-G, 18x24)	Agfa Health Care, Stockholm, Sweden

3.1.5. Machines

Product	**Manufacturer & sources of supply**
Analytical balance, Satorius MC 210P	Satorius AG, Göttingen, Germany
Automated solid-phase extraction ASPEC XLi (equipped with a 10-ml dilutor)	Gilson, Bad Camberg, Germany
Centrifugal evaporator system Alpha RVC	Christ, Osterode, Germany
Centrifuge, Avanti™ J-25, JA17 rotor	Beckman Coulter, Krefeld, Germany
CO_2-Inkubator, Inco II	Memmert GmbH, Schwabach, Germany
Corex tube for centrifuge, 30 ml	Corning Glassworks, Corning, NY, USA
Dissection microscope (equipped with low-voltage illumination, heat protection filter & green filter)	Wild Leitz, Rockleigh, NJ, USA
Electrophorese-System, Hoefer Migthy Small II	Amersham Pharmacia Biotech AB, Uppsala, Schweden
Film Developer, X-OMAT 1000Processor	Eastman Kodak Co., Rochester, NY, USA
Freezer (-20), Huskvarna	VSM Sverige AB, Huskvarna, Sweden
Freezer (-80 °C)	Forma Scientific Co., Marietta, Ohio, USA
Fridge, Huskvarna	VSM Sverige AB, Huskvarna, Sweden
HPLC-system	
LC-10AD pumps	Shimadzu, Duisburg, Germany
SCL-10AVP system controller	Shimadzu, Duisburg, Germany
SUS-mixer (0.5 ml)	Shimadzu, Duisburg, Germany
CTO-10AVP column oven	Shimadzu, Duisburg, Germany
UV-detector, SPD-10AVVP UV/Vis	Shimadzu, Duisburg, Germany
Autosampler, AS-4000	Merck, Darmstadt, Germany
Degaser, Degasys DG-1200	VDS Optilab, Montabaur, Germany
Fraction Controller, FRC-10A	Shimadzu, Duisburg, Germany
Homogenizer, Potter S	B. Braun Biotech International, Melsungen, Germany
Incubator for Bacteria, Minitron	Infors AG, Switzerland
Luminometer Rosys Anthos Lucy 2	Anthos Labtec Instruments, Krefeld, Germany
Micro centrifuge, Universal 32R and MIKRO 22R	Hettich Labinstruments, Sollentuna, Sweden
Microscope (cell work), Leica DMIL	Leica Microsystems Wetzlar GmbH, Wetzlar, Germany
Pipettor, Pipetboy	Integra Biosciences GmbH, Fernwald, Germany
Rocking platform, WT14	Biometra, Göttingen, Germany

RT-PCR System, iCyclerTM iQ	Bio-Rad Laboratories, Munich, Germany
RT PCR System, ABI Prism® 7500	Applied Biosystems, Stockholm, Sweden
Security guard	Phenomenex, Aschaffenburg, Germany
Spektrophometer, Ultrospec ® 3000 pro	Amersham Pharmacia Biotech, USA
Sterile hood	Holten LaminAir, Allerød, Denmark
Tablecentrifuge Universal 32 R	Hettich Labinstruments, Sollentuna, Sweden
Thermoblock, TB1	Biometra, Göttingen, Germany
Thermocycler, Gene Amp PCR System 2400	Applied Biosystems, Darmstadt, Germany
Thermomixer compact	Eppendorf, Hamburg, Germany
Transferpipette (10-100 µl)	BRAND GMBH + CO, Wertheim, Germany
Vacuum concentrator DNA-Speedvac, DNA110	Savant Instruments, Hicksville, NY, USA
Vacuum gel dryer, Slab Gel Dryer SGD2000	Savant Instruments, Hicksville, NY, USA
Vortexer	Janke & Kunkel IKA-Labortechnik VF2 Staufen, Gernamy
Water bath, A100	Lauda, Lauda-Königshofen, Germany

3.1.6. Enzymes

Enzyme	**Manufacturer & sources of supply**
DNAse I	Invitrogen, Karlsruhe, Gernamy
Lysozyme	ICN Biomedicals Inc., Aurora, OH, USA
RNAse A	Roche Diagnostics, Mannheim, Germany
RNAse T_1	Roche Diagnostics, Mannheim, Germany
RNase H	Invitrogen, Karlsruhe, Gernamy
RNasin	Invitrogen, Karlsruhe, Gernamy
Trypsin	Promega, Mannheim, Germany
T7 RNA Polymerase	Amersham Pharmacia Biotech, USA

3.1.7. Kits and ready-made material

Kit	**Manufacturer & sources of supply**
Galacto-Light Plus chemiluminescent - Reporter gene assay systems	Tropix, Bedford, USA
LipofectAMINE and Plus Reagent	Invitrogen, Karlsruhe, Gernamy
Luciferase Assay Kit	BioThema AB, Sweden
Power CyberGreen MasterMix (ABI)	Applied Biosystems, Stockholm, Sweden
Rabbit reticulocyte lysate cell-free system	Promega, Mannheim, Germany

RNeasy® Kit	Quiagen, Hilden, Germany
SuperScript II RT-PCR-System	Invitrogen, Karlsruhe, Gernamy
ThermoScriptTM RT-PCR-System	Invitrogen, Karlsruhe, Gernamy
TRIZOL®	Invitrogen, Karlsruhe, Gernamy

3.1.8. Bacteria strains and cell lines

Abbreviation	Full name & attributes	Origin/ ATCC ® -No.
E.coli XLBlue DH5α	Utilization for isolation of plasmids	Invitrogen, Karlsruhe, Germany
CV1	African green monkey kidney cells	CCL-70™
P19	Pluripotent mouse embryonic carcinoma cells	CRL-1825™
HeLa	Human cervical cancer cells	CCL-2.2™
Hepa-1	Murine Hepatoma cells	CRL-1830™
HC11-RARE	Mouse mammary gland epithelial cells Stable transfected with RARE	Malin Hedengren-Faulds *

* Department of Biosciences and Nutrition, Karolinska Institute Stockholm – Novum

3.1.9. Chicken Embryos

For the chicken embryo experiments, fertilised White Leghorn chicken eggs (VALO SPF-eggs, specified pathogen free) were purchased from Lohmann Tierzucht, Cuxhaven, Germany.

3.1.10. Oligo-nucleotides for gene expression analysis

The oligonuleotid primers for gene expression analysis in chick limb bud tissue were designed using the software Primer3 and optimised to an annealing temperature of 60 °C. These oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany). The forward and reverse PCR primers for *RARβ2* and *γ-actin* expression analysis in embryonic carcinoma cells (P19) were published in (POZZI *et al.*, 2006) and purchased from DNA Technology A/S (Aarhus, Denmark).

Tab.3.1: Oligo-nucleotid primers used for the qRT-PCR expression studies in chick limb bud tissue and in P19 cells.

Gene expression analysis in chick limb bud tissue			
Amplified gene		**Forward Primer (5' – 3')**	**Reverse Primer (5' – 3')**
Hoxb-8	Homeobox b 8	CTACCAGACGCTGGAACTGG	ACCTGCCTTTCTGTCAATCC
CYP26	cytochrome P450,family 26, subfamily A,polypeptide 1	CTTTCAGTGGGCTCTACCG	GCAGTGCATCCTTGTAGCC
RARβ2	RA receptor, beta2	GCATGCTTCAGTGGATTGG	AGTGGTGAAGGAGGGCTTG
shh	Sonic hedgehog	GGCCAGTGGAAGATATGAAGG	GCATTCAGCTTGTCCTTGC
bmp-2	Bone morphogenetic protein 2	CCTACATGTTGGACCTCTATCG	AAACTTCTTCGTGGTGGAAGC
TBP	TATA box binding protein	CTGGCAGCAAGGAAGTACG	GCTCATAGCTGCTGAACTGC
Gene expression analysis in P19 cells			
Amplified gene		**Forward Primer (5' – 3')**	**Reverse Primer (5' – 3')**
RARβ2	Retinoic acid receptor, beta2	CAGGCTTTTAGCTGGCTTGTCTGT	AATCCACTGAGGCAGGCTTTGAGA
γ-actin		GCCGGCTTACACTGCGCTTCTT	TTCTGGCCCATGCCCACCAT

3.2. Methods

3.2.1. HPLC-Analysis

3.2.1.1. Reversed phase HPLC

Retinoids were analysed according to a method developed by SCHMIDT *et al.* (2003). The HPLC system was composed of modules from Shimadzu (cf. chapter chapter 3.4.1.) controlled by the CLASS-VP 5.0 software with exception of the autosampler. The sample preparation procedure to analyse retinoids in liver-tissue starts with homogenising and extracting retinoids using a homogeniser and isopropanol. Polar retinoids were separated from apolar retinoids and neutral lipids using aminopropyl phase cartridges in an automated solid-phase extraction process. The eluates were concentrated to dryness via vacuum concentration in a centrifugal evaporator system, reconstituted in methanol and diluted 1:1 with PBS. The obtained polar fraction was injected into a HPLC-system using an autosampler equipped with a 200 µl

loop and a 0.5 ml dilutor. A multilinear binary gradient was generated from eluent A and B with the following gradient settings: 0 min, 20% B; 21 min, 80% B; 22.5 min, 100% B; 27.5 min, 100% B; 30.5 min, 20% B; and 40 min, 20% B. Eluent A was composed of 60 mM ammonium acetate buffer (see appendix), and methanol (1:1), whereas eluent B was pure methanol. Both mobile phases were degassed on-line. Additionally each eluent was purged for 20 min with helium and degassed for 20 min in an ultrasonic-water bath prior to use. The flow rate was 0.25 ml/min. Polar retinoids were separated on an analytical RP18 column (Spherisorb ODS2, 2.1 x 150 mm, 3 µm particle size), which was protected by a security guard equipped with a C18 ODS cartridge (4 mm L x 2 mm ID). The column oven was kept at 60°C. The HPLC system was coupled with an UV/Vis detector, equipped with a semi-micro flow cell (5 mm, 2.5 µl) and chromatograms were assessed using detection wavelength 340 nm (response 3, sampling frequency 2 Hz). Retinoids were quantitated against the appropriate internal standard (IS) all-*trans*-Acitretin (RO101670). The detection limit of the method for at-RA was 0.15 ng/ml or 0.3 ng/g, respectively.

3.2.1.2. Chiral phase HPLC

Chiral separation by HPLC was performed according to a method described by STEFAN (2006) using the above described HPLC system equipped with a Chiracel OJ column (10 µm, 0.46 x 25 cm) and normal phase conditions. The column and the developed separation method provide the opportunity to separate the 8 diastereomers (4-geometric isomers: all-*trans*, 9-*cis*, 9,11-di-*cis*, 11-*cis* – each of them with the two enantiomers *R/S*) of 4o-dh-RA. To obtain the specific isomers and enantiomers as pure material for the biological experiments the corresponding compounds were separated from the racemic mixtures. The enantiomers of 4o-9c-dh-RA were separated from a synthesis product, which was a racemic mixture of the two isomers 4o-9c-dh-RA and 4o-9,11dc-dh-RA (fig.3.2). The fractions were collected in beakers automatically, while the computer-integrated fraction controller “cut” the specific peaks at the defined time points out of the eluate. Each collected fraction was vacuum-concentrated using the centrifugal evaporator system. This procedure was repeated up to three times until the specific compound was pure. Using the same method the *R*- and

S-type enantiomers of the 4o-at-dh-RA isomer were selectively separated from a racemic mixture of 4o-at-dh-RA and 4o-11c-dh-RA (fig.3.2). The enantiomers of 4o-9,11dc-dh-RA and 4o-11c-dh-RA were not separated.

The eluent, composed of hexane/ isopropanol/ TFA (95/5/0.1), was degassed on-line and prior to use by purging 20 min with helium plus 20 min of ultrasonification. The isocratic flow rate was 0.9 ml/min. Each run took 100 min. The column oven was kept at 28°C. The UV/Vis detector was equipped with a standard flow cell (10 mm, 8 µl), and chromatograms were assessed using the detection wavelength of 320 nm (response 4, sampling frequency 2 Hz). The racemic mixtures were injected in concentrations from 300 mg/10ml to 2 g/10ml. The concentration of the obtained fractions containing the different isomers and enantiomers was adjusted gravimetrically.

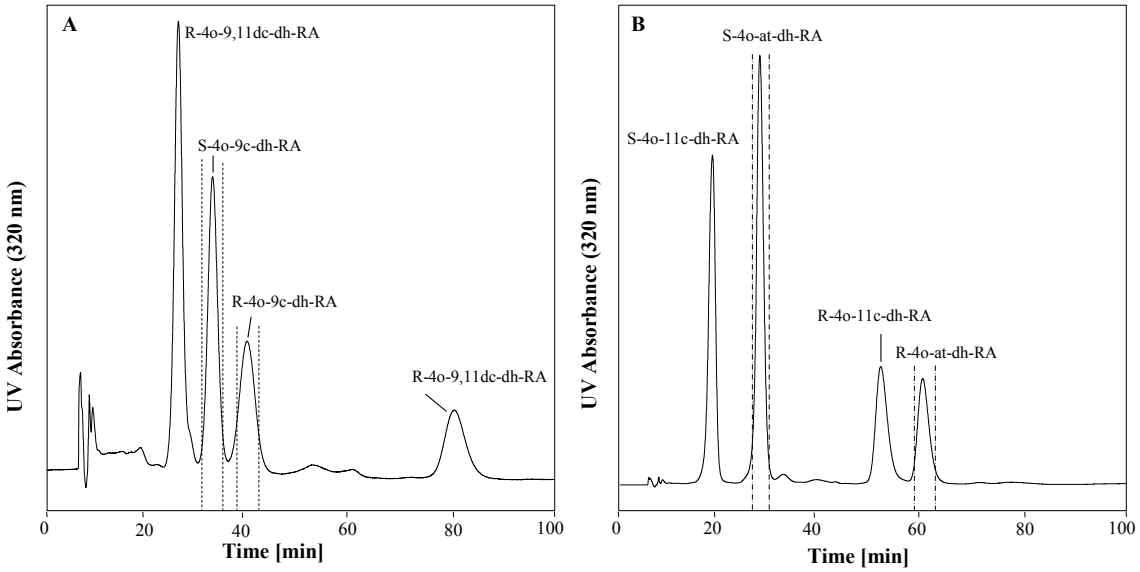

Fig.3.1: Chromatograms of synthetic racemic 4o-dh-RA mixtures.

The racemic mixtures of 4o-dh-RA, consisting of two different stereo-isomers, **(A)** 9,11dc & 9c and **(B)** 11c & at, in each case, were used as base material for the chiral separation of specific isomers and enantiomers, which have been tested in the biological experiments. The compounds were separated from the racemic mixtures via chiral phase HPLC and a fraction controller. **(A)** The *S*- and *R*-enantiomer of 4o-9c-dh-RA were separated from the 9,11dc- & 9c-4o-dh-RA mixture (dashed lines), whereas **(B)** the *S*- and *R*-enantiomer of 4o-at-dh-RA were separated from the 11c- & at-4o-dh-RA mixture (dashed-dot lines). (Chiracel OJ-H column, 4.6 x 250 mm, 10 µm particle size).

3.2.2. Standard methods in molecular and cell biology

3.2.2.1. Bacteria culture

Preparation of competent bacteria cells

A single colony of bacteria was inoculated into 50 ml LB media. 4 ml of this culture was added to 400 ml LB media in a 2 l flask and grown at 37°C in an incubator to an OD_{590} of 0.375, which takes approximately 3 h. This culture was aliquoted into 8x50 ml Falcon tubes and left on ice for 10 min, then it was spinned down at 3000 rpm for 7 min at 4°C. The supernatant was decanted and each pellet was gently re-suspended in 10 ml ice cold $CaCl_2$ solution (see appendix). The mixture was centrifuged at 2500 rpm for 5 min at 4°C and each pellet was gently re-suspended in 10 ml ice cold $CaCl_2$ solution and left on ice for 30 min. After centrifugation at 2500 rpm for 5 min at 4°C, each pellet was gently re-suspended in 2 ml ice cold $CaCl_2$ solution and left on ice for 6 h. Finally the bacteria suspension was aliquoted to eppendorf tubes (210 ml each) and immediately frozen in liquid N_2. Bacteria stocks were stored at minus 70°C.

Transformation of bacteria cells

Competent cells were thawed on ice. 10-50 ng of plasmid-DNA (1 µl) was mixed with 95 ml of competent bacteria suspension and incubated for 20 min on ice. Afterwards bacteria were incubated at 42°C for 90 sec in a thermoblock. Tubes were put back on ice for 2 min and 900 ml of LB media was added. Bacteria were incubated at 37°C for 60 min. Tubes were spinned down at 7000 rpm for 1 min and the supernatant was removed. The bacteria were re-suspended in 100ml LB media and plated out on LB media agar plates containing Ampicillin (100 µg/ml). Plates were incubated over night in an incubator at 37 °C.

3.2.2.2. Preparation and purification of plasmids

Bacteria were cultured in LB media containing 1 µl/ml Ampicillin. For preparation of the starter culture, 5 ml media was inoculated with transformed bacteria cells by

picking single colonies from the agar plates. The bacteria were incubated in a round-bottom tube for 12 h at 37°C on a shacking platform. For the preparation of the main culture, the starter culture was added to 200 ml LB media in a 1000-ml-Erlenmeyer flask. The main culture was incubated for another 12-15 h at 37°C. The bacteria were then centrifuged in a swing out rotor at 4500 rpm for 20-25 min at + 4°C. The supernatant was decanted and the pellet was re-suspended in 20 ml lysis solution consisting of $T_{50}E_{10}$ buffer with 0.5 ml Lysozyme (spatula tip / 10 ml). To thoroughly lyse bacteria the tubes were incubated for 30-60 min at 37°C in a water bath. Following the addition of 750 ml 10%-SDS, tubes were swirled and incubated for 20 min at RT. After adding 1.5 ml Kac (see appendix) (5 mM) tubes were swirled and incubated for 30 min on ice and centrifuged for 1 to 1 ½ h at 14.000 rpm and + 4°C. The supernatant was carefully collected in 50 ml tubes. 200 µl RNAse A and 10 µl RNAse Ti was added and the mixture was incubated for 45 min at 37°C in a water bath. After adding 2 ml sodium acetate (3 M, pH 7) and 10 ml buffered Phenol the mixture was vortexed and centrifuged at 3000 rpm for 30 min at 4°C. The top fraction was carefully transferred to new tubes and 10 ml chloroform/ isoamylalcohol (24:1) solution was added. The mixture was vortexed and centrifuged for 20-30 min at 3000 rpm and 4°C in a desk centrifuge. The top fraction (not more than 18 ml) was carefully transferred to 30 ml glass Corex tubes. After adding 9,7 ml (0,54 volume) isopropanol, tubes were wrapped tightly with Parafilm, vortexed and incubated for 20-30 min at RT. After removing the Parafilm, tubes were spinned for 1½ to 2 h at 11500 rpm in a swing out rotor at RT. The isopropanol was gently decanted and 15-20 ml ethanol (80%) was added. After another spin for 10 min at 11500 rpm in a swing out rotor at RT and decantation of the ethanol the DNA was dried in a Speedvac® vacuum concentrator. DNA pellets were dissolved in 400 µl $TE_{7,8}$ for 2 h in a water bath at 37-42°C. The DNA solution was transferred into sterile Eppendorf tubes and stored at -80°C.

3.2.2.3. Cell culture

All cell works were performed in a sterile hood with laminar air flow. The hood was sterilised with 70% ethanol before and after use and thoroughly once in a week. Cell

culture flasks, pipettes, pipettor and all materials which were taken under the hood were sprayed with 70% ethanol.

Maintenance of cells

CV1, P19 and Hela cells were grown in high Glucose DMEM supplemented with 10% (v/v) FBS, 1% (v/v) PEST, 1% (v/v) L-Glutamine and 1% NEA (non essential amino acids). Hepa-1 cells were grown in low glucose DMEM supplemented with 10% (v/v) FBS, 1% (v/v) PEST, 1% (v/v) L-Glutamine and 1% (v/v) Pyruvate. HC11-RARE cells were grown in RPMI 1640+ media supplemented with 10% (v/v) FCS, 50 µg/ml gentamycin, 1% (v/v) L-Glutamine, 240 µg/ml Geneticin® (G418), 10 ng/ml EGF, and 5 µg/ml insulin. All media were freshly prepared at least every week. Cells were cultured in an incubator at 37°C in a humidified atmosphere containing 5% CO_2. Media and other constituents were warmed up to 37°C in a water bath prior to use. Condition and density of cells was observed using a Leica DMIL light microscope. To avoid the pluripotent P19 cells to differentiate during growth, the cell culture plates were routinely coated with Gelatin 0.1% (w/v in water) before adding the cells. 4 ml of Gelatin was added to a 10 cm plate and spread evenly. Gelatin was allowed to sit at least 15 min in the incubator before it was removed. Culture media was added gently so that gelatine-film was not flushed away.

Cells were split at a confluency of 60-80% (dependent on cell line). Before splitting, cells were washed with PBS (10 ml per 10 cm plate) and treated with 0.6-0.7 ml Trypsin-EDTA until the cells detached (from 30 sec for P19 until 5 min for CV1). Immediately after cell detachment, media was added (10 ml per 10 cm plate) to stop the enzymatic reaction. To break up clusters, cells were pressed through the pipette against the bottom of the dish 2-5 times for CV1 cells or 8-10 times for P19 cells. The dilution factor varied depending on cell type and density. Plates were gently swirled to disperse cells in the media. The passage number never excided 5.

Cryokonservation and thawing of cells

Only cells from an early passage were frozen (one 10 cm plate in one cryo tube). The cells had a confluency of approximately 70%. First, freeze media was prepared composed of 80% FSB and 20% DMSO and kept on ice. The cells were split normally and collected in 50 ml Falcon tubes. The suspension was centrifuged at 1000 rpm for 10 min at RT. The media was removed and cells were re-suspended in fresh media (10 ml/ 10 cm plate). This step removes the Trypsin from the cells completely. Again the suspension was spinned at 1000 rpm for 10 min at RT. 0.5 ml of the pre-chilled freeze media was put to each cryo tube standing in a cool rack. The media was removed and cells were re-suspended in fresh media (0.5 ml/ 10 cm plate). 0.5 ml of the cell suspension was put to each cryo tube and well mixed using a 1 ml tip. In this final step the freezing media is diluted to give a final serum concentration of approximately 50% serum and 10% DMSO. The tubes were frozen immediately for 20-30 min at -20°C and then transferred to the -80°C for storage. Frozen stocks were warmed up in a 37°C warm water bath for 30-60 sec. instantly thawing; cells were transferred into warm cell media in cell culture plates. The plate was gently swirled to disperse the cells. Thawed cells stocks were split the following day.

Transient transfection

Cells were generally ready for transfection after the 3rd passage. The day before transfection, cells were seeded on 12- or 24-well culture plates so that they were 50-70% confluent the day of transfection. Transient transfections of plasmid DNA were performed using LipofectAMINE and Plus Reagent according to the manufactures protocol. The media that was used for the transfections was free of serum and antibiotics. The expression vectors were diluted to the appropriate concentrations. The plasmid dilutions and Plus Reagent (1 µl/well) were mixed with pre-warmed transfection media and incubated for 15 min at RT. While complexes were formed, cells were washed with serum-free media (1 ml/well on a 24-well-plate or 2 ml/well on a 12-well-plate) to wash away the serum. Cells were washed carefully not to flush cells from the culture dish. LipofectAMINE (0.7 µl/well) diluted in media was added to the plasmid mix and incubated for 20 min at RT. The volume of the transfection mix

was increased to a total of 0.5 ml/well and mixed well by pipetting. The washing media was removed from the cells of the first series to be transfected and 0.5 ml of the transfection mix was added gently to the cells. The procedure was continued with the next series of cells in the same manner. After adding the transfection media cells were removed back in the incubator. After 3 h of incubation the volume of media was increased by adding normal growth medium containing all referred additives and the retinoids (see 3.2.3.2.).

3.2.3. Retinoid receptor transactivation studies

3.2.3.1. Principle of the reporter assay

To analyse the possible transcriptional activity of *S*-4o-9*c*-dh-RA, several different cell lines were transfected with different luciferase reporter plasmids under the regulation of a RARE (fig.3.2), which is exclusively recognized by ligand activated RAR heterodimers. This provides the possibility to investigate if *S*-4o-9*c*-dh-RA is able to activate these retinoid receptors. The two different reporter plasmids, used in this study, share the same vector backbone but have different regulatory RARE sequences in front of the luciferase gene; the classic minimal RARE DR5, and a partial/regulatory region of the promoter of the natural retinoid target gene *RARβ2*. Additionally, the assays have been performed with a luciferase reporter construct under the control of a RXRE DR1, which responds to RXR homodimer activation, to investigate the ability of *S*-4o-9*c*-dh-RA to activate these retinoid receptors.

3.2.3.2. Plasmids

All used plasmids were constructed from either D. Wahlström or K. Pettersson (see tab.3.1). The pGL3b2xDR5luc reporter plasmid was designed and constructed by using the classic simple minimal RARE DR5 ($AGGTCAn_5AGGTCA$) in a direct repeat separated by a restriction site for SphI. The sequence was constructed with a minimal TATA-box in front and cleavage sites for NheI and XhoI, and cloned into a

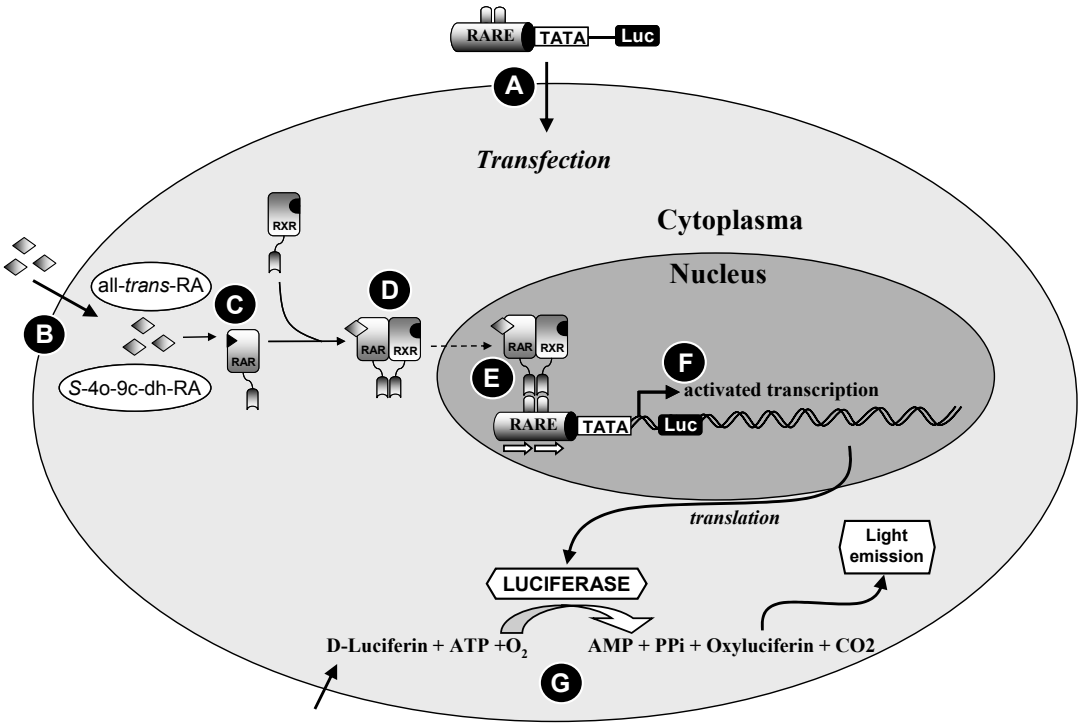

Fig.3.2: Schematic illustration of a luciferase assay.

A) Cells were transfected with a luciferase reporter plasmid which is regulated by a RARE and therefore RA-inducible. **B)** After transfection, cells were treated with *S*-4o-9*c*-dh-RA in various concentrations and at-RA [100 nM] as a positive control. **C)** RA binds to the LBD of RAR which **D)** forms a heterodimer together with RXR. **E)** The ligand-receptor-heterodimer complex binds to the RARE of the luciferase reporter and **F)** the transcription of the Luc-Gene is activated. The rate of transcription of Firefly Luciferase is dependent on the transactivation potency and concentration of the applied retinoid. **G)** After adding Luciferin and ATP substrate Firefly Luciferase catalyses the conversion of Luciferin to Oxyluciferin which emits light. The light emission of Oxyluciferin is linearly correlated with the rate of transcription.

pGL3basic vector in front of the luciferase promoter. The minimal mouse *RARβ*-promoter (-180 to + 83), was cloned into the pGL3basic vector in the same fashion as the 2xDR5 sequence. The plasmids expressing different retinoid receptors (RARα and β, RXRα and β) have a pSG5 backbone. pCMW-βGal and pRSV-βGal was used as an internal control.

3.2.3.3. Transfection of cell lines

Several different cell lines were transiently transfected with different reporter plasmids under variable conditions (tab.3.3), with the exception of the HC11 cell line,

Tab.3.2: List of plasmids used in retinoid receptor transactivation studies.

Name	Attributes	Reference
pGL3b-2xDR5luc	▪ synthetic minimal RARE-promotor ▪ drives luciferase-gene ▪ Ampicillin-Resistence	D. Wahlström *
pGL3b-*RARβ*luc	▪ region of RARE-promotor from natural RARβ gene ▪ drives luciferase-gene ▪ Ampicillin-Resistence	D. Wahlström *
pGl3b-DR1luc	▪ RXRE-promotor ▪ drives luciferase-gene ▪ Ampicillin-Resistence	D. Wahlström *
pSG5-*RARα*	▪ contain mouse *RARα* ▪ Ampicillin-Resistence	K. Pettersson *
pSG5-*RARβ*	▪ contain mouse *RARβ* ▪ Ampicillin-Resistence	K. Pettersson *
pSG5-*RXRα*	▪ contain mouse *RXRα* ▪ Ampicillin-Resistence	K. Pettersson *
pSG5-*RXRβ*	▪ contain mouse *RXRβ* ▪ Ampicillin-Resistence	K. Pettersson *
pCMW-bGal	▪ CMV-driven β-Galactosidase ▪ Ampicillin-Resistence	K. Pettersson *
pRSV-βGal	▪ RSV-driven β-Galactosidase ▪ Ampicillin-Resistence	K. Pettersson *

* Department of Biosciences and Nutrition, Karolinska Institute Stockholm – Novum

which has the RARE DR5 element stably integrated. Cells were transfected with the luciferase reporter plasmids pGL3basic2xDR5, pGL3b-*RARβ*2luc or pGL3b-DR1Luc that allow the expression of the reporter gene Firefly Luciferase upon activation by the proper ligand activated receptor complex, i.e. RAR/RXR heterodimer complex. To normalise the variations in transfection efficiency pCMW-βGal and pRSV-βGal, expressing β-Galactosidase, were used as internal controls. CV1 cells were transiently co-transfected with the reporter-plasmid pGL3basic2xDR5 in combination with the retinoid receptor- expression vectors RARα and RXRβ. Transfection was carried out as described above. Stable transfected HC11 cells were kindly provided from M. Hedengren-Faulds (Department of Biosciences and Nutrition, Karolinska Institute Stockholm – Novum). Therefore, HC11-RARE cells were grown in 10-cm plates and transfected with 10 µg of the reporter plasmid pGL3b2xDR5luc using Lipofectamine reagent. Stable clones were selected in 240 µg/ml Geneticin in RPMI 1640 medium.

Tab.3.3: Transfection scheme of the different cell lines.

Cell line	Plasmids							
	Luc-Reporter	[ng/well]		Retinoid-receptor expression-vector	[ng/well]		β-Galactosidase reporter-enzyme	[ng/well]
CV1	pGL3basic2xDR5	10	+	pSG5-*RARα* & pSG5-*RXRβ*	2 2	+	pCMW-βGal	30
	pGL3basic2xDR5	100	+	pSG5-*RARβ* & pSG5-*RXRβ*	2 2	+	pCMW-βGal	30
	pGL3b-DR1Luc	100	+	pSG5-*RXRα*	20	+	pCMW-βGal	30
	pGL3b-DR1Luc	100	+	pSG5-*RXRβ*	20	+	pCMW-βGal	30
HeLa	pGL3basic2xDR5	200		---	---	+	pCMW-βGal	30
Hepa-1	pGL3b-*RARβ2*luc	100		---	---	+	pCMW-βGal	30
P19	pGL3basic2xDR5	400		---	---	+	pRSV-βGal	100
HC11-RARE	pGL3basic2xDR5	400		---	---	+	---	100

3.2.3.4. Retinoid treatment

3 h after transfection, cells were treated with the appropriate retinoids. All experimental procedures involving treatment with retinoids were light-protected (light was shut off, rooms were dimmed, all mix tubes containing retinoids were wrapped with aluminium foil). Prior to each experiment, the retinoid stock solutions were diluted in the culture media to the final concentration indicated (see tab.3.4). 0.5 ml media containing all referred additives and the retinoids was added per well. The final concentration of ethanol did not exceed 1% in the culture media, which should not disturb cell growth.

Tab.3.4: Overview about retinoid treatments of the different cell lines.

Cell line	Transfection	Treatment		
		single/ double	retinoid	concentration
CV1	2xDR5/RARα/RXRβ	single	ethanol	-
			at-RA	100 nM
			S-4o-9c-dh-RA	1 nM; 10 nM; 100 nM; 1 µM, 10 µM
			R-4o-9c-dh-RA	1 nM; 10 nM; 100 nM; 1 µM, 10 µM
			S-4o-at-dh-RA	1 nM; 10 nM; 100 nM; 1 µM, 10 µM
			R-4o-at-dh-RA	1 nM; 10 nM; 100 nM; 1 µM, 10 µM
	2xDR5/RARβ/RXRβ	single	ethanol	-
			at-RA	100 nM
			S-4o-9c-dh-RA	1 nM; 10 nM; 100 nM; 1 µM, 10 µM
			R-4o-9c-dh-RA	1 nM; 10 nM; 100 nM; 1 µM, 10 µM
			S-4o-at-dh-RA	1 nM; 10 nM; 100 nM; 1 µM, 10 µM
			R-4o-at-dh-RA	1 nM; 10 nM; 100 nM; 1 µM, 10 µM
	DR1/RXRα	single	ethanol	-
			9c-RA	100 nM
			S-4o-9c-dh-RA	10 nM; 1 µM, 10 µM
			S-4o-at-dh-RA	10 nM; 1 µM, 10 µM
	DR1/RXRβ	single	ethanol	-
			9c-RA	100 nM
			S-4o-9c-dh-RA	10 nM; 1 µM, 10 µM
			S-4o-at-dh-RA	10 nM; 1 µM, 10 µM
HeLa	2xDR5	single	ethanol	-
			at-RA	100 nM
			S-4o-9c-dh-RA	1 nM; 10 nM; 100 nM; 1 µM
			R-4o-9c-dh-RA	1 nM; 10 nM; 100 nM; 1 µM
			S-4o-at-dh-RA	1 nM; 10 nM; 100 nM; 1 µM
			R-4o-at-dh-RA	1 nM; 10 nM; 100 nM; 1 µM
Hepa-1	RARβ2	single	ethanol	-
			at-RA	100 nM
			S-4o-9c-dh-RA	100 nM; 1 µM; 10 µM
	RARβ2	double	ethanol	-
			at-RA	1 nM
			at-RA + *S*-4o-9c-dh-RA	1 nm + 1 nM; 10 nM; 100 nM; 1 µM
P19	2xDR5	single	ethanol	-
			at-RA	100nM
			S-4o-9c-dh-RA	1 nM; 10 nM; 100 nM; 1 µM, 10 µM
			R-4o-9c-dh-RA	1 nM; 10 nM; 100 nM; 1 µM, 10 µM
			S-4o-at-dh-RA	1 nM; 10 nM; 100 nM; 1 µM, 10 µM
			R-4o-at-dh-RA	1 nM; 10 nM; 100 nM; 1 µM, 10 µM
	2xDR5	double	ethanol	-
			at-RA	10 nM
			at-RA + *S*-4o-9c-dh-RA	10 nM + 1 nM; 10 nM; 100 nM; 1 µM; 10 µM
HC11-RARE	2xDR5	single	ethanol	-
			at-RA	100 nM
			S-4o-9c-dh-RA	10 nM; 100 nM; 1 µM, 10 µM
			R-4o-9c-dh-RA	10 nM; 100 nM; 1 µM, 10 µM
			S-4o-at-dh-RA	10 nM; 100 nM; 1 µM, 10 µM
			R-4o-at-dh-RA	10 nM; 100 nM; 1 µM, 10 µM

3.2.3.5. Harvesting of cells

The cells were harvested 24 h after treatment by removing the media from the wells and adding the lysis buffer (100 µl/well for 24 well plates or 130 µl/well for 12 well plates, respectively). For the preparation of lysis buffer, 0.5 µl 1M DTT was added to 1 ml lysis-solution (Tropix®). The plates were incubated with the lysis buffer at RT until cells clearly disintegrate (dependent on cell line between 10 and 60 min). To avoid evaporation, plates were wrapped in Parafilm.

3.2.3.6. Reportergene-assays

Measurement of luciferase activity

Luciferase activity in the reporter gene studies was measured using a Luciferase Assay Kit (BioThema) according to the manufactures protocol at RT. First the cell extracts were homogenised by pipetting, followed by transferring the samples (40 µl/well) from the 12- or 24-well plates to 96-well microplates. Luciferin and ATP were delivered lyophilised and were reconstituted by adding Tris-EDTA-buffer (delivered). Luciferin substrate was added to the wells with the cell extracts (100 µl/well). The plate was placed in the luminometer (Lucy 2) and after adding the same volume of reconstituted ATP substrate (100 µl/well), light emission was measured.

Measurement of β-Galactosidase activity

β-Galactosidase activity of transfected cells was detected using the Tropix® Galacto-Light Plus chemiluminescent reporter gene assay systems according to the manufactures protocol using the luminometer Lucy 2. The assays were performed at RT. First the reaction buffer was prepared by diluting Galacton-Plus® substrate with reaction buffer diluent at a rate of 1:100. The cell extract was transferred to a 96-microplate (20 µl/well) and the reaction buffer was added (70 µl/well) using a transfer pipette. The microplate was covered with aluminium foil and incubated for 60 min. 2 µl H_2O_2 was added to the Accelerator-II. To stop the reaction, Accelerator-II solution was added to the samples (100 µl/well). Galacton Plus® substrate emits light at a near

constant level with a half life of approximately 180 min after addition of Accelerator-II. The plate was placed in the luminometer and light signal was measured.

Evaluation of luciferase activity

Ratios of luciferase activity to β-galactosidase activity were determined to control transfection efficiency. The relative induction of luciferase activity is defined as a quotient of luciferase levels between treated and untreated control samples. Relative luciferase induction/ activity was calculated as follows: [Normalised luciferase level $_{\text{treated sample}}$ (luciferase level $_{\text{treated sample}}$ / b-galactosidase level $_{\text{treated sample}}$) / Normalised luciferase level $_{\text{untreated sample}}$ (luciferase level $_{\text{untreated sample}}$ / b-galactosidase level $_{\text{untreated sample}}$)].

3.2.4. *RARβ2* gene expression analysis in P19 cells

3.2.4.1. Treatment of P19 cells with retinoids

P19 cells were cultured on Falcon 6 well culture plates two days before the start of each experiment so that they reach 80% confluency at the day of treatment. Each experiment started by treating all cells at the same time point during dark conditions. Cells were treated with increasing amounts of *S*-4o-9c-dh-RA (100 nM, 1 μM and 10 μM) and at-RA (100 nM) as positive control diluted in culture media 2 ml/well). Prior to each experiment, the retinoid stock solutions were diluted in the culture media to the final concentration. The final concentration of ethanol did not exceed 0.5 ppm in culture media.

3.2.4.2. Harvesting P19 cells and Isolation of RNA

The cells were treated from 2 to 24 h. The cell lysis was combined in a single-step with the RNA isolation by using TRIZOL® according to the manufacturer's protocol. Cells were lysed and homogenised by adding 1 ml of TRIZOL Reagent per well and passing the cell lysate a several times through a pipette. For the phase separation homogenised samples were incubated for 5 minutes at 15 to 30°C. After adding 0.2 ml

of chloroform to each well, culture plates were caped securely and vigorously shaked by hand for 15 seconds, followed by incubation at RT for 2 to 3 minutes. Samples were centrifuged at 12,000 × g for 15 min at 4°C. For RNA precipitation aqueous phase was transferred to a fresh tube and 0.5 ml of isopropyl alcohol was added. Samples were incubated for 10 min at RT and centrifuged at 12,000 × g for 10 min at 4°C. After supernatant was removed, the RNA pellet was washed once with 1 ml 75% ethanol. Sample were mixed by vortexing and centrifuged at 7,500 × g for 5 min at 4°C. At the end RNA pellet was dried and redissolved in RNase-free water by passing the solution a few times through a pipette tip, and incubated for 10 min at 55 to 60°C. In order to eliminate genomic DNA 2µg of total RNA from each extracted sample was treated with DNAseI.

3.2.4.3. Reverse transcription of RNA into cDNA

Total RNA was transformed to cDNA using SuperScript II. 1-2 µg of total RNA was DNase treated (together with 1 ul 10x reaction buffer and water in a total volume of 10 ul) in RT for 15 min. The reaction was stopped by adding 1 ul of [25 mM] EDTA and heated at 65°C for 15 min. After adding 1 ul random hexamer primers [50 ng/µl] and 1 ul dNTP [10 mM] to each sample, the volume was adjusted to 11 µl with RNase free water. The samples were heated for 5 min at 65°C to denature primers and RNA and put immediately on ice. After a brief centrifugation in a table top centrifuge specialised for PCR strips for a few sec, the following additives were supplemented (in a master mix prepared in advance): 4 µl of 5x 1st strand buffer (delivered with Superscript II enzyme), 2 µl 0.1M DTT [0,1M] (delivered with Superscript II enzyme), 1 µl RNase OUT (recombinant Ribonuclease inhibitor). The samples were gently mixed with the additives and incubated for 10 min at 25°C and equilibrated for 2 min at 42°C. After adding 1 µl of Superscript II, samples were incubated at 42°C for 50 min in a thermocycler and reaction was inactivated at 70°C for 15 min. In the end, 1 ul of RNase H was added to each cDNA sample followed by incubation for 20 min at 37°C. To limit variations all RNA samples were reverse transcribed simultaneously.

3.2.4.4. Absolute quantification of RARβ2 transcript levels in P19 cells using qRT-PCR analysis

Prior to q-RT-PCR analysis the cDNA stocks was diluted 5x in water. The quantitative Real-Time PCR was performed on 96-well-PCR-plates using an ABI Prism 7500 Real-Time PCR System (Applied Biosystems) with the software version 1.3 in combination with Power CyberGreen MasterMix (ABI). The reaction volume per well was 12 µl in total and contained the following components: 0.36 µl Foreward primer [300 nmol], 0.36 µl Reverse primer [300 nmol], 6 µl Power CyberGreen MasterMix, 3.28 ul water and 2 µl 5x diluted cDNA-template. To minimise the pipetting errors a master mix containing primers, CyberGreen MasterMix and water was prepared in advance. The PCR-plate was closed with tape, centrifuged and placed into the ABI Prism 7500 rotor. A dilution curve was added to each run in order to trace artefacts in individual samples.

Tab.3.5: qRT-PCR reaction profile for *RARβ2* and *γ-actin* transcript amplification.

Program		Temperature (°C)	Time
Initial denaturation		95	10 min
Amplification & quantification (40 cycles)	*Denaturation:*	95	15 sec
	Annealing:	60	60 sec
Melting curve		60-95	15 sec
Cooling step		4	∞

The amplification and quantification program was performed with a single fluorescence measurement. The specificity of the qRT-PCR products was determined by performing the melting curve analysis after each PCR. The dissociation curve was: 95°C in 15 sec down to 60°C for 1 min and again up to 95°C for 15 sec with an increasing set point temperature after cycle 2 by 0.5 ºC per second and a continuous fluorescence measurement. The melting temperatures of *RARβ2* and *γ-actin* transcripts were approximately 83°C and 86°C, respectively. Oligonuleotid primers (see

tab.3.2) were optimised to an annealing temperature of 60°C. *γ-actin* is a housekeeping gene that should be equally expressed in all cells and was used as an internal standard (endogenous control).

3.2.4.5. Mathematical model for the calculation of the relative expression ratios

The expression levels of the *RARβ2* gene was determined based on the relative quantity levels using the ΔΔct-method (comparative threshold method). The determination of the Ct values for each transcript and the calculation of the qRT-PCR efficiencies (E) from the given slopes were performed using the ABI Prism 7500 Real-Time PCR System software version 1.3. The relative expression ratio (RER) of the target gene *RARβ2* is calculated based on E and Ct deviation of a treated sample (sample) versus a control (ctrl), and expressed in comparison to the reference gene *γ-actin* (Equation 1). All values are expressed in arbitrary units relative to the control treated cells from 1h time point (the calibrator).

$$\mathrm{RER} = \frac{\left(E_{\mathrm{target}}\right)^{\Delta \mathrm{Ct}_{\mathrm{target}}(\mathrm{control}-\mathrm{sample})}}{\left(E_{\mathrm{ref}}\right)^{\Delta \mathrm{Ct}_{\mathrm{ref}}(\mathrm{control}-\mathrm{sample})}}$$

Fig.3.3: Equation for the calculation of the relative expression ratios.

E_{target}: real-time PCR efficiency of the target gene transcript
E_{ref}: real-time PCR efficiency of the reference gene transcript
ΔCt_{target}: Ct deviation of the control minus sample from the target gene transcript
ΔCt_{ref}: Ct deviation of the control minus sample from the reference gene transcript

3.2.5. Limited proteolytic digestion assays

3.2.5.1. *In vitro* translation of RARα and RARβ proteins

Mouse RARα and RARβ protein was synthesised *in vitro* using the rabbit reticulocyte lysate cell-free system coupled with T7 RNA polymerase and incorporation of [^{35}S]methionine. Expression vectors pSG5-*RARα* and -*RARβ* were used as DNA-templates. TNT Rabbit Reticulocite Lysate, TNT Reaction buffer, T7 RNA Polymerase, Amino Acid Mix, RNasin and expression vectors were stored at -70°C,

[^{35}S]methionine at 5°C. Upon removal from storage, all components were immediately placed on ice. "Lysate-reaction-mix" (tab.3.6) was prepared on ice and incubated at 30°C for 90 min in a Thermomixer.

Tab.3.6: Composition of a "lysate-reaction-mix".

Components	Standard reaction (50 µl)
TNT Rabbit Reticulocite Lysate	25 µl
TNT Reaction buffer	2 µl
T7 RNA Polymerase	1 µl
Amino Acid Mix –Methionine	1 µl
[^{35}S]-Methionine	2 µl
RNasin	1 µl
DNA-Template[a)] (1 µg/µl)	1 µl
Water (to final volume of 50 µl)	17 µl

[a)] pSG5-*RARα* and –*RARβ*

3.2.5.2. Ligand incubation and limited proteolysis reactions with Trypsin

A 3.5 µl aliquote of the "lysate-reaction-mix" containing [^{35}S]Methionine labelled retinoid receptor was incubated with 1.5 µl retinoid (100 nM at-RA or 10 µM *S*-4o-9c-dh-RA, respectively) dissolved in ethanol for 45 min at 30°C in a Thermomixer (lids were covered with tinfoil to keep reactions light-protected). Controls were incubated with ethanol alone. Final concentration of ethanol was < 1%. To 5 µl aliquots of retinoid-treated receptor proteins, 19 µl "digestion-mix" (tab.3.7) and 4 µl "enzyme-buffer-mix" (Trypsin dissolved in 50 mM acetic acid) in concentrations of 30, 60, 120, 240 ng/µl) was added and incubated for 10 min at 25°C in a Thermomixer. This step includes a second ligand-incubation. The reaction was stopped by adding 6 µl 5xSDS-loading buffer (see appendix) containing 500 µM EDTA and boiling for 5 min in a water bath.

Tab.3.7: Composition of "digestion-mix" and 2nd ligand-Incubation.

Components	Single Reaction Mixture
2 x BSB (see appendix)	10 µl
DTT (0,1 M)	2 µl
$CaCL_2$ (40 mM)	3 µl
WCEB (see appendix)	2 µl
H_2O	1 µl
+ EtOH	1 µl
+ retinoid-solution (to final conc. of 100 nM)	1 µl

3.2.5.3. Separation of protein-fragments using SDS-PAGE

Protein fragments were separated using SDS-polyacrylamide gel electrophoresis on 10% (w/v) polyacrylamide gels (see appendix). Gels were gently filled into a chamber between two 10x10 cm glass-sheets parted by two 3 mm wide spacers jammed into the electrophorese-system. After filling the separation gel into the chamber, it was covered with a layer of water to obtain a horizontal border of the gel. After polymerisation, the water was removed and the stacking gel (see appendix) was poured. To create wells for loading the samples onto the gel, a comb to create 1 cm wide chambers was plugged into the gel. Proteins were separated in a vertical apparatus and 1x running buffer (see appendix) at 120 mV for approximately 1 ½ h. For fixation of the proteins, gels were soaked in 25% isopropyl alcohol and 10% acetic acid aqueous solution for 30 min followed by Amplify for 30 min to intensify the signal. Gels were gently put on absorbent paper, covered with cling film and dried in a vacuum drier for 30 min at 60°C, followed by 45 min at 80°C. The dried gels were autoradiographed over night at -80 °C. The X-ray-film was developed in an automatic process using a Film Developer.

3.2.6. Chicken embryo limb bud model

The procedure of the chicken experiments is schematically illustrated in fig.3.3 and the appropriate experimental conditions are summarised in tab.3.8.

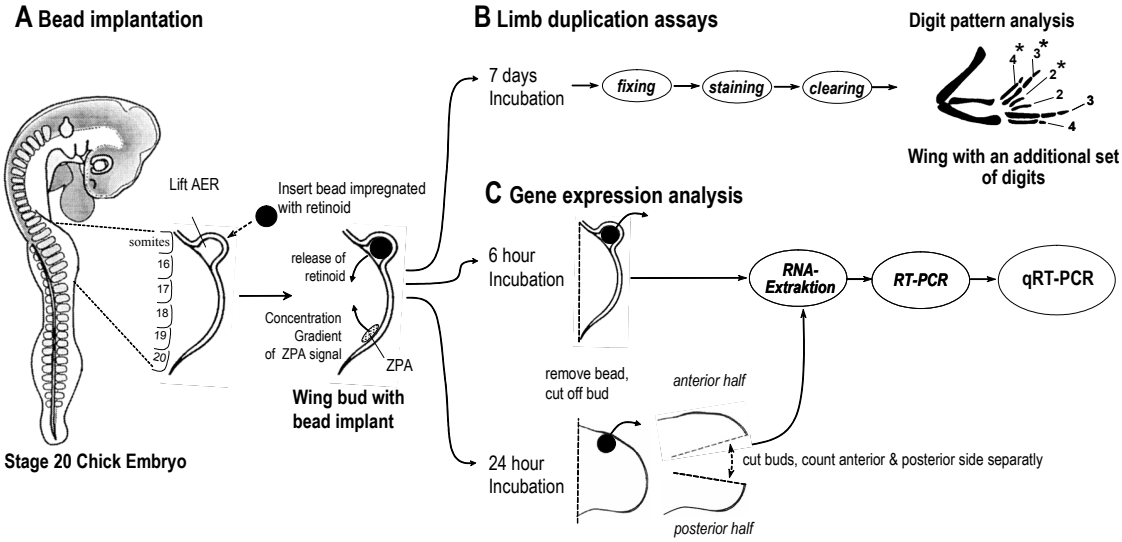

Fig.3.4: Schematic representation of A) bead-implantation-experiments and the following procedures for B) limb duplication assays and C) gene expression analysis.

Fig.3.4A) Ion-exchange beads were soaked in ethanolic solution containing different concentrations of *S*-4o-9c-dh-RA or at-RA (see tab.3.8) and implanted in a created hole under the AER at the anterior margin of a right wing bud from a stage 20 chick embryo (3 ¼ days of incubation). Retinoids diffuse continuously into the mesenchymal tissue and set up a concentration gradient, which results in the induction of the expression of certain genes. By placing the bead opposite to the ZPA, the RA-gradient is formed mirror-symmetrically to the endogenous ZPA-signal gradient, which disturbs the patterning process of the skeletal limb elements. The embryos were returned back into the incubator and left to develop for a certain time depending on the purpose of the experiment. Embryos where the influence of the novel RA-metabolite on the patterning process was investigated (limb duplication experiments)

were incubated for 7 days followed by sacrificing the embryo and inspecting their skeletal pattern **(fig.3.3B)**. Embryos were sacrificed and their limbs stained to visualise the skeletal pattern. The treatment of the limb buds with bioactive retinoids leads to an aberrant skeletal pattern. The normal digit pattern 234 (from anterior to posterior) is invariable changed in a pattern with one to three additional digits (marked by asterisks) arranged in a mirror-symmetrical manner. In a second set of experiments it was examined whether *S*-4o-9c-dh-RA can regulate the expression of certain at-RA target genes in the limb bud tissue using qRT-PCR analysis. Therefore buds were cut off after 6 or 24 h of incubation, respectively **(fig.3.3C)**. Transcript levels of the direct target genes *RARβ2, Cyp26*, and *Hoxb-8* were analysed after 6 h of incubation, whereas levels of the indirect target genes *shh* and *bmp-2* were analysed after 24 h. Buds removed after 6 h were homogenised completely, whereas buds from 24 hour time-point were cut into anterior and posteroir part.

3.2.6.1. Incubation of eggs

Fertilised White Leghorn chicken eggs (VALO SPF-eggs) were incubated at 37.5°C, and a humidity of ~ 60% in a horizontally orientation.

3.2.6.2. Preparation of embryos

After 65 h of incubation the eggs were taken out of the incubator and flipped twice by 180 ° (embryo detaches from the inner shell membrane). This was done while the eggs were disinfected with ethanol spray from all sides. Dissection needles were used to make first small holes into the blunt end of the eggshell followed by creating another 3-4 mm wide hole on the flat eggshell above the embryo, which ought to float on the top. This step was done very carefully not to injure the embryo. After the underlying opalescent membrane was cautiously punctured the embryo instantly detached from the shell membrane and sunk, and an airspace formed. The hole on the flat top of the egg was sealed with tape and a hole of app. 12 mm diameter was created with the help of small scissors. The embryos should be in the center of the hole.

Tab.3.8: Experimental conditions of the chicken embryo experiments.

Experiment	Treatment	Soaking conc. [mg/ml]	Embryos per group *n*	Incubation time after bead implantation
Limb duplication assays	at-RA	0.025	12	7 d
		0.1	8	7 d
		0.2	9	7 d
		0.5	8	7 d
	S-4o-9c-dh-RA	0.2	8	7 d
		0.5	7	7 d
		1	10	7 d
		2.5	9	7 d
		5	11	7 d
		10	13	7 d
	ethanol	-	8	7 d
Gene expression analysis	at-RA	0.2	3	6 h
		0.2	3	6 h
		0.2	3	6 h
		0.2	2	24 h
		0.2	2	24 h
		0.2	2	24 h
	S-4o-9c-dh-RA	2	3	6 h
		2	3	6 h
		2	3	6 h
		2	2	24 h
		2	2	24 h
		2	2	24 h
	ethanol	-	3	6 h
		-	3	6 h
		-	3	6 h
		-	2	24 h
		-	2	24 h
		-	2	24 h

If not, the eggs were slightly jiggled until the embryos were centered. The embryos were staged according to Hamburger & Hamilton (HAMBURGER & HAMILTON, 1951) and should be in the stage of 17. In order to provide the embryos in the same stage for the operations, they were synchronised by leaving them at RT for up to 5 h. After staging the hole was covered with another piece of tape and the eggs were returned back into the incubator for another 15-18 h to bring them in the stage of 20.

3.2.6.3. Preparation of material before operating embryos

The surgical manipulations of the limb buds were accomplished with the help of a electronically sharpened tungsten needle. Therefore a 3 cm long piece of tungsten wire (0.3 mm thick) was clamped into a bracket and one outlet of a transformer (set on 6 Volt) is connected to the tungsten wire with a clip. The other outlet is connected to a solution of 2 N sodium hydroxide. While dunking the tungsten wire into the hydroxide solution up and down the tungsten wire form a sharp, fine needle. All embryo works were carried out under a dissecting microscope with a magnification range from x10 to x50 on a clean bench, which was cleaned with 70% ethanol together with all necessary tools prior to the experiments.

3.2.6.4. Impregnation of the beads with retinoids

All works with retinoids were carried out under shade conditions. Approximately 15 AG1-X2 ion-exchange beads of 200 – 250 µm diameters were placed by forceps into a 1.5 ml micro centrifuge polypropylene tube and soaked in 30 µl ethanolic solution containing the retinoids. The soaking concentration for at-RA ranged from 25 to 500 µg/ml for limb duplication experiments and was 200 µg/ml for gene expression analysis, whereas the soaking concentration for *S*-4o-9c-dh-RA was 0.2 to 10 mg/ml for limb duplication experiments and 2 mg/ml for gene expression analysis (for details see tab.3.8). As controls, beads were soaked in ethanol. The micro centrifuge tubes containing beads and soaking solution were vigorously shaken in a microtube shaker for 20 min at RT. After removing the retinoid solution, beads were washed twice for 20 min in 200 µl of phenol red-containing phosphate-buffered saline (100 ml PBS and 500 µl of a 2 mg/ml phenol red solution in ethanol) while tubes were shacked. Beads were placed with the last washing solution in form of a drop on a 35 mm Petri dish.

3.2.6.5. Bead-Implantation at the anterior wing bud margin

The embryos in stage 20 were taken out of the incubator. After removing the tape the serosa and amnion membrane were torn away with two watchmaker's forceps. Using

the electronically sharpened tungsten needle a slit was cut between the AER and the underlying mesenchyme (cf. chapter fig.3.3). The ridge was carefully lifted up to create a loop in which the bead was then carefully maneuvered using the forceps. The method is precisely described in TICKLE *et al.* (1985) and WEDDEN *et al.* (1990). Fig.3.4 shows an image of a stage 20 chick embryo with a bead implant immediately after implantation. The eggs were sealed with tape and returned back to the incubator for either 6 or 24 h for gene expression analysis and 7 days for analysis of wing patterns (cf. chapter fig.3.3B, C and tab.3.8).

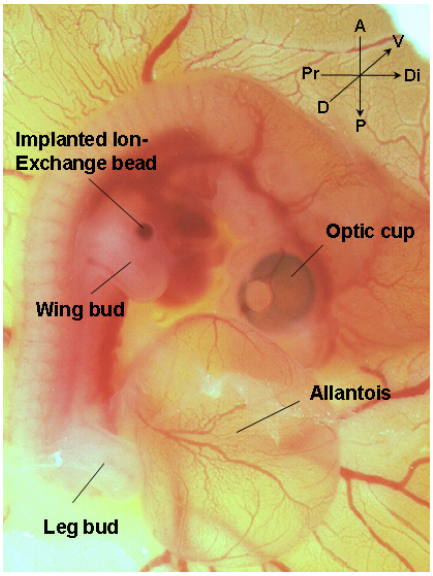

Fig.3.5: Picture of a HH-stage 22 old chick embryo with bead-implant.

3.2.6.6. Continuing procedures for limb duplication assays

Sacrificing, fixing, staining and clearing of the embryos

After another 7 days of incubation embryos were removed out of the egg with blunt forceps and sacrificed by decapitation. The specimens were washed three times in water, fixed over night in 5% (w/v) trichloroacetic acid, stained for 8 h in Alcian Blue dye solution (0.5 g of dye in 500 ml 70% (v/v) ethanol containing 1% HCl) and washed for 6 h in acidic ethanol (70% ethanol containing 1% HCl) to remove excess dye. Subsequently the embryos were dehydrated in absolute ethanol for 6 h. Finally specimens were dropped in methyl salicylate to clear the embryos and enable the

inspection of the skeletal pattern under a dissecting microscope. All listed steps were performed in densely closed glass vials slightly shaking on a rocking platform.

Analysis of wing patterns and data analysis

In order to generate dose-response curves that quantitatively reflect the effects of retinoids on the digit pattern, the extent of pattern duplication is stated in percentage respecification values (PRV) in which the wing patterns are expressed in numerical terms. Patterns were scored as follows: A pattern with the anteriormost additional being a digit 4 scored 100%. A wing with an additional digit 3 anteriorly scored 66%, while a wing with an additional digit 2 scored 33%. A digit of equivocal identity obtained a score of 0%. For the calculation of a PRV of a group treated with a certain concentration the scores of specimens treated with particular dose are added and divided by embryos per group (n). These values, which range from 0 to 100%, were plotted against the soaking concentration in the retinoid solution to generate a dose-response curve. The validation of limb duplication results in the form of a dose-response-curve is exemplarily shown in fig.3.5.

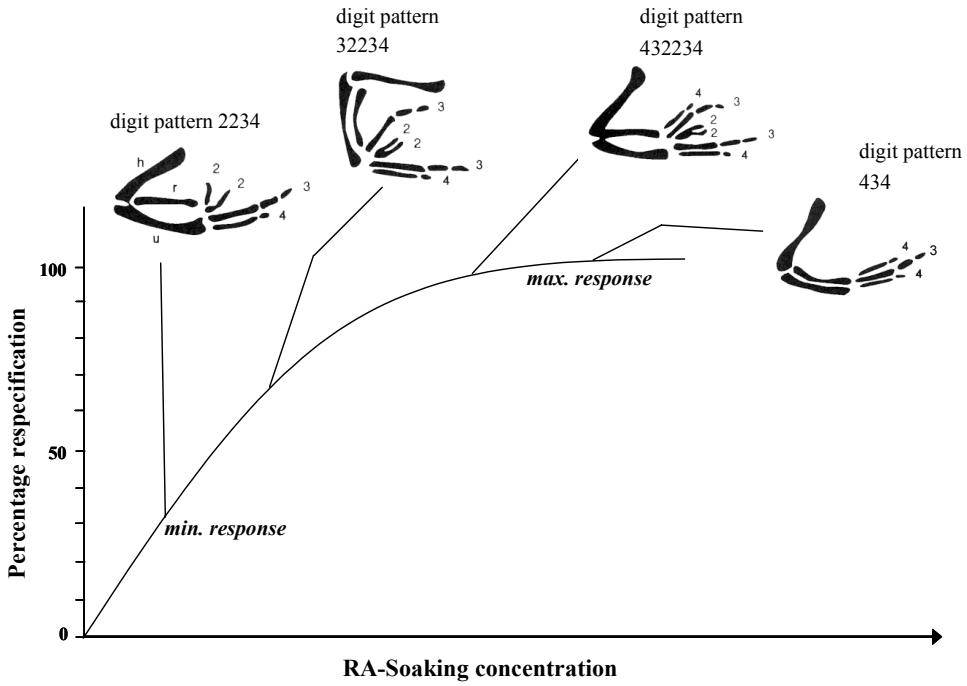

Fig.3.6: Validation of the limb duplication results in a dose-response curve.

3.2.6.7. Continuing procedures for gene expression assays

Dissection of limb bud tissue

After 6 and 24 h of incubation embryos were dissected out of the egg and rinsed in ice-cold PBS. Before cutting off the right buds with bead implantion using small scissor the beads were removed off the bud (see fig.3.3C). From each embryo the untreated left buds were collected additionally to obtain tissue material for analysis of endogenous expression levels of the target genes. In addition, buds from the 24 hour time point were cut into anterior and posterior parts using a fine tungsten needle (see fig.3.3C.), whereas whole buds were used from the 6 hour time point. The partition was a quite delicate procedure since the buds possess a size of a small pinhead (~ 2-2.5 mm diameter). For each time point 3 buds were pooled, and experiments were carried out in triplicate. Dissected buds were collected in sterile micro centrifuge vials. Before tissue was rinsed in 600 µl disruption buffer (RLT-lysis buffer from RNeasy® kit containing 10 µl β-Mercaptoethanol/ ml), the left-over PBS was removed by pipetting. After 10 min of incubation on ice the tissue was homogenised by pushing the extract a several times through a needle with a syringe.

RNA-Isolation from limb bud tissue

Total RNA was extracted and purified using universal tissue RNeasy® Kit according to the manufacturer's instructions. 1 Volume (600 µl) of 70% ethanol was added to the homogenised lysate and mixed by well pipetting. Samples were applied in two steps (600 µl each time) to RNeasy mini columns (containing silica gel membrane) and centrifuged for 15 sec at 10000 rpm in a micro centrifuge. The flow-through was discarded. In a washing step 700 µl RW1 buffer was added to the columns, followed by 15 sec centrifugation at 10000 rpm and discarding the flow-through together with the collection tubes. The columns were placed in new sterile RNase free tubes and 500 µl RPE buffer was added. Columns were centrifuged for 15 sec at 10000 rpm and flow-through was discarded. This step was repeated with 2 min centrifugation time. The flow-through was discarded. Columns were placed in new sterile RNase free tubes and centrifuged for 1 min at full speed. For the elution of RNA columns were placed again in new 1.5 ml RNase free tubes and 50 µl of RNase-free DEPC-

H_2O was added and columns were centrifuged for 1 min. at 10000 rpm. The step was repeated with the same eluate.

Reverse transcriptase reaction (cDNA-synthesis)

Total RNA was reverse transcribed using ThermoScript™ RT-PCR-System (Invitrogen) with oligo-$(dt)_{20}$ primers. For priming polyadenylated RNA 1 µg total RNA was combined with 1 µl oligo-(dt)20 primers (50 pmol), 2 µl dNTP Mix (10 mM) and DEPC-H_2O (up to volume of 12 µl). To denature primers and RNA the samples were incubated at 65°C for 5 min in a thermocycler followed by chilling on ice. A master-mix for the reverse transcription was produced (see fig.3.9). cDNA synthesis buffer, RNaseOUT & ThermoScriptRT are very temperature-sensitive, therefore solvents were thawed directly before use. 5 X cDNA synthesis buffer was vortexed immediate before pipetting. All pipetting steps were performed on ice. The sample was reversed transcribed at 52°C for 60 min in a thermocycler. After cDNA synthesis and termination of the reaction by incubating at 85°C for 5 min, the reaction mixture was incubated with RNase H (1 µl per sample) at 37°C for 20 min. To limit variations all RNA samples were reverse transcribed simultaneously.

Tab.3.9: Composition of master-mix for qRT-PCR.

Components	per Reaction
5 X cDNA synthesis buffer	4 µl
0,1 M DTT	1 µl
RNaseOUT (40 U/µl)	1 µl
DEPC-H2O	1 µl
ThermoScriptRT (15 units/µl)	1 µl

Absolute quantification of RA-target gene transcript levels in limb bud tissue

The expression rates of the corresponding RA-target genes in proportion to the endogenous controls were analysed using qRT-PCR performed on an iCycler™ in 20 µl reaction mixtures (conditions see fig.3.10). For the reactions a master mix of the following reaction components was prepared to the indicated end-concentration: 2,5

µl Foreward primer (350 nmol), 2,5 µl Reverse primer (350 nmol) and 10 µl Farst start iQ^{TM} SYBR Green Supermix. The SYBRGreen-Primer-Mix (15 µl) was filled in 96-well-PCR-plates and 5 µl cDNA was added as PCR template. The PCR-plate was closed with tape, centrifuged and placed into the iCycler^{TM} rotor.

Tab.3.10: qRT-PCR conditions for *Hoxb-8, RARβ2, Cyp26, shh,* and *bmp-2* transcript amplification.

Program		Temperature (°C)	Time
Initial denaturation		95	10 min
Amplification & quantification (50 cycles)	*Denaturation:*	94	15 sec
	Annealing:	60	25 sec
	Extension:	72	20 sec
Melting curve		50-94	10 sec
Cooling step		4	∞

The amplification and quantification program was performed with a single fluorescence measurement. The specificity of the qRT-PCR products was determined by performing the melting curve analysis after each PCR from 50-94°C with an increasing set point temperature after cycle 2 by 0.5 ºC per second and a continuous fluorescence measurement. Oligonuleotid primers (see tab.3.2) were optimised to an annealing temperature of 60°C. TBP (TATA box binding protein) is a housekeeping gene that should be equally expressed in all cells and was used as an internal standard.

Calculation of relative expression ratios

Expression levels of target genes were determined by the standard curve method. The standard curve of each target gene is performed with coincidental samples over 3.5 log levels on each plate. The absolute quantity of target RNA was determined by the iCycler^{TM} iQ Optical Software. The expression level of each target gene was normalised by dividing it by the TBP expression level. All assays were performed in triplicate and the results represent three repeated experiments. The RER of each target gene is defined as a quotient between treated and untreated samples, based on the absolute quantity levels, and is expressed in arbitrary units (RER = [absolute target

gene quantity $_{\text{treated sample}}$ (absolute quantity $_{\text{target gene}}$ / absolute quantity $_{\text{TBP}}$)] / [absolute target gene quantity $_{\text{untreated sample}}$ (absolute quantity $_{\text{target gene}}$ / absolute quantity $_{\text{TBP}}$)]). Since *shh* is a gene which is not expressed endogenously in the anterior section of the limb buds, the RER for *shh* (RER_{shh}) is determined as a quotient between at-RA and *S*-4o-9c-dh-RA-treated samples (RER_{shh} = [absolute *shh* quantity $_{\text{at-RA-treated sample}}$ (absolute quantity $_{shh}$ / absolute quantity $_{TBP}$)] / [absolute *shh* quantity $_{S\text{-4o-9c-dh-RA-treated sample}}$ (absolute quantity $_{shh}$ / absolute quantity $_{TBP}$)]). The difference in RER levels between retinoid treated and untreated samples were assessed using the unpaired t-test. The level of significance was selected as $p<0.05$.

3.2.7. Statistical analysis of transactivation and qRT-PCR results

The statistical analysis of the data from the transactivation studies (relative luciferase activity/ induction) as well as the data from the gene expression studies (relative expression ratios) were assessed by paired *t*-tests using Sigmastat Statistical software (Jandel Scientific, Erkath, Germany). The reported data are the arithmetric mean ± standard deviation (SD) for individual groups of different treatments. When the data were normally distributed (Kolmogorov-Smirnov test) the paired *t*-tests was used to test the statistic significance between control vs. treated sample. Group sizes are indicated in result figures.

4. Results

4.1 Chemical purity of the synthetic *S*-4-oxo-9-*cis*-13,14-dihydro-retinoic acid used in biological experiments

For purity check aliquots of the standard stock solutions were injected in concentrations between 10 and 100 ng/ml. Fig.4.1 shows three typical chromatograms of polar retinoids separated by reversed phase HPLC. Fig.4.1A shows the typical polar fraction of liver retinoids from NRMI-mice and displays the high endogenous concentration of the new RA-metabolite in comparison to at-RA. The purity of the synthetic

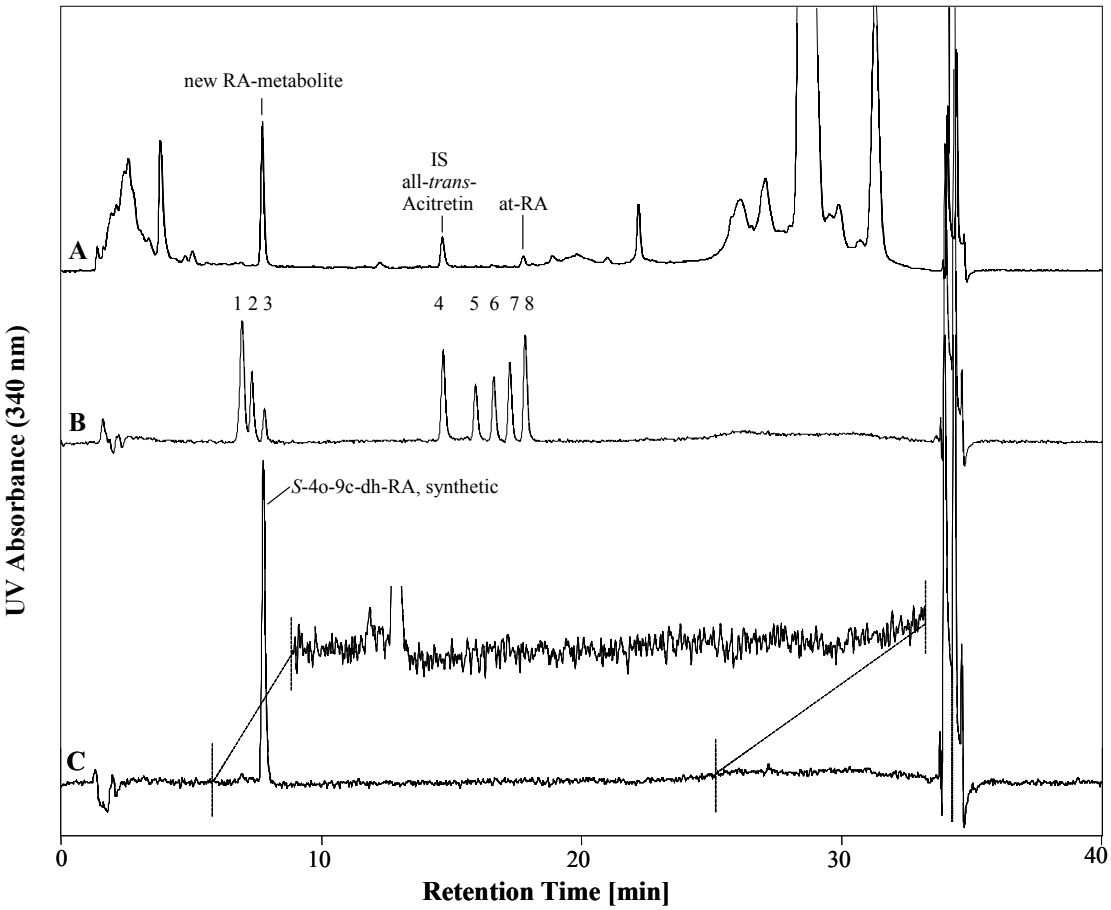

Fig.4.1: Chromatograms of polar retinoids separated by reversed phase HPLC.

A) Polar fraction of liver retinoids from NRMI-mice. **B)** Standard mixture consisting of several RA derivatives 1: 4-oxo-13-*cis*-RA , 2: 4-oxo-all-*trans*-RA, 3: *S*-4o-9c-dh-RA, 4: internal standard (IS) all-*trans*-Acitretin, 5: all-*trans*-3,4-didehydro-RA, 6: 13-*cis*-RA, 7: 9-*cis*-RA, 8: at-RA. **C)** Aliquot of the purified synthetic *S*-4o-9c-dh-RA-stock solution used for biological investigations. The 50-times magification of the signal demonstrates the purity of the stock solution. (RP18 column, Spherisorb ODS 2 mm, 2.1 x 150 mm, 3 µm particle size).

material used in the biological experiments is demonstrated by comparing the chromatograms of an aliquot of the synthetic *S*-4o-9c-dh-RA-stock solution (fig.4.1C) with a standard mixture containing a number of RA derivatives (fig.4.1B). The tiny peak right in front of the *S*-4o-9c-dh-RA-peak (fig.4.1C) cannot be assigned to a certain compound, since the signal is smaller than the signal-to-noise ratio (S/N) of 2. Fig.4.2 demonstrates the purity of the specific enantio-selective isomers after three separation steps in comparison to the initial racemic mixtures.

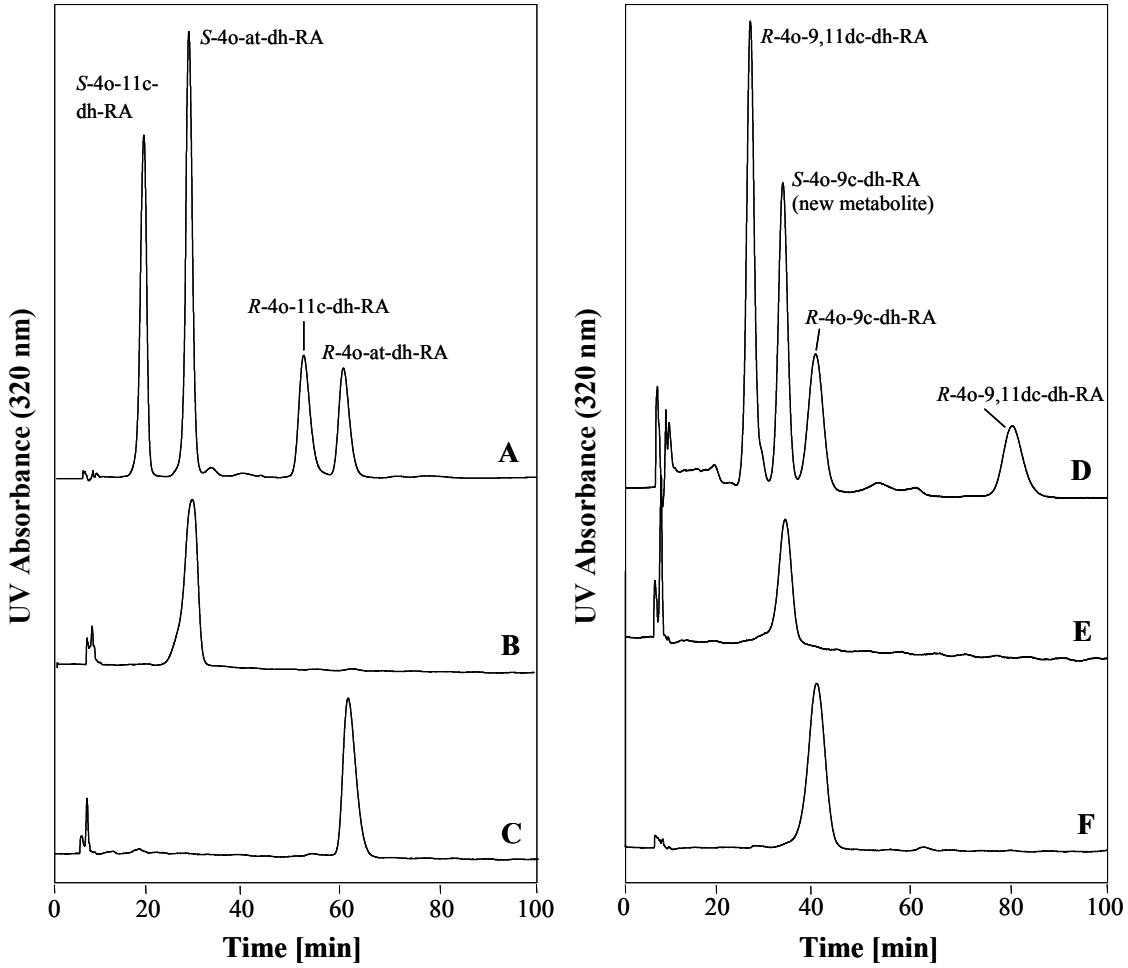

Fig.4.2: Chromatograms of purified 4o-dh-RA stock solutions separated by chiral phase HPLC.

A) Aliquot of synthesis product containing both enantiomers of 4o-11c-dh-RA and 4o-at-dh-RA. **B)** Aliquot of *S*-4o-at-dh-RA and **C)** *R*-4o-at-dh-RA after purification with chiral phase HPLC. **D)** Aliquot of synthesis product containing both enantiomers of 4o-9,11dc-dh-RA and 4o-9c-dh-RA. **E)** Aliquot of *S*-4o-9c-dh-RA (new metabolite) and **F)** *R*-4o-9c-dh-RA after purification with chiral HPLC. Chromatograms of B, C, E, and F) demonstrate the purity of stock-solutions, which were used in the biological investigations. (Chiracel OJ-H column, 4.6 x 250 mm, 10 µm particle size).

4.2 Transcriptional activation of retinoid receptor dependent luciferase reporter plasmids transfected to different cell lines

To analyse the possible transcriptional activity of 4o-dh-RAs several different cell lines were transfected with different luciferase reporter plasmids. The luciferase synthesis from these plasmid constructs is under the regulation of a RARE or RXRE, which are exclusively recognised by either ligand activated RAR/RXR heterodimers or ligand activated RXR/RXR homodimers, respectively. The retinoid-receptor-dimer-complexes bind to the corresponding regulatory sequence of the plasmids, which is the 2xDR5 element for RAR/RXR-heterodimers or the DR1 element for RXR/RXR-homodimers, respectively. This results in transcriptional activation and the expression of the luciferase gene (for details see methods). The level of luciferase expression is positively correlated to the rate of transcription, which is in turn related to the specific capacity of a ligand to activate signaling through the retinoid receptors. In all experiments at-RA was used as a positive control. The control treated cells were set to one, meaning all the treated sample values are relative to untreated cells.

4.2.1. Transcriptional regulation of the synthetic 2xDR5 element in HC11-RARE, Hela, and P19 cells

HC11-RARE, HeLa and P19 cells express several retinoid receptors and thus have a functioning retinoid signaling system. These cell lines are thus suitable model systems for investigating retinoid dependent signaling. The pGL3basic2xDR5luc reporter vector was stably transfected to HC11 cells (fig.4.3) and transiently to Hela (fig.4.4) and P19 cells (fig.4.5). After transfection, cells were subsequently treated with increasing doses of 4o-9c-dh-RA or 4o-at-dh-RA, respectively. In each case both enantiomers were tested to observe, whether differences exist between the *S*- and the *R*-type.

Transcriptional activity in HC11-RARE cells

The first picture (A) in figure 4.3 shows the stable transfected HC11-RARE cells, treated with four concentrations of *S*-4o-9c-dh-RA for 24 hours. Beside a 3.2-fold

induction of the at-RA treated cells (fig.4.3A lane 2), a dose dependent increase in transcriptional activity from the luciferase reporter upon treatment with *S*-4o-9c-dh-RA was observed. The increase in transcriptional activity among the *S*-4o-9c-dh-RA -treated cells compared to the control treated became significant from a concentration of 1 µM of *S*-4o-9c-dh-RA, with a 1.7-fold increase, whereas 10 µM resulted in a 2.4-fold increase (fig.4.3A lanes 5-6). At lower concentrations (10 nM and 100 nM), only a slight increase in transcriptional activity was observed (fig.4.3A lanes 3 and 4). The effect of *R*-4o-9c-dh-RA on transcriptional activation was very weak and failed to show any statistically significance (fig.4.3B lane 3-6). Whereas at-RA treatment induced the luciferase activity 2.1-fold, the highest induction of *R*-4o-9c-dh-RA was 1.2-fold at 10 µM (fig.4.3B lane 6).

The situation in HC11-RARE followed by 4o-at-dh-RA treatment was different. Both enantiomers, and in particular the *S*-type, were able to induce the luciferase reporter activity. *S*-4o-at-dh-RA treatment of the cells showed a significant induction of the luciferase activity already at the lowest tested concentration with 1.8-fold at 10 nM (fig.4.3C lane 3 & 4) and reached a 3.3-fold induction at 10 µM (fig.4.3B lane 6) compared to a 4.2-fold induction of at-RA treatment. *R*-4o-at-dh-RA (fig.4.3D lane 3-6) was less active and didn't show the same clear dose-response compared to *S*-4o-9c-dh-RA or *S*-4o-at-dh-RA. The fold-induction of the luciferase activity followed by *R*-4o-at-dh-RA was 1.4 at 10 nM and 2.0 at 10 µM compared to 3.0 after at-RA treatment.

Transcriptional activity in Hela cells

Figure 4.4 shows transiently transfected Hela cells, which were treated in a similar shape but already at lower concentrations. The transcriptional activity of *S*-4o-9c-dh-RA in Hela cells is similar to the results from HC11-RARE cells. The luciferase activity at low concentrations (between 1 and 100 nM) was not significantly induced (fig.4.4A lanes 3-5). However, *S*-4o-9c-dh-RA treatment in concentration of 1 µM led to a 2-fold increase of luciferase activity (fig.4.4A lane 6), compared to a 3.7-fold increase followed by control treatment with at-RA (fig.4.4A lane 2).

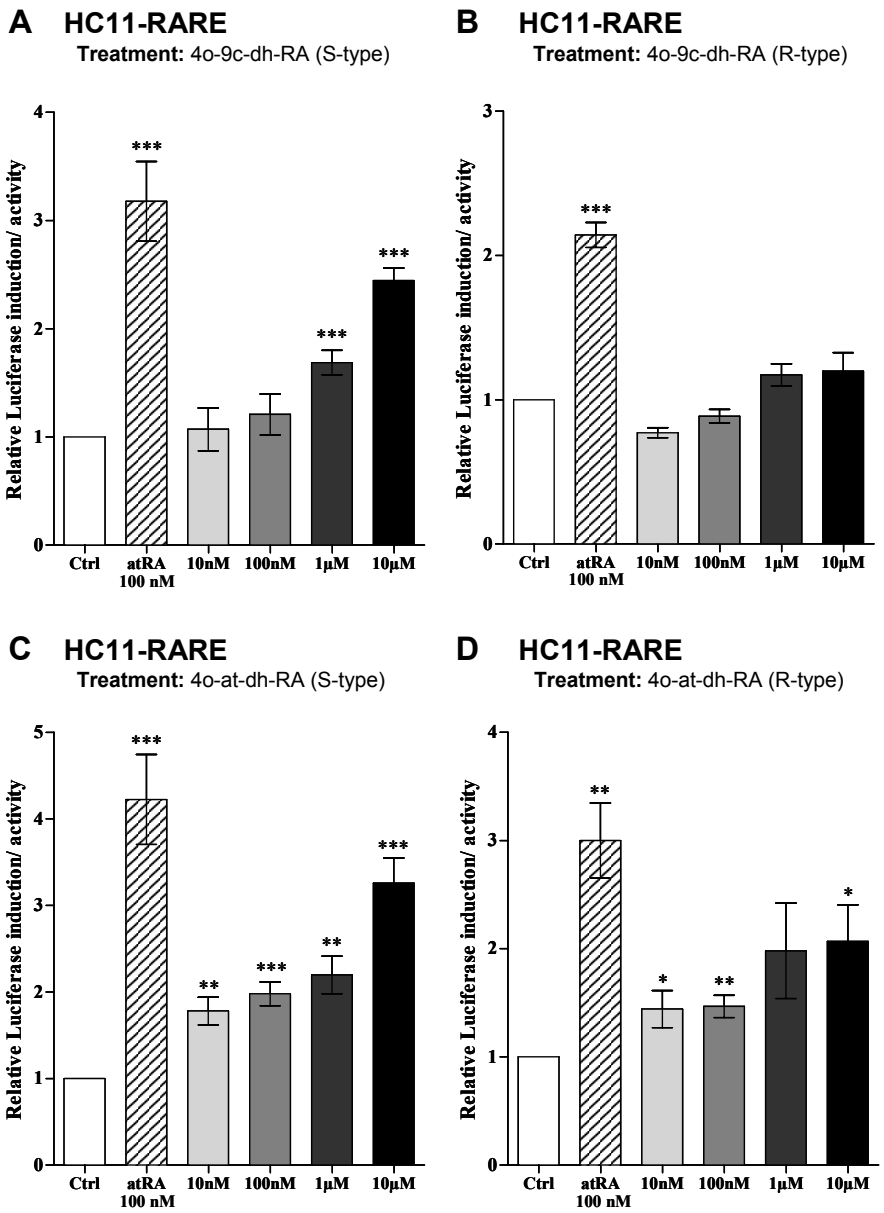

Fig.4.3: Transcriptional activation of the minimal RARE (2xDR5) by 4o-9c-dh-RA and 4o-at-dh-RA, in HC11-RARE cells.

HC11 were stable transfected with a simple RARE in direct repeat (2xDR5), which has been cloned into a pGL3basic-luc vector. Luciferase expression (represented in relative amounts by the different bars) results only upon transcriptional activation of the luciferase reporter plasmids by ligand activated RAR/RXR heterodimers (for details see methods). After 3 h of transfection cells were treated with **A)** *S*-4o-9c-dh-RA, **B)** *R*-4o-9c-dh-RA, **C)** *S*-4o-at-dh-RA, or **D)** *R*-4o-at-dh-RA in the indicated concentrations. In each experiment at-RA [100 nM] was used as a positive control. After 24 h of incubation, cells were collected by lysis and assayed for luciferase activity as described in methods. The relative luciferase induction is defined as a quotient of luciferase levels between treated and untreated control samples. Presented results are mean values of three experiments carried out in duplicates. Statistical analyses are described in methods. Stars indicate significant difference from controls (Ctrl), whereas * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respectively.

Similar to the results from HC11-RARE cells in fig.4.3B, *R*-4o-9c-dh-RA showed only marginal or no transcriptional activity on luciferase expression in Hela cells at any concentrations (fig.4.4B lane 3-6) compared to the robust induction followed by at-RA control treatment (fig.4.4B lane 2). As can be seen in fig.4.4C, *S*-4o-at-dh-RA had a distinct effect on RAR-mediated transcription and induced luciferase activity already at low concentrations (fig.4.4C lane 3-6). The effect was dose-dependent and reached – with 3.4-fold at 1 µM – a noticeable induction compared to the effect of at-RA. In contrast, the transcriptional activity of *R*-4o-at-dh-RA (fig.4.4D lane 3-6) in Hela cells was again weaker compared to *S*-4o-at-dh-RA and did not reach its potency.

Transcriptional activity in P19 cells

In P19 cells *S*-4o-9c-dh-RA treatment induced the luciferase reporter activity already at low concentrations from 1 nM or 10 nM (fig.4.5A lane 3 and 4). The induction – 1.2-fold at 1 nM and 1.3-fold at 10 nM – was weak but statistically significant and reached a 2.8-fold increase at the highest treatment concentration of 10 µM (fig. 4.5A lane 7) compared to a 6.8-fold increase followed by at-RA treatment (fig. 4.5A lane 2). The *R*-type enantiomer of 9c-4o-dh-RA (fig. 4.5B lane 3-7) was also able to induce luciferase activity in this system at low concentrations, whereas the effect was a bit less strong compared to *S*-4o-9c-dh-RA treatment thought. In contrast, a clear dose-dependent effect on luciferase activity was observed following *S*-4o-at-dh-RA (fig.4.5C lane 3-7) treatment.

S-4o-at-dh-RA induced the luciferase reporter activity at the lowest treatment concentration of 1 nM (1.6-fold, fig. 4.5C lane 3) and reached a 4.1-fold induction at 10 µM (fig. 4.5C lane 7). Similar to the results from HC11-RARE and Hela cells shown in fig.4.3D and 4.4D, *R*-4o-at-dh-RA had a weak(er) effect on transcriptional activation of luciferase expression, with the exception of the highest concentration (10 µM), where the luciferase activity is 4.6-fold induced compared to the untreated controls (fig. 4.5D lane 7).

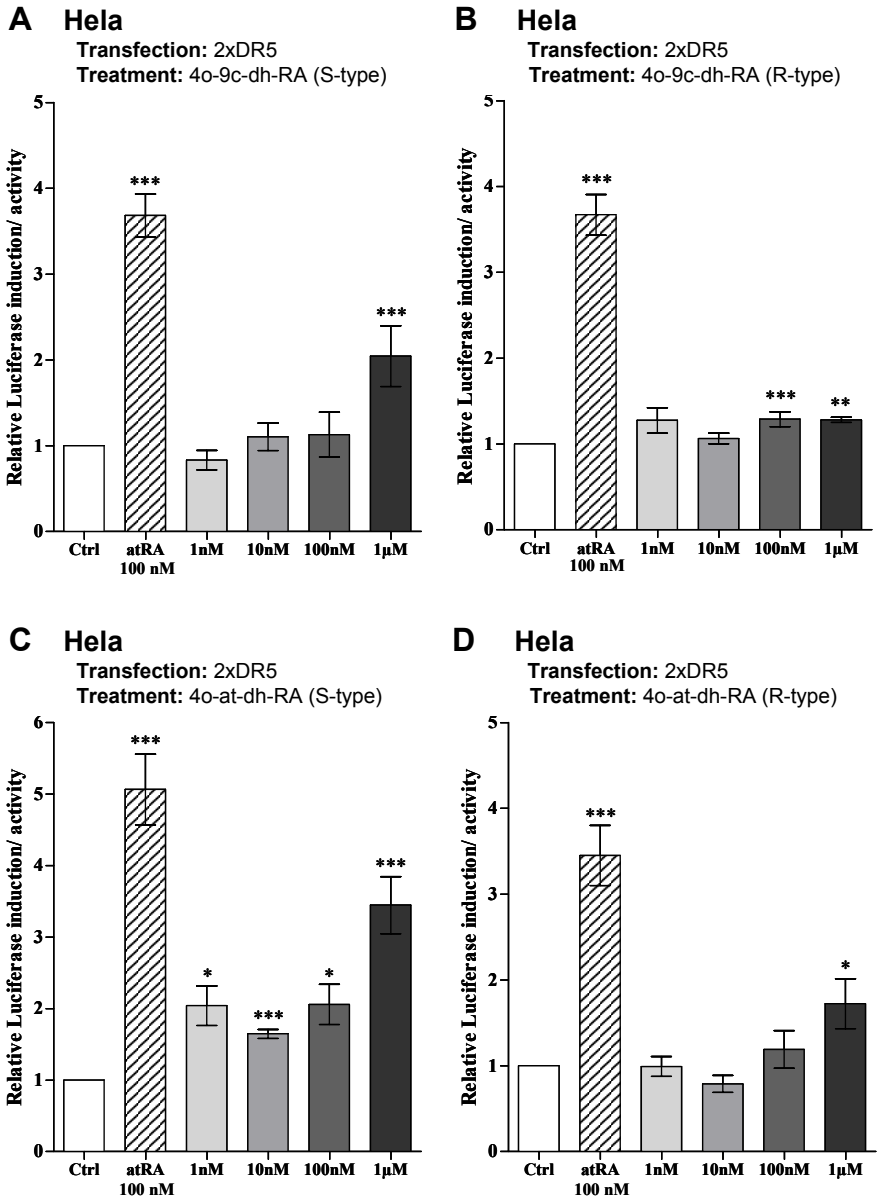

Fig.4.4: Transcriptional activation of the minimal RARE (2xDR5) by 4o-9c-dh-RA and 4o-at-dh-RA, in transfected Hela cells.

Hela were transiently transfected with a simple RARE in direct repeat (2xDR5), which has been cloned into a pGL3basic-luc vector. Luciferase expression (represented in relative amounts by the different bars) results only upon transcriptional activation of the luciferase reporter plasmids by ligand activated RAR/RXR heterodimers (for details see methods). After 3 h of transfection cells were treated with **A)** *S*-4o-9c-dh-RA, **B)** *R*-4o-9c-dh-RA, **C)** *S*-4o-at-dh-RA, or **D)** *R*-4o-at-dh-RA in the indicated concentrations. In each experiment at-RA [100 nM] was used as a positive control. After 24 h of incubation, cells were collected by lysis and assayed for luciferase activity as described in methods. The relative luciferase induction is defined as a quotient of luciferase levels between treated and untreated control samples. Presented results are mean values of three experiments carried out in duplicates. Statistical analyses are described in methods. Stars indicate significant difference from controls (Ctrl), whereas * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respectively.

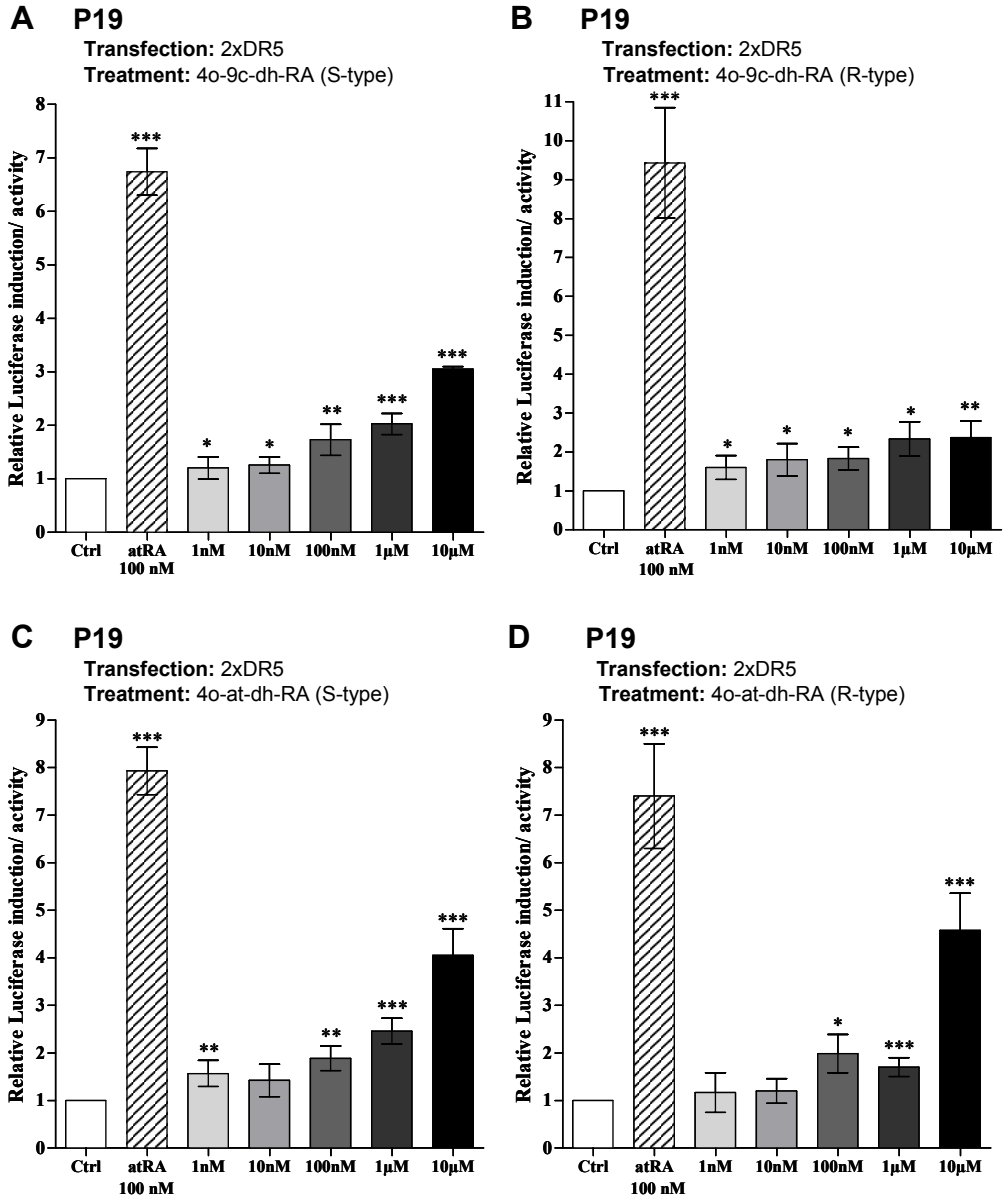

Fig.4.5: Transcriptional activation of the minimal RARE (2xDR5) by 4o-9c-dh-RA and 4o-at-dh-RA, in transfected P19 cells.

P19 were stable transfected with a simple RARE in direct repeat (2xDR5), which has been cloned into a pGL3basic-luc vector. Luciferase expression (represented in relative amounts by the different bars) results only upon transcriptional activation of the luciferase reporter plasmids by ligand activated RAR/RXR heterodimers (for details see methods). After 3 h of transfection cells were treated with **A)** *S*-4o-9c-dh-RA, **B)** *R*-4o-9c-dh-RA, **C)** *S*-4o-at-dh-RA, or **D)** *R*-4o-at-dh-RA in the indicated concentrations. In each experiment at-RA [100 nM] was used as a positive control. After 24 h of incubation, cells were collected by lysis and assayed for luciferase activity as described in methods. The relative luciferase induction is defined as a quotient of luciferase levels between treated and untreated control samples. Presented results are mean values of three experiments carried out in duplicates. Statistical analyses are described in methods. Stars indicate significant difference from controls (Ctrl), whereas * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respectively.

The results show similarities for all three cell lines: *S*-4o-9c-dh-RA is actually able to induce transcriptional activity in a similar manner as at-RA compared to control treated cells. The transcriptional activity induced by *S*-4o-9c-dh-RA from the pGL3b-2xDR5luc reporter does not reach exactly the same level as at-RA in these experiments. Even though, the clearly observable increase in transcriptional activity induced by *S*-4o-9c-dh-RA compared to controls showed to be statistically significant in concentrations from of 1 nM in P19 cells (fig.4.5A lane 3) and 1 μM in HC11-RARE (fig.4.3A lane 5) and Hela cells (fig.4.4A lane 6). Although the effect at lower concentrations is not statistically significant altered in some cases, the data clearly show a trend suggesting that the effect is dose-dependent. The effect of *R*-4o-9c-dh-RA on the transcriptional activation of the pGL3b-2xDR5luc reporter was throughout weaker compared to *S*-4o-9c-dh-RA in all three cell lines and failed to show a clear dose-dependency. In this respect the difference in the effectiveness between the *S*- and the *R*-enantiomer was also observed among the two 4o-at-dh-RA enantiomers. Whereas *S*-4o-at-dh-RA was thus able to transactivate luciferase expression in a dose-dependent fashion, *R*-4o-at-dh-RA was consistently less active in all three cell systems.

Additionally, the possibility that *S*-4o-9c-dh-RA could have antagonistic or synergistic effects against at-RA, regarding ligand properties towards RAR/RXR heterodimers was investigated. To investigate this, P19 cells were transfected with the pGL3b-2xDR5luc reporter in the same fashion and subsequently co-treated with at-RA together with different doses of *S*-4o-9c-dh-RA, ranging from 1 nM to 1 μM (fig.4.6 lanes 3-6). All retinoid treatments with at-RA or *S*-4o-9c-dh-RA induced the luciferase reporter activity statistically significant ($P < 0.001$) between 4.2- and 5.0-fold. The results from these experiments failed to show any statistically significant difference (marked by #) between the effects from co-treated cells (fig.4.6 lane 3-6) compared to cells treated only with at-RA (fig.4.6 lane 2).

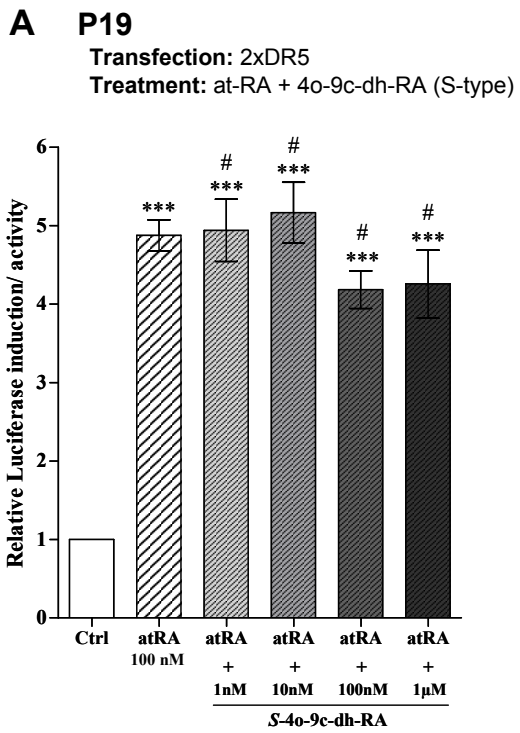

Fig.4.6: Transcriptional activation of the minimal RARE (2xDR5) in transfected P19 cells after co-treatment with at-RA and *S*-4o-9c-dh-RA.

P19 were stable transfected with a simple RARE in direct repeat (2xDR5), which has been cloned into a pGL3basic-luc vector. Luciferase expression (represented in relative amounts by the different bars) results only upon transcriptional activation of the luciferase reporter plasmids by ligand activated RAR/RXR heterodimers (for details see methods). In this set of experiments P19 cells were double-treated with at-RA and increasing concentrations of *S*-4o-9c-dh-RA to investigate antagonistic or synergistic effects of *S*-4o-9c-dh-RA towards at-RA. In each experiment at-RA single treatment [100 nM] was used as a positive control. After 24 h of incubation, cells were collected by lysis and assayed for luciferase activity as described in methods. The relative luciferase induction is defined as a quotient of luciferase levels between treated and untreated control samples. Presented results are mean values of three experiments carried out in duplicates. Statistical analyses are described in methods. Stars indicate significant difference from controls (Ctrl), whereas * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respectively. # indicates no statistical significant difference between double vs. at-RA single treatment.

Resume of the results from HC11-RARE, Hela, and P19 cells

To sum up the results briefly, all three cell lines that were transfected with the pGL3b-2xDR5luc reporter showed a robust dose dependent increase in transcriptional activity upon treatment with *S*-4o-9c-dh-RA, compared to controls. Co-treatment with at-RA and increasing dose of *S*-4o-9c-dh-RA in P19 cells did not show either antagonistic or synergistic effects.

4.2.2. Transcriptional regulation of the natural *RARβ2* gene promoter in Hepa-1 cells

Hepa-1 cells also express several retinoid receptors, and are therefore also appropriate for studying retinoid signaling. Hepa-1 cells were transiently transfected with the luciferase reporter plasmid pGL3b-RARβluc, regulated by a partial *RARβ2* promoter sequence, which is more complex than the simple 2xDR5 RARE. As seen in figure 4.7A, treatment of the transfected Hepa-1 cells with *S*-4o-9c-dh-RA induced transcriptional activity also from this more naturally regulated RARE sequence. The treatment of 10 μM *S*-4o-9c-dh-RA was followed by a 1.6-fold induction of the luciferase reporter activity compared to the controls (fig.4.7.A lane 1 & 5), whereas the lower concentrations of *S*-4o-9c-dh-RA had no effect (fig.4.7A lane 3 and 4). At-RA treated cells showed a 2.8-fold increase (fig.4.7A lane 2).

Again, the possibility of antagonistic or synergistic effects between at-RA and *S*-4o-9c-dh-RA in activating the retinoid receptors was investigated (see figure 4.7B). The treatment condition was similar as for the P19 cells in fig. 4.6. First of all, at-RA treatment induced the luciferase reporter activity 3.9-fold compared to untreated controls (fig. 4.7B lane 2). Co-treatment with 1 nM and 10 nM of *S*-4o-9c-dh-RA led to a slightly increased luciferase expression, which was induced approximately 4.5-fold (fig. 4.7B lane 3-4). Co-treatment with 1 and 10 μM (fig. 4.7B, lanes 5-6) led to a greater induction, approximately 6-fold (1.5 times higher than at-RA single treatment). The variations were greater between the repeated experiments here compared to those in fig. 4.6. Nevertheless, an interesting trend is clearly observable, whereas double-treatment of these cells resulted in a more robust transcriptional activation than at-RA single treatment, especially at concentrations from 1 μM *S*-4o-9c-dh-RA. This effect of double treatment was not seen in P19 cells. This difference is more likely to be dependent on difference between the cell types rather than the regulatory sequences of the transfected reporter plasmids. Any how, there is a clear difference between the results from these two double treatment experiments (fig. 4.6 & 4.7B).

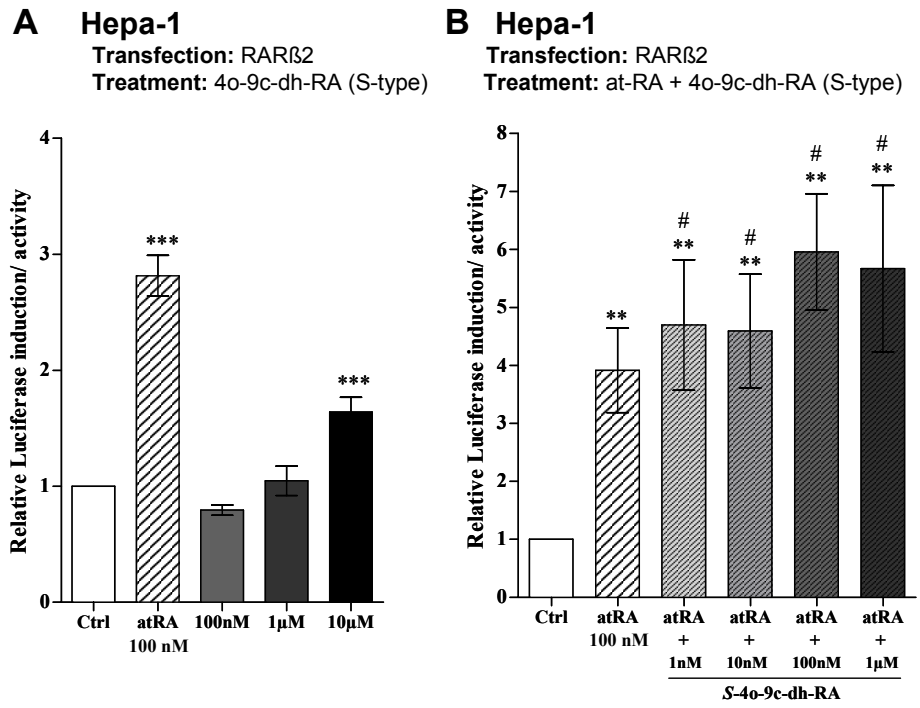

Fig.4.7: Transcriptional activation of the natural RARE by *S*-4o-9c-dh-RA, in transfected Hepa-1 cells.

Hepa-1 cells were transfected with the natural RARE from the *RARβ2* promoter, which has been cloned into a pGL3basic-luc vector. Luciferase expression results only upon transcriptional activation of the luciferase reporter plasmids by ligand activated RAR/RXR heterodimers (for details see methods). **A)** After 3 h of transfection cells were treated with *S*-4o-9c-dh-RA in the indicated concentrations and at-RA [100 nM] as a positive control. **B)** In comparative experiments cells were treated with at-RA together increasing concentrations of *S*-4o-9c-dh-RA to investigate antagonistic or synergistic effects of *S*-4o-9c-dh-RA towards at-RA. After 24 h of incubation, cells were collected by lysis and assayed for luciferase activity as described in methods. The relative luciferase induction is defined as a quotient of luciferase levels between treated and untreated control samples. Presented results are mean values of three experiments carried out in duplicates. Statistical analyses are described in methods. Stars indicate significant difference from controls (Ctrl), whereas ** $P < 0.01$ and *** $P < 0.001$. # indicates no statistical significant difference between double vs. at-RA single treatment.

4.2.3. Transcriptional activation of the RARE element (2xDR5) in CV1 cells via RARα or RARβ

CV-1 cells lack expression of retinoid receptors except small amounts of RARα. This makes them a useful tool to investigate whether the 4o-9c-dh-RA metabolites distinguish between certain combinations of retinoid receptor isoforms. The experiments using CV-1 cells were carried out by transfecting cultured cells with vector plasmids expressing certain RAR and RXR isoforms in different combinations, together with the reporter plasmid pGL3b-2xDR5luc and pCMW-βGal as internal controls to cor-

rect for intercellular variations. CV1 cells were transfected with a combination of RARα & RXRβ (fig.4.8A&B; fig.4.9A&B) and in a combination of RARβ & RXRβ (fig.4.8C&D; fig.4.9C&D). The cells were thereafter treated in the same pattern as in the other experiments, with untreated control, at-RA as positive control and *S*-4o-9c-dh-RA (fig.4.8A & C), *R*-4o-9c-dh-RA (fig.4.8B & D), *S*-4o-at-dh-RA (fig.4.9A & C), and *R*-4o-at-dh-RA (fig.4.9B & D) in increasing concentration, respectively.

Transcriptional effect of 4o-9c-dh-RAs in CV1 reporter cells

As seen in figure 4.8A and 4.8C, *S*-4o-9c-dh-RA induced transcriptional activation from the 2xDR5-reporter in both combinations in a dose dependent fashion compared to controls. Cells treated with 1 nM *S*-4o-9c-dh-RA did not differ from controls (Fig.4.8A & C, lanes 1 & 3), but the transcriptional activity increases with raised concentration, starting at concentrations from 100 nM in the cells with RARα & RXRβ (fig.4.8A, lanes 5-7), ranging from 1.3 to 3-fold change. 10 nM *S*-4o-9c-dh-RA led to a 1.4-fold change when RARβ & RXRβ was present and dose dependently ranges up to 3.1 (fig. 4.8C, lanes 4-7). These results show that *S*-4o-9c-dh-RA can mediate transcriptional activity from both combinations of retinoid receptors equally. The figures 4.8B and 4.8D demonstrate clearly another time, that the effect of *R*-4o-9c-dh-RA on transcriptional activation of the 2xDR5 element, this time solely mediated via either RARα (fig. 4.8B) or RARβ (fig. 4.8B), was weaker compared to *S*-4o-9c-dh-RA treatment. The effect (fold-induction) was poor at all treatment concentration and no dose-dependency was observed in both series of experiments compared to those seen in fig.4.8A & C.

Transcriptional effect of 4o-at-dh-RAs in CV1 reporter cells

Both the *S*- and *R*-type enantiomer of 4o-at-dh-RA was able to regulate RARE-dependent transcription in transfected CV1 cells and thus to induce luciferase activity via either RARα/RXRβ (fig.4.9A & B) or RARβ/RXRβ heterodimers (fig.4.9C & D). A statistically significant increase of the luciferase reporter activity followed by treatment of RARα/RXRβ transfected cells with *S*-4o-at-dh-RA was already observed at 10 nM (2.1-fold, fig.4.9A lane 4), whereas a concentration of 10 μM had a

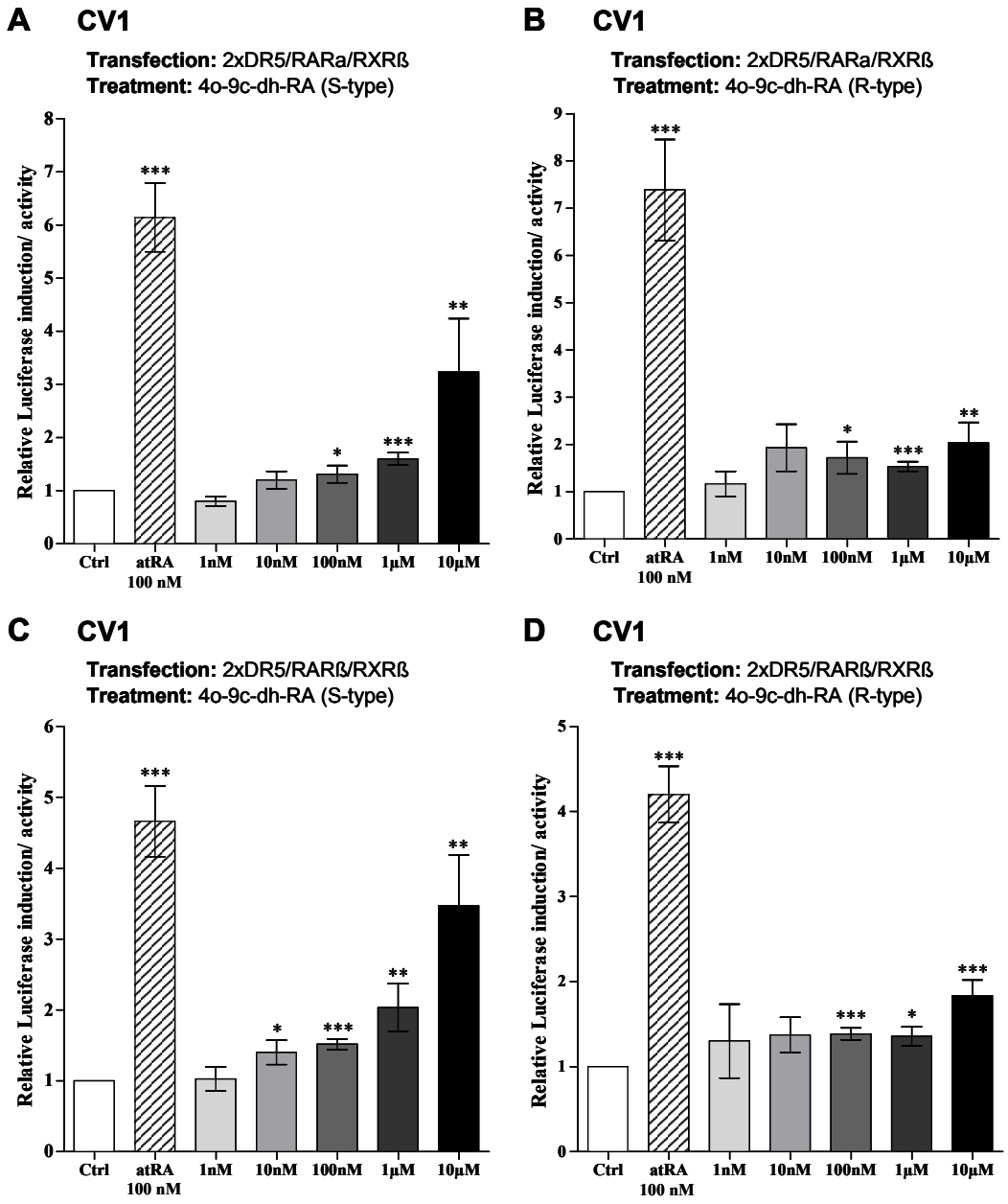

Fig.4.8: Differences in transcriptional activation of the 2xDR5-Reporter by *S*-4o-9c-dh-RA, *R*-4o-9c-dh-RA and at-RA in CV1 cells transfected with RARα/RXRβ or RARβ/RXRβ.

CV1 cells were transiently co-transfected with the pGL3basic2xDR5luc reporter vector and expression vectors for RXRβ together with either RARα **(A & B)** or RARβ **(C & D).** Cells were subsequently treated with either *S*-4o-9c-dh-RA **(A & C)** or *R*-4o-9c-dh-RA **(B & D)** in concentrations ranging from 10 nM to 10 µM. At-RA [100 nM] was used as a positive control. Cells were harvested after 24 h of incubation for assaying luciferase activity as described in methods. The relative luciferase induction is defined as a quotient of luciferase levels between treated and the untreated control samples. Presented results are mean values of seven experiments carried out in duplicates. Statistical analyses are described in methods. Stars indicate significant difference from controls (Ctrl), whereas * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respectively.

distinct effect and was followed by a 5.6-fold increase (fig.4.9A lane 7). Similar to the effects in RARα/RXRβ transfected cells seen in fig.4.9A, *S*-4o-at-dh-RA also induced the luciferase reporter activity in RARβ/RXRβ transfected cells already at low concentrations (1.4-fold increase at 1 nM, fig.4.9C, lane 3). The variations between the treatments and the repeated experiments were greater; therefore the effects were not statistically significant. However, the effect was dose-dependent and reached with a 3.8-fold increase a relative high induction compared to at-RA (4.8-fold, fig.4.9C, lane 2). Equally, *R*-4o-at-dh-RA had the same inducing effect on the luciferase reporter activity via both RARα/RXRβ (fig.4.9B lane 3-7) and RARβ/RXRβ (fig.4.9D lane 3-7) transfected cells. The effect was observable already at low concentrations and showed a clearly dose-dependency.

4.2.4. Transcriptional activation of the RXRE element (DR1) in CV1 cells via RARα or RARβ

To investigate the ability of *S*-4o-9c-dh-RA or *S*-4o-at-dh-RA to induce the transcription of the RXRE-regulated luciferase expression in reporter plasmids via RXR mediated transactivation, CV1 cells were co-transfected with the luciferase reporter plasmid pGl3b-DR1luc together with either RXRα or RXRβ expression vectors. The regulatory sequence, DR1, is exclusively activated by ligand activated RXR/RXR dimers which lead to the expression of the luciferase gene. In these set of experiments 9-*cis*-RA [100 nM] was used as a positive control for retinoid induced signaling, since 9-*cis*-RA is the natural ligand for RXR receptors. The results showed significant luciferase induction after treatment with 9-*cis*-RA in both RXRα (fig.4.10A&C lane 2) and RXRβ (fig.4.10B & D lane 2) transfected cells, whereas neither *S*-4o-9c-dh-RA (fig.4.10A&B lanes 3-5) nor *S*-4o-at-dh-RA (fig.4.10C&D lanes 3-5) was able to induce this effect in the RXRE-regulated luciferase reporter cells. Only RXRα transfected cells showed a slightly increase of the luciferase induction followed by treatment with *S*-4o-9c-dh-RA (fig.4.10A lanes 3-5) with a 1.6-fold increase at 10 μM. However, the effect was extremely weak compared to 9-*cis*-RA treatment and did not reveal a statistically difference in the luciferase activity values compared to the untreated control cells.

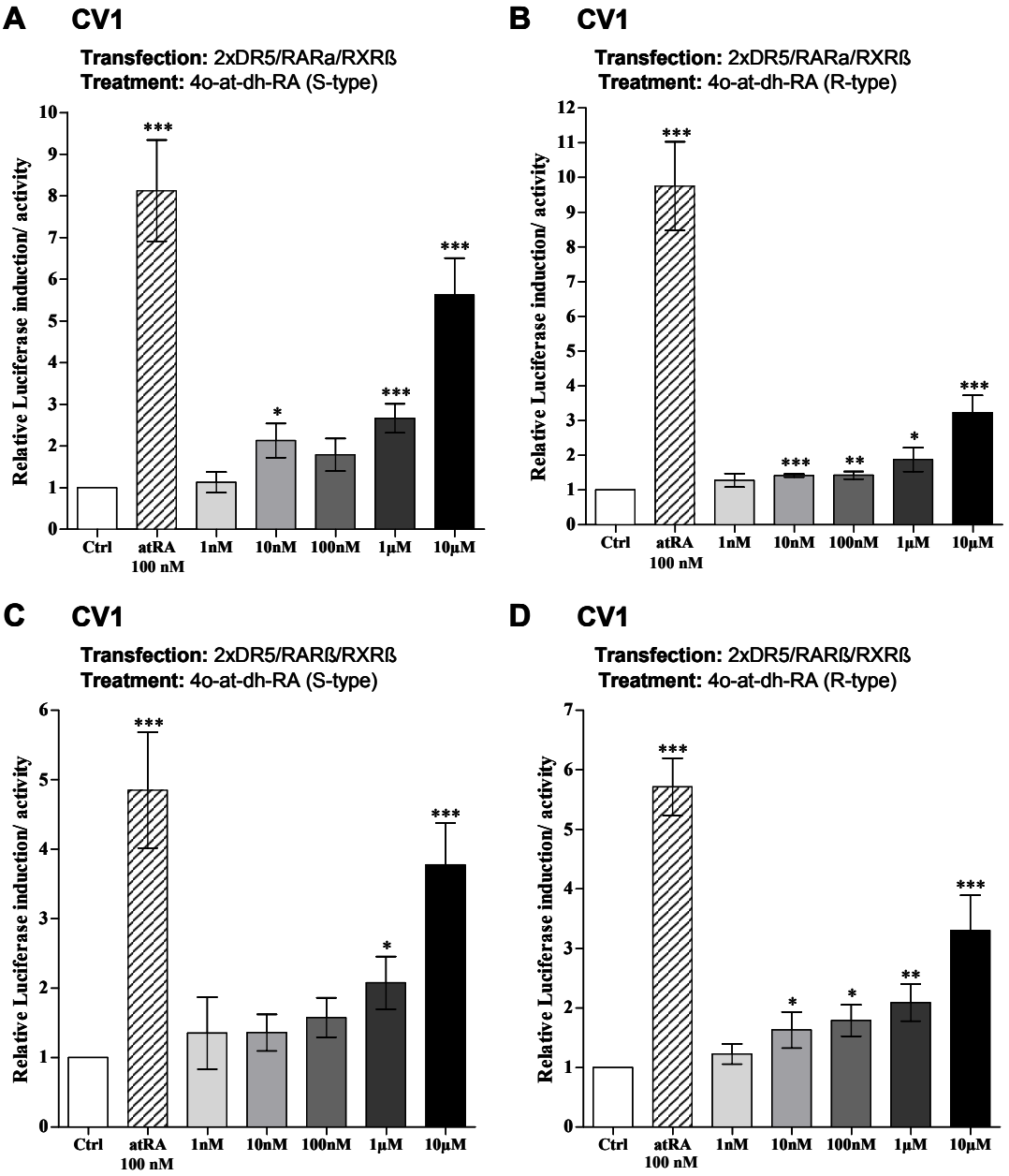

Fig.4.9: Differences in transcriptional activation of the 2xDR5-Reporter by *S*-4o-at-dh-RA, *R*-4o-at-dh-RA and at-RA in CV1 cells transfected with RARα/RXRβ or RARβ/RXRβ.

CV1 cells were transiently co-transfected with the pGL3basic2xDR5luc reporter vector and expression vectors for RXRβ together with either RARα **(A & B)** or RARβ **(C & D).** Cells were subsequently treated with either *S*-4o-at-dh-RA **(A & C)** or *R*-4o-at-dh-RA **(B & D)** in concentrations ranging from 10 nM to 10 µM. At-RA [100 nM] was used as a positive control. Cells were harvested after 24 h of incubation for assaying luciferase activity as described in methods. The relative luciferase induction is defined as a quotient of luciferase levels between treated and the untreated control samples. Presented results are mean values of seven experiments carried out in duplicates. Statistical analyses are described in methods. Stars indicate significant difference from controls (Ctrl), whereas * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respectively.

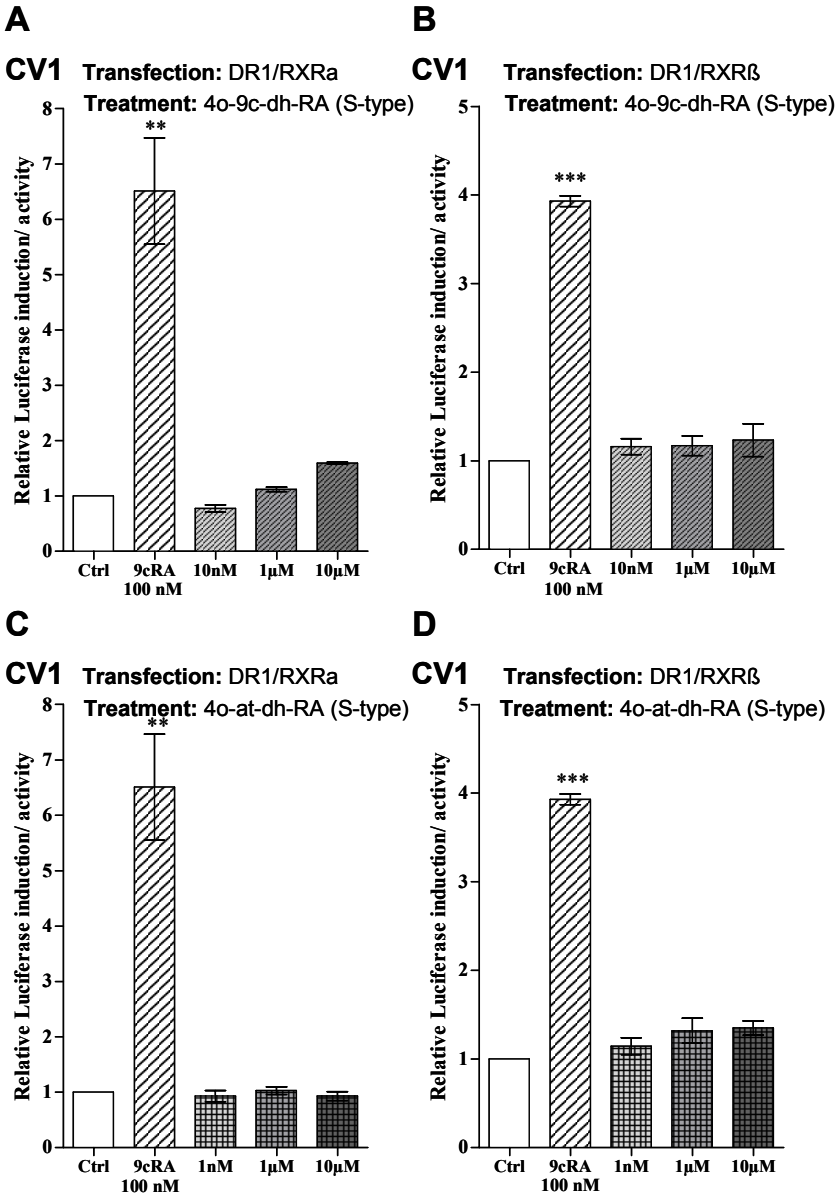

Fig.4.10: Transcriptional activation of the DR1 element by *S*-4o-9c-dh-RA and *S*-4o-at-dh-RA, in transfected CV1 cells.

CV1 cells were transiently transfected with the DR1 element together with either RXRα **(A & C**) or RXRβ **(B & D)** expression vectors. The DR1 element has been cloned into a pGL3basic-luc vector, which allows the luciferase expression only upon transcriptional activation of the reporter plasmids by ligand activated RXR/RXR homodimers (for details see methods). Transfected cells were treated with either *S*-4o-9c-dh-RA **(A & B)** or *S*-4o-at-dh-RA **(C & D)** in the indicated concentrations and 100 nM 9-cis-RA (9cRA) as a positive control. Cells were harvested after 24 h of incubation for assaying luciferase activity as described in methods. The relative luciferase induction is defined as a quotient of luciferase levels between treated and untreated control samples. Presented results are mean values of three experiments carried out in duplicates. Statistical analyses are described in methods. Stars indicate significant difference from controls (Ctrl), whereas ** $P < 0.01$, and *** $P < 0.001$, respectively.

4.3 *S*-4-oxo-9-*cis*-13,14-dihydro-retinoic acid induces the expression of RARβ2 mRNA levels in P19 cells

In order to investigate whether *S*-4o-9c-dh-RA is able to affect endogenous gene expression in cells, the mRNA expression levels of a well characterised RA target gene, *RARβ2*, was measured. P19 cells were cultured two days before the start of each experiment and subsequently treated with increasing amounts [1μM and 10μM] of *S*-4o-9c-dh-RA and [100 nM] at-RA as positive control. Thereafter the cells were sequestered at the indicated time points (2 & 24 hours). Total RNA was extracted and the specific mRNA expression levels of *RARβ2* and *γ-actin* (endogenous control) were analysed by qRT-PCR. No primer-dimers were generated during the applied 40 real-time PCR amplification cycles. The indicated expression levels in the figure were corrected against endogenously expressed *γ-actin*, the control treated cells at the 1 hour time point was used as calibrator (set to 1) for the other samples.

Figure 4.11 shows the resulting relative transcription of *RARβ2* mRNA following at-RA and *S*-4o-9c-dh-RA treatment. Already after 2 hours of treatment, 1 μM and 10 μM of *S*-4o-9c-dh-RA induced transcription of endogenous *RARβ* mRNA approximately 2 and 4-fold, compared to controls (fig.4.11, lanes 1,3-4), The fold change increased significantly with time and did not reach any plateau phase or down regulation within the 24 hour time span. After 24 hours of 10 μM *S*-4o-9c-dh-RA treatment, the *RARβ* transcription reached a 32-fold change (fig.4.11, lane 8), 1 μM treatment did not increase transcription equally much over time and reaches a fold change of 3.2 (fig.4.11, lane 7). One interesting notion was that after 24 hours of treatment the difference in fold change between positive control (at-RA) and *S*-4o-9c-dh-RA – visible at the early time point – decreased dramatically. The 10 μM concentration of *S*-4o-9c-dh-RA led to a high transcriptional activity, exceeding half of the fold change seen for at-RA. In summary, these results further strengthen the evidence that *S*-4o-9c-dh-RA is able to activate gene transcription through the retinoid receptors, both over a transfected RARE construct, but more importantly also from endogenous genes by a simple treatment regimen.

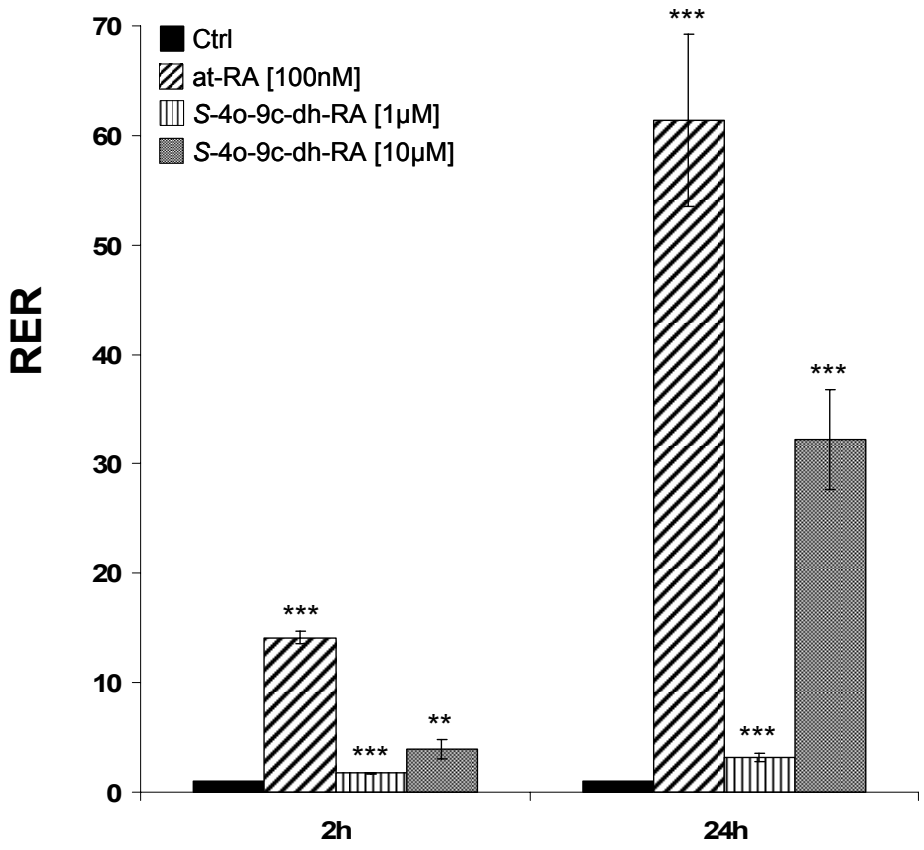

Fig.4.11: Induction of endogenous gene transcription in P19 cells by *S*-4o-9c-dh-RA.

P19 cells were simultaneously treated with 1µM and 10µM of *S*-4o-9c-dh-RA, and incubated for 2 and 24 h. As a positive control for induction of endogenous *RARβ2* transcripts, cells were treated in parallel with at-RA [100 nM]. PCR primers for *RARβ2* and *γ-actin* were used in quantitative realtime-PCR to measure the endogenous levels of *RARβ2* mRNA after the different treatments (see methods). The relative expression ratio (RER) of *RARβ2* showed in the diagram is relative to *γ-actin* (endogenous control) within each sample. The presented results are mean values ±SEM from three experiments. Statistical analyses are described in methods. Stars indicate significant difference from controls (ctrl), whereas *** $P < 0.001$.

4.4 Induction of conformational changes in RARα and RARβ proteins by *S*-4-oxo-9-*cis*-13,14-dihydro-retinoic acid

Hormone binding to nuclear receptor induces conformational changes in the receptor, which renders the ligand binding domain to become resistant to protease digestion. In these experiments it was investigated whether the new metabolite can induce a distinct conformational change in RARα and RARβ proteins. [^{35}S]Methionine-labeled

RARα and RARβ were translated *in vitro*, incubated with retinoids and digested in limited proteolysis reactions with Trypsin (fig.4.12). The labeled receptors were incubated with the retinoid carrier alone (ethanol), *S*-4o-9c-dh-RA or at-RA, and then digested with Trypsin. The digestion products were separated on a 10% SDS-polyacrylamide gel (see methods). Trypsin digestion of a control-treated RARα and RARβ produced a 25-kDa fragment (lane 4 in fig.4.12A & B), which was not detectable in samples where RARα and RARβ had been preincubated with at-RA or *S*-4o-9c-dh-RA (lane 5,6 in fig.4.12A & B). In the presence of either at-RA or *S*-4o-9c-dh-RA, the receptors were only partially digested, resulting in the accumulation of a 30-kDa resistant proteolytic fragment. The results show that *S*-4o-9c-dh-RA – similar to at-RA – induces a conformational change or a stabilisation of a particular conformation of both RARα and RARβ, and as a consequence alters the pattern of degradation by Trypsin. These results add proof to what was seen when the CV1 cells were transfected with RARα or RARβ together with RXRβ; that *S*-4o-9c-dh-RA was able to perform signal transduction mediated by both RAR subtypes (see fig.4.5).

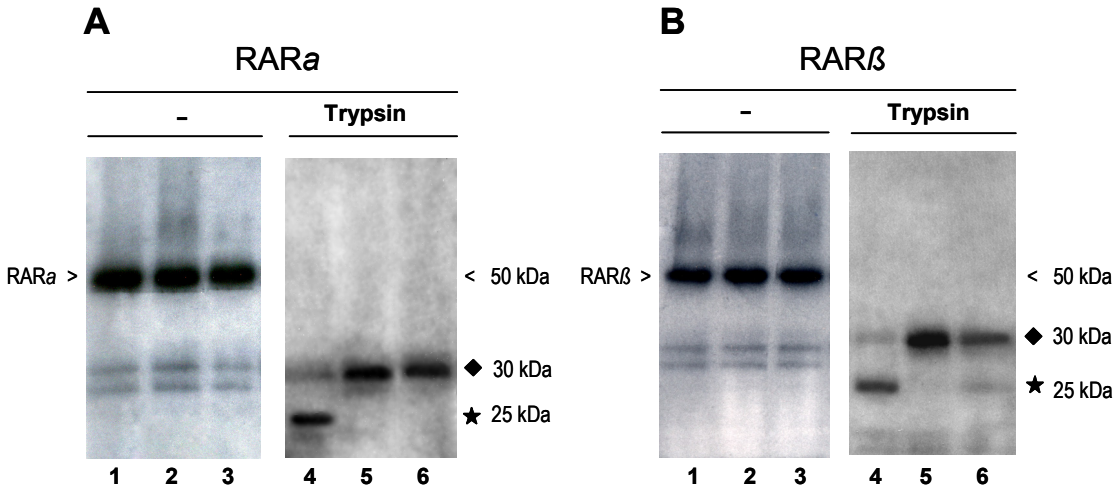

Fig.4.12: *S*-4o-9c-dh-RA inhibits limited Trypsin digestion of RARα and RARβ.

In vitro translated [^{35}S]methionine-labeled RARα **(A)** and RARβ **(B)** samples were preincubated with ethanol alone (lanes A1,4 & B1,4) or together with 100 nM at-RA (lanes A2,5 & B2,5) or 10 µM *S*-4o-9c-dh-RA (lanes A3,6 & B3,6), followed by incubation with Trypsin or only buffer as indicated (for details see methods). Samples were separated via 10% SDS-PAGE. For both RARα and RARβ, the 30 kDa proteolytic fragments (marked by a diamond) of the receptors were protected from digestion by the presence of either retinoids (lanes A5,6 & B5,6) in comparison to the samples treated with ethanol only (lanes A4 & B4). The 25 kDa fragments of the Trypsin digested receptors (marked by asterisk) were only present in the samples treated as controls (ethanol; lanes A4 & B4).

4.5 *S*-4-oxo-9-*cis*-13,14-dihydro-retinoic acid evokes digit pattern duplications in chicken embryos

S-4o-9c-dh-RA induces digit pattern duplications in a dose-dependent fashion. Ion-exchange beads were soaked in ethanolic solutions of *S*-4o-9c-dh-RA at concentrations that ranged from 0.2 to 10 mg/ml and were implanted at the anterior margin of wing buds of Hamburger-Hamilton stage 20 chick embryos (cf. chapter 3.2.6.). At concentrations of 0.2 and 0.5 mg/ml wing patterns were mostly normal or had an additional digit 2 (fig.4.13A,B; tab.4.1). Patterns with additional digit 3 and 4 (43234), some with truncations of digit 2 (4334), became most prevalent as soon as the soaking concentrations was equal or greater that 1 mg/ml (fig.4.13C, tab.4.1). Thus within a five-fold change in the soaking concentration there was a dramatic change in effect.

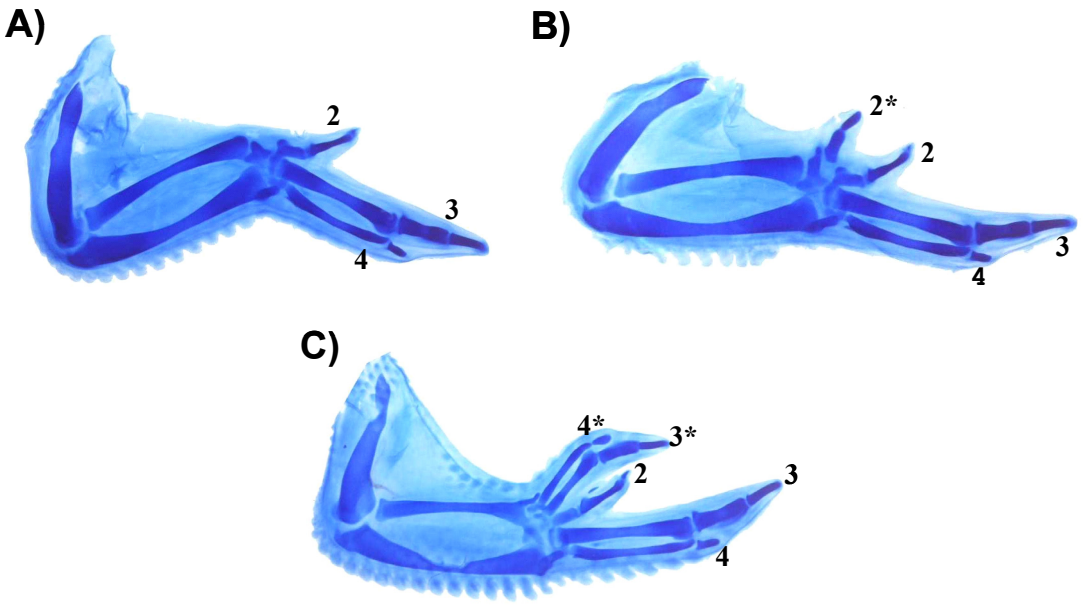

Fig.4.13: Effect of different doses of locally applied *S*-4o-9c-dh-RA on the chick wing pattern.

Beads were soaked in ethanolic *S*-4o-9c-dh-RA solution and implanted at the anterior margin of right wing buds of stage 20 chick embryos. The images display the most frequent wing digit patterns of the chick embryos in the different treatment groups. **A)** Normal 234 pattern (untreated control and soaking concentration of 0.2 mg/ml); **B)** 2234 pattern [conc. 0.5 mg/ml]; **C)** 43234 pattern [conc. 1 mg/ml]; Digit identities 2, 3, 4 are read from anterior to posterior, additional digits are marked by asterisks.

The pattern of additional digits was quantified in form of a percent respecification value (see methods for a definition) allowing plotting of data in a dose-response curve. The efficacy of at-RA in the limb pattern duplication assay has been extensively documented (e.g. TICKLE *et al.*, 1985; SUMMERBELL, 1983). As can be seen in the dose-response curves (fig.4.14), the profile for *S*-4o-9c-dh-RA is shifted towards higher soaking concentrations indicating that this metabolite has a lower potency than at-RA by a factor of ~10.

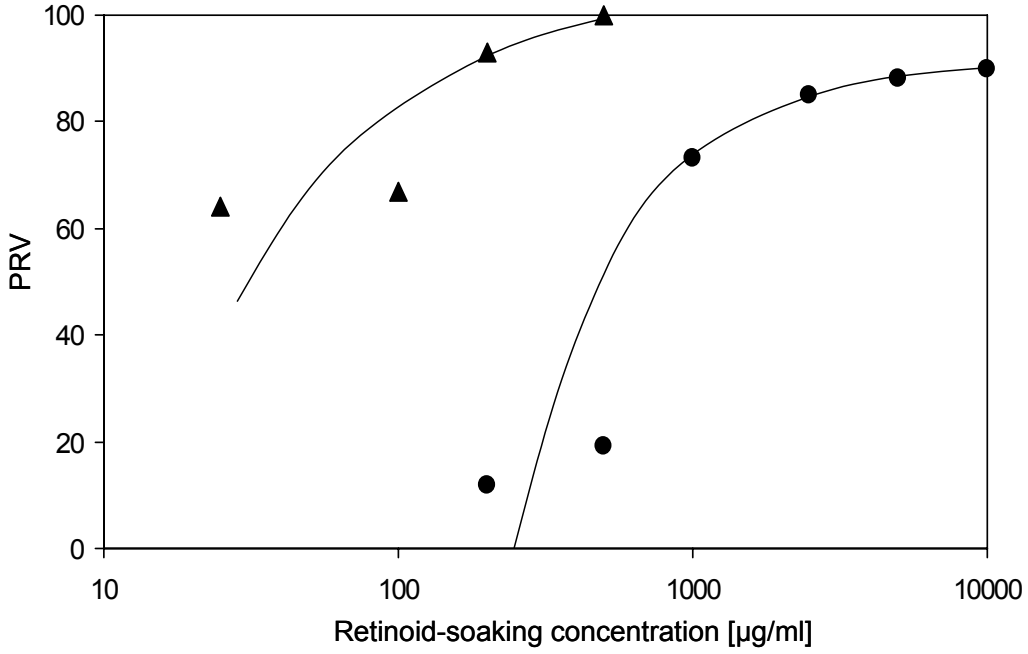

Fig.4.14: Dose-response curves for *S*-4o-9c-dh-RA (circles) & at-RA (triangles).
The percentage respecification value was plotted against the soaking concentration and is a measure for the extent of pattern duplication (for definition see methods). The percentage respecification value is an average value of each set. The sum of the scores of each wing is divided by the number of limbs in each set.

S-4o-9c-dh-RA did not evoke the loss of the hand plate or forearm elements, a result frequently seen with high doses of at-RA (tab.4.1 and TICKLE *et al.*, 1985). Thus the new RA-metabolite is less embryotoxic than at-RA. Control bead implants immersed in ethanol had no effect on the wing digit pattern (tab.4.1).

Tab.4.1: Digit patterns following local application of at-RA or *S*-4o-9c-dh-RA to stage 20 chick wing buds.

Treatment	Soaking conc. [mg/ml]	Embryos per group *n*	Digit pattern *	Number of cases	PRV
at-RA	0.025	12	234 (normal)	1	64
			d234	1	
			dd234, dd234, d3234	3	
			4334, 43234	7	
	0.1	8	2234, 2234	4	67
			43234, 4334	4	
	0.2	9	2234	1	93
			4334	2	
			4334	6	
	0.5	8	234	1	100
			4334, 4334	3	
			434	1	
			humerus only	3	
***S*-4o-9c-dh-RA**	0.2	8	234 (normal), d32	6	12
			2234	2	
	0.5	7	234 (normal)	3	19
			2234, d234	4	
	1	10	2234	3	73
			dd234	1	
			43234	5	
			4334	1	
	2,5	9	2234	1	85
			dd234	1	
			4d234	1	
			43234, 4334, 4334	6	
	5	11	2234, 2234	2	88
			43234, 43234, 4334	7	
			4d234	1	
			43d234	1	
	10	13	dd234	2	90
			43234, 4334	10	
			4334	1	
Ethanol		8	234 (normal)	8	0

* Digit identities are read from anterior to posterior; digits which are not clearly identifiable are marked as d, digits which are proximal fused are indicated by underlining

4.6 *S*-4-oxo-9-*cis*-13,14-dihydro-retinoic acid regulates the transcription of RA target genes in the chick limb bud

To assess induction of genes mediating normal limb development, beads, soaked in 0.2 mg/ml at-RA or 2 mg/ml *S*-4o-9c-dh-RA, were implanted. These concentrations were selected because they evoke pattern duplications to a similar extent (tab.4.1 and fig.4.14). The transcript levels of the direct at-RA target genes *RARβ2, Cyp26* and *Hoxb-8* were determined by qRT-PCR in whole buds removed after 6 hours of retinoid treatment, whereas the transcripts of the indirect at-RA target genes *shh* and *bmp-2* were quantified in buds treated for 24 hours since their induction by at-RA is known to occur only after prolonged treatment (HELMS *et al.*, 1994; FRANCIS *et al.*, 1994).

Because endogenous *shh* is expressed only in the posterior part of the limb bud (RIDDLE *et al.*, 1993), buds were dissected into posterior and anterior half prior to RNA isolation and induction was assessed in both halves independently. *Bmp-2* transcript levels were also measured in both halves because in the HH-stages between 17 and 26 the occurrence of *bmp-2* transcripts is also restricted to the posterior mesenchyme, except for a certain extent in the apical ridge of the anterior half (FRANCIS *et al.*, 1994).

The PCR-efficiancy was consistently about 100±5% in all experimental qRT-PCR runs of each gene (see appendix ch.7.2). No primer-dimers were generated during the applied 50 real-time PCR amplification cycles, observed in the melting curve analysis (see appendix ch.7.3). The transcript levels of all investigated retinoid regulated target genes were significantly increased in limb bud tissue treated with either retinoid (fig.4.15). The target genes *RARβ2, Cyp26* and *Hoxb-8* are induced by both retinoids in a range between 2- to 9-fold (fig.4.15A: Induction of *RARβ2*: 2.1-fold by *S*-4o-9c-dh-RA and 2.3-fold by at-RA; fig.4.15B: Induction of *Cyp26*: 5.7-fold by *S*-4o-9c-dh-RA and 8.9-fold by at-RA; fig.4.15C: Induction of *Hoxb-8*: 2.3-fold by *S*-4o-9c-dh-RA and 2.2-fold by at-RA. Whereas in the case of *RARβ2* and *Cyp26 S*-4o-9c-dh-RA was slightly less active (fig.4.15A, B), *Hoxb-8* expression is somewhat stronger induced by *S*-4o-9c-dh-RA (fig.4.15C).

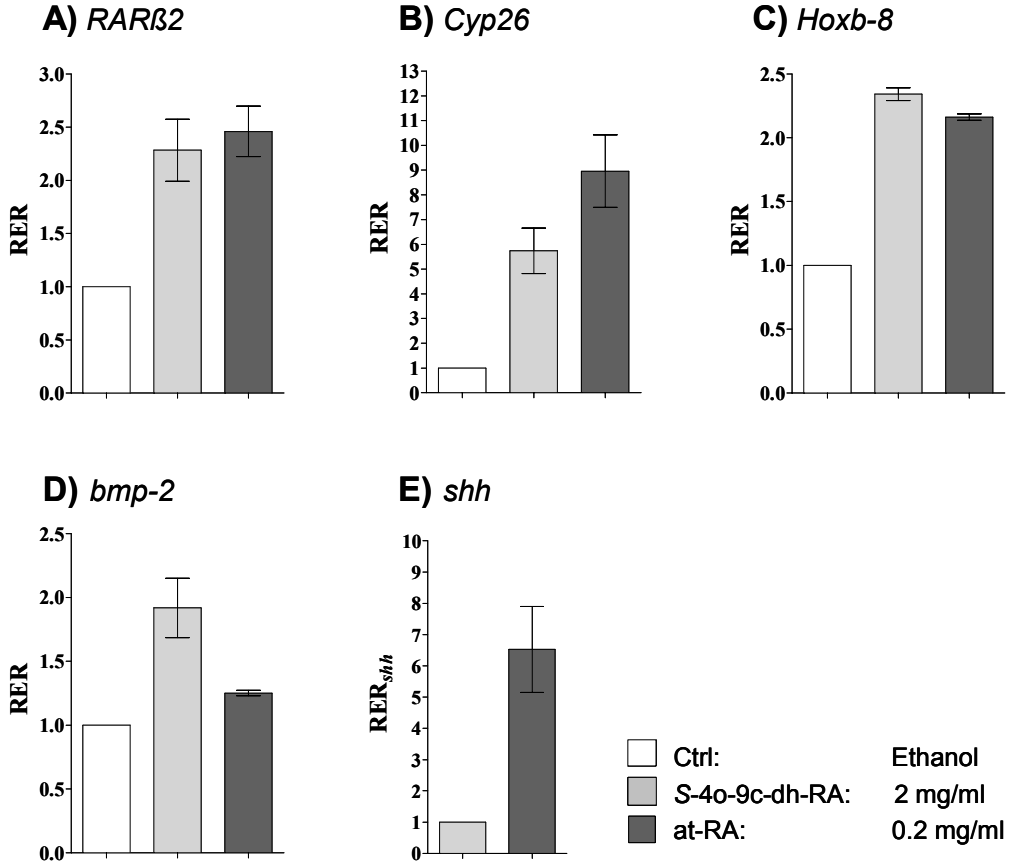

Fig.4.15: Transcript levels of RA-induced genes in limb bud tissue.

Transcript levels of direct at-RA target genes (**A-C**: *RARß2, Cyp26, Hoxb-8*) and indirect at-RA target genes (**D,E**: *bmp-2, shh*) were significantly induced in limb buds treated with *S*-4o-9c-dh-RA or at-RA. Note that beads were soaked in a solution of 2 mg/ml *S*-4o-9c-dh-RA or 0.2 mg/ml at-RA, respectively. Absolute expression levels were determined by the standard curve method (see methods). RER of target genes were normalised to TBP (target gene/TBP). **A-D)** Transcript levels, expressed as relative expression ratios (RER), of treated buds were compared to the endogenous expression levels of the appropriate genes in untreated buds (Ctrl). **E)** Relative expression ratio of *shh* (RER_{shh}) is determined as a quotient between at-RA and *S*-4o-9c-dh-RA treated samples (see methods). Presented results are mean values of three experiments carried out in duplicates. Statistical analyses are described in methods. Stars indicate significant difference from controls (ctrl), whereas * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respectively.

The indirect target genes *bmp-2* and *shh* are also induced by either retinoids (fig.4.15D, E). In the case of *bmp-2* there was a weak but significant induction in the anterior limb bud half with *S*-4o-9c-dh-RA (fig.4.15D: 1.9-fold) being slightly more efficient than at-RA (fig.4.15D: 1.3-fold by at-RA). *Shh* is not endogenously expressed in the anterior section of the limb bud and therefore the RER in fig.4.15E is determined as a quotient between at-RA and *S*-4o-9c-dh-RA (RER_{shh}). By this criterion, at-RA is a 6.5-fold

stronger inducer of *shh* expression than *S*-4o-9c-dh-RA. There was no difference found in the expression of target genes in untreated limb bud samples and samples treated with ethanol soaked beads.

In conclusion, *S*-4o-9c-dh-RA can control the expression of genes which are involved in limb morphogenesis, such as *shh* (RIDDLE *et al.*, 1993), *Hoxb-8* (IZPISUA-BELMONTE & DUBOULE, 1992; CHARITE *et al.*, 1994) and *bmp-2* (FRANCIS *et al.*, 1994), and likewise induces the expression of direct at-RA regulated target genes, such as *RARβ2, Cyp26* and *Hoxb-8*, which are known to contain a RARE in their promotor region. However, the relative potency of *S*-4o-9*c*-dh-RA to induce the expression of these RA target genes is less compared to at-RA.

5. Discussion

The number of identified endogenous retinoids in plasma and tissues of various species including the human is limited. The new metabolite, *S*-4o-9c-dh-RA, was recently discovered and characterised by SCHMIDT *et al.* (2002). It was shown that *S*-4o-9c-dh-RA is a new abundant endogenous vitamin A metabolite occurring in particularly high levels in the livers of rats and mice. Over the last years several groups aimed at discovering new endogenous RA metabolites (MOISE *et al.*, 2005; PIJNAPPEL *et al.*, 1998; SHIRLEY *et al.*, 1996). In contrast to the findings of other groups, which reported the occurrence of novel RA metabolites, especially in transgenic animals or after providing certain retinoids exogenously, *S*-4o-9c-dh-RA is occurring endogenously in wild type mice and rats. Additionally, it was found in one human liver sample. Whereas SHIRLEY and co-workers (1996) described the reduction of 9-*cis*-RA to 9-*cis*-13,14-dihydro-RA in rats after administration of 9-*cis*-RA, MOISE and co-workers (2005) reported the occurrence of all-*trans*-13,14-dihydro-RA (at-dh-RA) in liver of transgenic mice supplemented with retinyl palmitate. Moreover, these studies did not take the chirality aspect of dihydro-RAs into consideration. 4o-9c-dh-RA is characterised by a chiral carbon at C13, while it was shown that 4o-9c-dh-RA isolated from mouse liver is the *S*-enantiomer.

The identification of *S*-4o-9c-dh-RA in some tissues of mice, rats, and humans is very remarkable because of several other reasons: Based on the state of knowledge it is the first time that a 9-*cis* configurated isomer of RA has been detected endogenously in considerable concentrations. Indeed, some research groups reported the occurrence of 9-*cis*-RA and/or other 9-*cis*-RA-metabolites *in vivo* as mentioned above. However, in most cases after liver consumption or providing certain retinoids exogenously and in levels that were slightly above the detecting limit. Other researchers were not able to detect 9-*cis*-RA or any other 9-*cis*-RA-isomers in any tissue. Contrary, the endogenous levels of *S*-4o-9c-dh-RA in serum, kidney and liver of mice and rats were found to be great, and especially in liver significantly higher than the at-RA levels. The second major interesting finding was that the metabolism of *S*-4o-9c-dh-RA appears to be highly

regulated and dependent on the retinol intake. The endogenous levels increased dramatically in the liver following vitamin A supplementation in mice. The fluctuation in the endogenous levels of *S*-4o-9c-dh-RA depending on the vitamin A provision is an interesting sign for an important function of the new metabolite in the maintenance of the retinoid metabolism within the body. Furthermore the metabolism of *S*-4o-9c-dh-RA seems to be disturbed following exposure to environmental pollutants such as TCDD. The hepatic levels of *S*-4o-9c-dh-RA decreased dramatically in mice (HOEGBERG *et al*., 2005) and rats (SCHMIDT *et al*., 2003) in consequence of TCDD exposure. The evaluation of the biological activity of *S*-4o-9c-dh-RA is of great concern to identify and clarify the function of this new major RA metabolite in diverse RA-signaling pathways and represents considerable progress towards understanding the physiological role of this compound in the body.

5.1. *S*-4-oxo-9-*cis*-13,14-dihydro-retinoic acid is a new ligand for RARα and RARβ

The diverse effects of RA action in controlling miscellaneous cellular processes are thought to be due to the multiplicity of retinoid receptors and gene pathways influenced by these receptors. Due to differences between these receptors, ligands may also differentially influence the individual receptor isoforms. Therefore it is an important issue to examine whether the different retinoid receptors are only activated by at-RA, or if other endogenous ligands exist, which may selectively activate a subset of receptors.

S-4o-9c-dh-RA had activating effects on signal transduction compared to control treated cells in all of the tested reporter cell systems. This was partly unexpected since it was anticipated that a different endogenous setup of receptor isoforms, co-factor proteins, metabolism, differential stage, tissue type and certainly other factors could possibly be a limiting factor in some case. Nevertheless, the results show that *S*-4o-9c-dh-RA functions as an activator for RAR-dependent signal transduction in all of these transfected cell systems. Further on, no limitations in the capacity to acti-

vate different combinations of retinoid receptors were observed. *S*-4o-9c-dh-RA did not show particular selectivity between the different combinations of RARs and RXRs tested in this study. In the transfection studies, *S*-4o-9c-dh-RA showed a transactivating function via the retinoid receptors from luciferase reporter plasmids under regulation of both a minimal but also a more natural and complex RARE, in the same fashion as at-RA, the positive control. Mechanistically, *S*-4o-9c-dh-RA induced conformational changes to both RARα and RARβ in the limited proteolysis assay, in a similar manner as at-RA. More precisely the observed ligand induced accumulation of a resistant proteolytic fragment is a result of an altered LBD conformation of these receptors in consequence of ligand binding. On the basis of these facts it is obvious that *S*-4o-9c-dh-RA binds to the LBD of RARα & RARβ and hence activates retinoid dependent signal transduction.

The interpretation of the transactivation results could also take the metabolic stability of *S*-4o-9c-dh-RA into consideration, which for the moment is not known. Most probably, various cell lines metabolise *S*-4o-9c-dh-RA different after administration. For example it could be expected that a hepatic cell line (like Hepa-1) have a greater enzymatic activity compared to an embryonic cell line (like P19) and thereby a greater potential to metabolise retinoid metabolites such as *S*-4o-9c-dh-RA to other metabolites. Anyway, the transactivating capacity of *S*-4o-9c-dh-RA during transfections was similar between the entire cell lines tested in this study, indicating a relative strong stability of *S*-4o-9c-dh-RA. It is unlikely that none of the tested cell lines is not able to metabolise *S*-4o-9c-dh-RA during the 24 hour period of a single experiment. Taken together the data suggest that the transactivating function of *S*-4o-9c-dh-RA, seen in this study, could be expected also *in vivo*, either by administered or endogenously produced *S*-4o-9c-dh-RA. In essence, to gain further information about the metabolic stability of *S*-4o-9c-dh-RA, it would be necessary to measure the clearance of *S*-4o-9c-dh-RA in comparison to at-RA using radiolabeled compounds. However, radiolabeled *S*-4o-9c-dh-RA is currently not available.

The data clearly show that *S*-4o-9c-dh-RA closely mimics at-RA with regard to transcriptional activation of RARE-regulated genes in diverse cell systems. The efficacy of *S*-4o-9c-dh-RA compared to at-RA in these systems was approximately 200-fold lower dependent on the cell line (133 to 240-fold). Since it is known that some RA metabolites exhibit biological activity in some systems, it should be investigated whether there is a specific significance in the fact that most RA metabolites, exhibit a lower potency compared to at-RA. On the other hand, the discussion about the relative potency of the new metabolite in comparison to at-RA should also consider the relative high tissue concentration of the new metabolite observed in mice, which exceeds the corresponding at-RA concentration considerably at least in the liver and increases drastically as a consequence of high all-*trans*-retinol intake with the diet (SCHMIDT *et al.*, 2002). For instance the hepatic levels of *S*-4o-9c-dh-RA increased from 11.4 up to 117.0 ng/g in NMRI mice fed with feed containing either 15,000 or 150,000 IU retinyl palmitate/kg diet, respectively (SCHMIDT *et al*., 2002). The at-RA levels increased only from 5.6 to 7.6 ng/g in the tissue of the same animals. Thus the level of *S*-4o-9c-dh-RA is 2-fold higher compared to at-RA and increases up to a 15-times higher content. Regarding to this fact there is a dramatic change in the relative efficacy of the new metabolite to transactivate RAR-dependent transcriptional activation. An arithmetical estimation of the difference in potency between the two compounds can be obtained by dividing the 200-fold difference in responsiveness seen in the transactivation assays in this study with the factor 2 and 15 that reflect the difference in the actual tissue concentrations of both retinoids in the liver. According to this, it appears that *S*-4o-9c-dh-RA is simply 100-fold or even just 13-fold less active than at-RA.

5.2. *S*-4-oxo-9-*cis*-13,14-dihydro-retinoic acid could not transactivate RXRα and RXRβ

The data confirms that the new metabolite is a novel endogenous ligand for at least the RARα and β isoforms. The fact that *S*-4o-9c-dh-RA transactivates RARs was a bit surprising. It was assumed that *S*-4o-9c-dh-RA would bind to the LBD of RXR

isoforms because of the 9-*cis* configuration and preliminary results from molecular modeling studies, which suggested binding-affinities to the LBD of certain RXR isoforms (data not shown). The ability of *S*-4o-9c-dh-RA to induce the transactivation of RXR isoforms was investigated in CV1 cells. The retinoid receptor free CV1 cells were transfected with a luciferase reporter plasmid under the control of a DR1 sequence, which is characterised as an RXRE. Cells were simultaneously transfected with expressing vectors for RXRα or RXRβ, and subsequently treated with 9-*cis*-RA as positive control. The result showed significant luciferase induction after treatment with 9-*cis*-RA, which was expected since 9-*cis*-RA is characterized as the strongest endogenous ligand for the RXRs so far. Both the new metabolite *S*-4o-9c-dh-RA and *S*-4o-at-dh-RA were not able to induce the same effect in these reporter cells. The results led to the conclusion that neither *S*-4o-9c-dh-RA nor *S*-4o-at-dh-RA primarily function as endogenous ligands for RXR homodimers.

5.3. Potential of other 4-oxo-13,14-dihydro-retinoic acid metabolites to transactivate RARα and RARβ

It was of interest to examine whether there is a difference in the potential between both the *R*- and the *S*-enantiomer to transactivate RARα and RARβ. Therefore *R*-4o-9c-dh-RA has been tested in the same reporter cell systems. Additionally the potency of 4o-at-dh-RA (again *R*- and *S*-type) to activate the transcription of RARE-regulated genes was tested. 4o-at-dh-RA was not detected in any sample from various tissues and species in our laboratory. Based on the state of knowledge from the literature it cannot be confirmed, that 4o-at-dh-RA is a new endogenous occurring retinoid metabolite. However, as mentioned above MOISE and co-workers (2005) reported the occurrence of 4o-at-dh-RA traces in liver of transgenic mice supplemented with all-*trans*-13,14-dihydro-retinol. It is likely that 4o-at-dh-RA is occurring endogenously besides *S*-4o-9c-dh-RA only in consequence of high vitamin A intake.

All tested compounds were able to regulate RARE-dependent transcription and thus induced luciferase activity in most of the tested systems to a different extent. Inter-

estingly, the data clearly demonstrated that the *R*-type enantiomers of both the 9-*cis*- and the all-*trans*-form showed very little till no activity in all of the tested cell systems. Beside other factors, which are not known so far, the difference in the activity could be an explanation for the predominance of the *S*-type compared to the *R*-type. The potency of *S*-4o-at-dh-RA to induce luciferase reporter activity was equal to *S*-4o-9c-dh-RA, and in some cases even stronger, which can be possibly explained by differences among the cell types. The results show similarities to the findings from MOISE and co-workers (2005), who described at-dh-RA as a metabolite which can transactivate RAR/RXR heterodimers but nor RXR/RXR homodimers in reporter cell assays. At-dh-RA is possibly a precursor metabolite of other 4o-dh-RAs, including *S*-4o-9c-dh-RA.

5.4. *S*-4-oxo-9-*cis*-13,14-dihydro-retinoic acid induces endogenous gene transcription *in vitro*

After discovering the widespread capacity of *S*-4o-9c-dh-RA to activate transfected reporter plasmids, it was of interest to evaluate how *S*-4o-9c-dh-RA influences the endogenous gene expression. As expected, the gene expression analysis of mRNA transcripts from *S*-4o-9c-dh-RA-treated P19 cells showed a significant 2 to 30-fold increase of endogenous *RARβ2* expression, dependent on treatment-doses and -time. This gene is well known as a direct target gene for the RARs. The regulatory part in the promoter region of this gene contains a direct repeat of a AGGTCA motif spaced by five nucleotides (DR5) (DE THE *et al.*, 1990; SUCOV *et al.*, 1990). The *RARβ2* gene serves as one of the master regulators in many RA-induced cellular events, such as proliferation, differentiation, and apoptosis, by regulating a number of downstream effector genes (BAIN *et al.*, 1994; ROY *et al.*, 1995). Compared to at-RA, *S*-4o-9c-dh-RA is 200-fold less active at inducing *RARβ2*-mRNA levels in the P19 cells. The reason for this circumstance is not known. Trying to value these results is probably also relatively irrelevant, given to the fact that a system with a specific cell line can never reflect the environment and the circumstances, where the new metabolite is meant to play a role. The important fact is that *S*-4o-9c-dh-RA is potently acti-

vating the retinoid receptors and consequently induces gene transcription out of its normal environment. Anyway, this result further establishes the evidence that this novel and major retinoid metabolite has the capability to regulate gene transcription, mediated through nuclear retinoid receptors.

5.5. *S*-4-oxo-9-*cis*-13,14-dihydro-retinoic acid evokes digit pattern duplications in chick wings

Using the chicken limb bud model, this study demonstrated that *S*-4o-9c-dh-RA is morphogenetically active in this system, suggesting *in vivo* activity of this compound in general. It was shown that *S*-4o-9c-dh-RA closely mimics at-RA with regard to pattern respecification and also in the induction of previously characterised at-RA effector genes in the limb bud, which include *Hoxb-8, RARβ2, shh, Cyp26* and *bmp-2*. The data suggest that *S*-4o-9c-dh-RA is less active than at-RA. This conclusion is based on the assumption that the release kinetics from the slow release beads and the clearance from the tissue are similar for at-RA and *S*-4o-9c-dh-RA. *In vitro* release studies (EICHELE *et al.*, 1984) showed that different retinoids are released from the used type of ion-exchange beads at similar rates, suggesting that the difference in activity is not caused by unequal release kinetics. The RA receptor agonist, 4-(E-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl) benzoic acid (TTNPB) is apparently more potent than at-RA in duplication assays when using solution concentrations as a reference value (EICHELE *et al.*, 1985). However, it was shown that the synthetic retinoid is much more metabolically stable and based on the actual tissue concentrations of TTNBP and at-RA, both agents have similar efficacy (EICHELE & THALLER, 1987). Therefore the difference in clearance may account for the difference in efficacy in the present study. To investigate the metabolic stability, it will be again necessary to measure the clearance of *S*-4o-9c-dh-RA in comparison to at-RA using radiolabeled *S*-4o-9c-dh-RA, which is presently not available. However, the discussion about the potency of *S*-4o-9c-dh-RA in this system is only feasible in a theoretical sense in view of the fact that *S*-4o-9c-dh-RA was not detectable in chick limbs (data not shown).

5.6. *S*-4-oxo-9-*cis*-13,14-dihydro-retinoic acid induces the expression of RA-regulated genes in the chick limb bud

It is likely that *S*-4o-9c-dh-RA induces additional digits in a way similar to that reported for at-RA (SUMMERBELL, 1983; THALLER *et al.*, 1993; THALLER & EICHELE, 1990; TICKLE *et al.*, 1982; TICKLE *et al.*, 1985). The role of at-RA during the complex interactions and morphogenetic processes in the limb development is most likely the initiation of a cascade of signaling molecules via regulating the expression of genes coding for such signaling molecules (HELMS *et al.*, 1994). The finding that *S*-4o-9c-dh-RA can control expression of genes which are involved in limb morphogenesis, such as *shh* (RIDDLE *et al.*, 1993), *Hoxb-8* (CHARITE *et al.*, 1994; IZPISUA-BELMONTE & DUBOULE, 1992) and *bmp-2* (FRANCIS *et al.*, 1994) is supporting the assumption that *S*-4o-9c-dh-RA provokes digit duplication in the same way as at-RA mediates its function. It was already demonstrated in embryonic carcinoma cells (P19) that the new metabolite is able to regulate the expression of the direct at-RA target gene *RARβ2 in vitro*. Nevertheless, *S*-4o-9c-dh-RA induced beside *RARβ2* other direct target genes, such as *Cyp26* and *Hoxb-8,* also *in vivo* in the chick limb buds. *Cyp26* and *Hoxb-8* are also known to contain a RARE in their promoter region (DE THE *et al.*, 1990; LOUDIG *et al.*, 2000; OOSTERVEEN *et al.*, 2003; SUCOV *et al.*, 1990).

5.7. Potential role of *S*-4-oxo-9-*cis*-13,14-dihydro-retinoic acid in physiology

It is unlikely that *S*-4o-9c-dh-RA is a simple degradation product of at-RA without a biological function. Rather the results taken together suggest that *S*-4o-9c-dh-RA is a biologically active retinoid metabolite. It is likely that the new metabolite could have important transactivation properties under certain physiological circumstances and perhaps play an important role in cellular physiology. To establish the physiological role of *S*-4o-9c-dh-RA in controlling gene expression more studies are necessary. For example, the micro array technique could be useful to screen for differences in the regulation of genes by at-RA compared to *S*-4o-9c-dh-RA.

The previous study revealed *S*-4o-9c-dh-RA as a major retinoid metabolite *in vivo*, with hepatic levels that correlate with increasing retinyl palmitate content in the diet (SCHMIDT *et al.*, 2002). This apparent correlation to dietary intake is not seen for at-RA. The at-RA levels within the organism are very stringent regulated. The enzymatic pathway responsible for the formation of *S*-4o-9c-dh-RA and the possible precursor retinoids are not known. The metabolism of vitamin A is a highly regulated process which includes conjugation, decarboxylation, oxidation, double bond isomerisation and reduction, carried out by a well-organised interplay of enzymes, such as LRAT, ARAT, REHs, MDHs, RALDHs, and P450s, as well as inter- and extracellular retinoid binding proteins, such as RBP, CRBP, and CRABP. It is of interest to examine enzymes, binding proteins and other factors involved in the metabolism of the new metabolite. The use of recombinant enzymes, which can possibly be involved in the formation of *S*-4o-9c-dh-RA, could be a suitable technique to reconstitute the pathway of the new metabolite *in vitro*. Knock out animals, deficient in certain enzymes involved in the metabolism of retinoids, could also be an appropriate way to answer these questions. The use of siRNA against specific enzymes involved in retinoid metabolism could be another way to point out responsible proteins for the metabolic pathway of *S*-4o-9c-dh-RA and other 13,14-dihydro-RAs.

MOISE *et al.* (2004) described a novel enzyme in mice, which could possibly catalyse the key step in the formation of 13,14-dihydro-RAs. All-*trans*-retinol:13,14-dihydroretinol Saturase (RetSat) converts all-*trans*-retinol to all-*trans*-13,14-dihydroretinol. Their further studies showed that the same enzymes involved in the oxidation of all-*trans*-retinol to at-RA and then to oxidised RA metabolites can also catalyse the oxidation of all-*trans*-13,14-dihydro-retinol to oxidised dihydro-RAs (MOISE *et al.*, 2005). These enzymes, CYP26s together with ADHs, SDRs and RALDHs, are involved in the regulation of desirable at-RA levels and could therefore be also involved in the formation of *S*-4o-9c-dh-RA in certain physiological circumstances. Adh1 and Raldh1 are somehow involved in protective mechanisms in response to pharmacological doses of all-*trans*-retinol, since *Adh1-/-* and *Raldh1-/-* mice showed to be more sensitive to retinol-induced toxicity than their wild type counterparts

(MOLOTKOV *et al.*, 2004; NIEDERREITHER *et al.*, 2003). MOISE *et al.* (2005) identified increasing levels of at-dh-RA, 4o-at-dh-RA, and other 13,14-dihydro-retinoids in the tissues of lecithin:retinol acyltransferase (LRAT)-deficient (*Lrat -/-*) mice supplemented with retinyl palmitate. LRAT is basically involved in the esterification of all-*trans*-retinol (RUIZ *et al.*, 1999). Whereas most of the ingested all-*trans*-retinol is converted to esters in wild type mice, the uptake and storage of all-*trans*-retinol is heavily affected in *Lrat -/-* mice (BATTEN *et al.*, 2004). Taken these results together the following assumption of MOISE *et al.* (2005) seems to be likely: The formation of 13,14-dihydro-retinoid metabolites could be a further degradation pathway of all-*trans*-retinol to protect the body against pharmacological doses of all-*trans*-retinol due to fluctuations in the nutritional vitamin A (predominantly all-*trans*-retinol) levels, under circumvention of the formation of at-RA, which is also generally known to exhibit toxic effects in excess levels. This could be a reasonable explanation of the heavily increasing *S*-4o-9c-dh-RA and the relative stable at-RA levels in mice gavaged with retinyl palmitate at high doses (SCHMIDT *et al.*, 2002).

5.8. Perspective

S-4o-9c-dh-RA had basically positive effects in mimicking the action of at-RA in all of the tested systems. The data suggests that it may not be such a strong activator of the retinoid receptors as at-RA, but nevertheless it functions as an activator for retinoid dependent signal transduction in these systems. The results demonstrate that the new RA-metabolite is a biologically active retinoid and possibly can be involved in other signal transduction pathways. With regard to the heavily fluctuating hepatic levels in mice after retinol-supplementation on the one hand or TCDD-treatment on the other hand, *S*-4o-9c-dh-RA apparently plays an important role in cellular physiology. However, many questions concerning the action, metabolism, and the general role of the new RA-metabolite in the body remain still unanswered.

At-RA is known to regulate the expression of hundreds of different genes through the activation of nuclear transcription factors. Other mechanisms of action of retinoids

apart from the known transactivation effect of retinoids continue to be discovered. It has been found out in the latest past that at-RA regulates a large number of noncoding RNAs (reviewed in BLOMHOFF & BLOMHOFF, 2006). Furthermore, extranuclear mechanisms of action of retinoids are also being identified. Therefore the role of at-RA and other bioactive RAs may extend beyond the regulation of gene transcription. In this study all results reveal that *S*-4o-9c-dh-RA acts as at-RA in all essential aspects, but it is possible that *S*-4o-9c-dh-RA actually has specific biological roles apart from acting in the same manner as at-RA. An obvious suggestion for future studies is therefore to evaluate if this metabolite also has specific biological roles different from at-RA.

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7. Appendix

Composition of general solutions, buffers and gels

Preparation of 60 mM ammonium acetate buffer (2 l):

- weight out 9,25 g Ammonium acetate
- fill slightly less than 2 l of ddH_2O in a beaker
- dissolve the salt in the water
- adjusted to pH 5.7 with acetic acid (~ ½ Pasteur pipette)
- fill up with Aqua-bidest to 2 l in a graduated cylinder
- filtrate the buffer through a membrane filter

Composition of $CaCl_2$ solution:

- prepare 60 mM $CaCl_2$
- add 15% glycerol and 10 mM PIPES (pH7.0)
- Autoclave solution

Composition of PIPES buffer (pH 6.5):

- 140 mM NaCl
- 5 mM KCL
- 0.6 mM $MgCl_2$
- 1.0 mM $CaCl_2$
- 5.5 mM glucose
- 0.1% BSA
- 10 mM PIPES (pH 7.4)

Composition of 5 mM Kac (100 ml):

- 60 ml 5 M Kac
- 11,5 ml Hac
- 28,5 ml ddH_2O

Composition of 2 x BSB (10 ml):

- 6 ml (50%): Glycerol
- 4 ml (1 M): 40 mM Hepes 7.90
- 0.1 ml (1 M): 10 mM $MgCl_2$
- 1 ml (1 M): 100 mM KCL
- 20 µl: 0.02% Triton X-100
- ddH_2O up to 10 ml final volume

Composition of WCEB (10 ml):

- 1 ml (1 M): 10 mM Hepes 7.90
- 0.8 ml (5 M): 0.4 M NaCl
- 2 µl (0.5 M): 0.1 mM EDTA
- 1 ml (50%): 5% Glycerol
- ddH_2O up to 10 ml final volume

Composition of 5xSDS-loading-buffer (10 ml):

- 0.5 ml (1 M): 50 mM Tris-HCL pH 6.8
- 1 ml (1 M): 100 mM DTT
- 2 ml (10%): 2 % SDS
- 1 ml (1%): 0.1 % BFB
- 2 ml (50%): 10% Glycerol
- ddH_2O up to 10 ml final volume

Composition of running-buffer:

- 1 l: H_2O
- 94 g: Glycine
- 83 ml: 1,5 M $Tris_{8.8}$
- 50 ml: 10% SDS

Composition of 10%-separation gel (25 ml):

- 12 ml: ddH_2O
- 6,31 ml: 5 M $Tris_{8.8}$
- 6,25 ml: 40% Acrylamide
- 250 µl: 10% SDS
- 250 µl: 10% APS
- 30 µl: TEMED

Composition of stack gel (~10 ml):

- 7,2ml: ddH_2O
- 1,25ml: 1.5M $Tris_{8.8}$
- 1,25ml: 40% Acrylamide
- 100 µl: 10% SDS
- 100 µl: 10% APS
- 10 µl: TEMED

Standard curve graphs from qRT-PCR analysis of mRNA transcripts in chick limb bud tissue

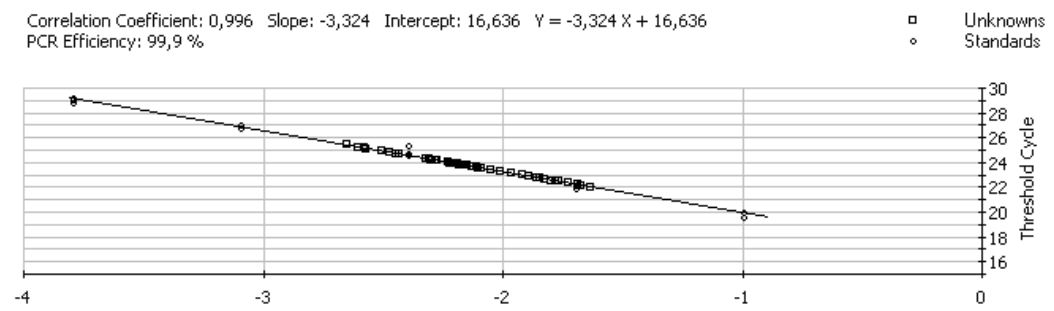

Fig.7.1: Standard curve graph from qRT-PCR analysis of the target gene *Hoxb-8*.

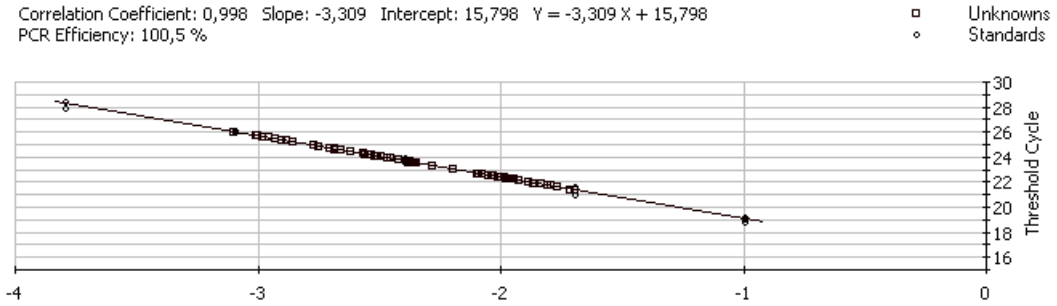

Fig.7.2: Standard curve graph from qRT-PCR analysis of the target gene *RARβ2*.

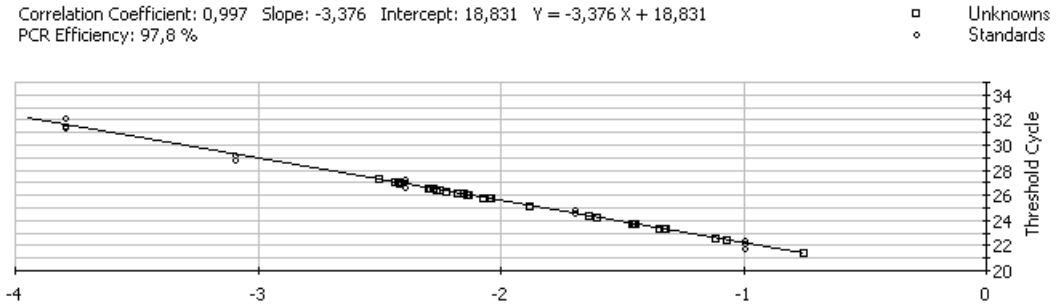

Fig.7.3: Standard curve graph from qRT-PCR analysis of the target gene *Cyp26*.

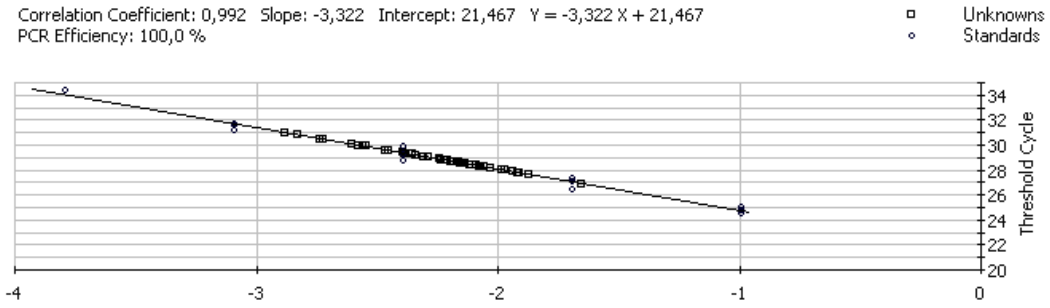

Fig.7.4: Standard curve graph from qRT-PCR analysis of the target gene *bmp-2*.

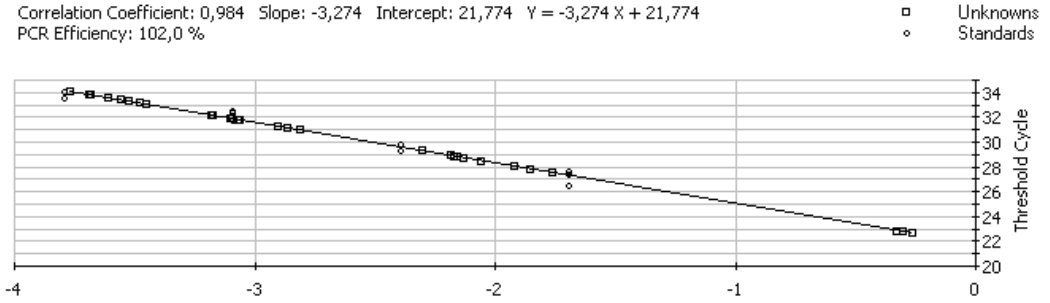

Fig.7.5: Standard curve graph from qRT-PCR analysis of the target gene *shh*.

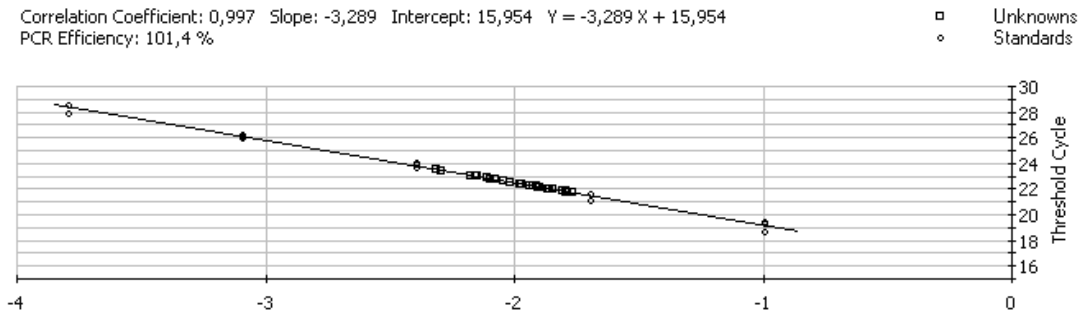

Fig.7.6: Standard curve graph from qRT-PCR analysis of the housekeeping gene *TBP*.

Melt curve graphs from qRT-PCR products of mRNA transcripts in chick limb bud tissue

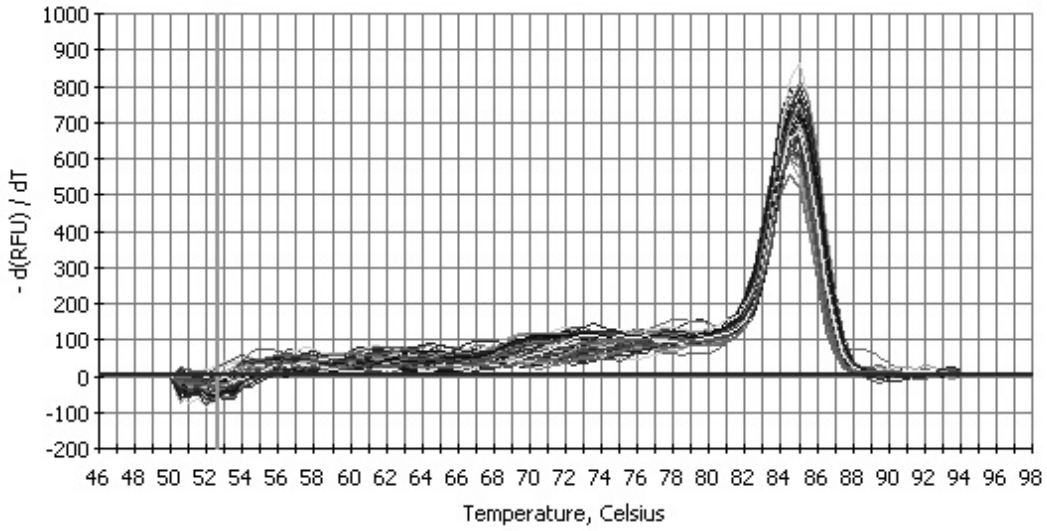

Fig.7.7: Melt curve graphs from the qRT-PCR products of *Hoxb-8* transcript amplification.

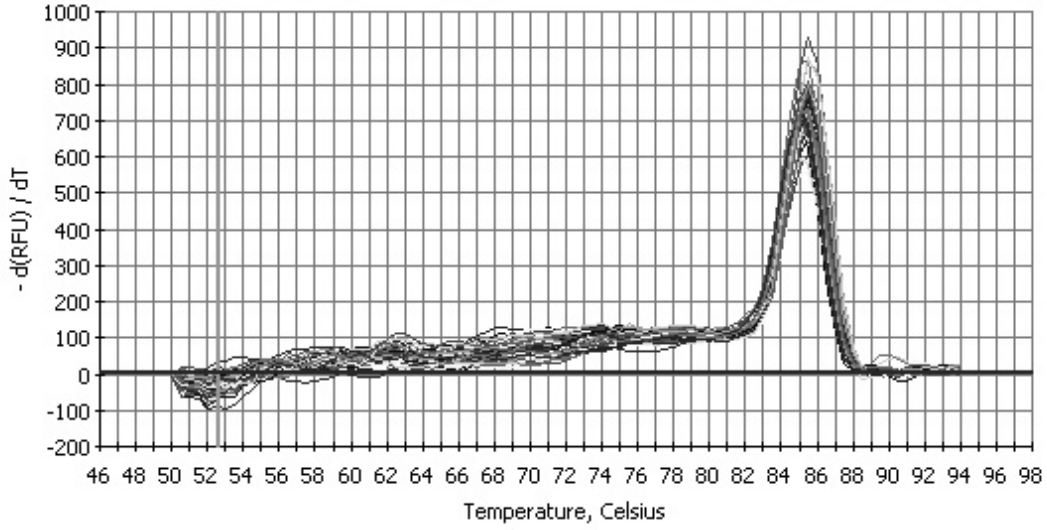

Fig.7.8: Melt curve graphs from the qRT-PCR products of RARβ2 transcript amplification.

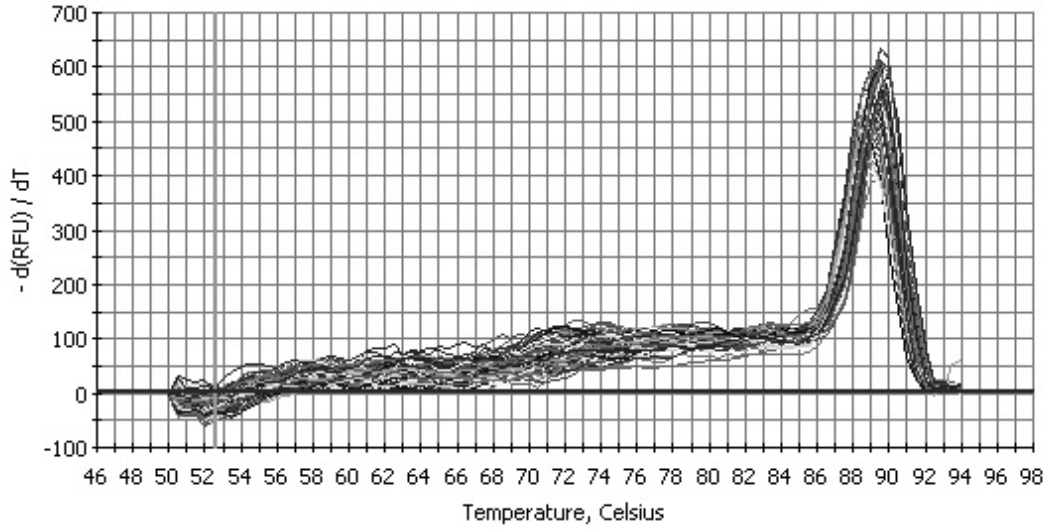

Fig.7.9: Melt curve graphs from the qRT-PCR products of Cyp26 transcript amplification.

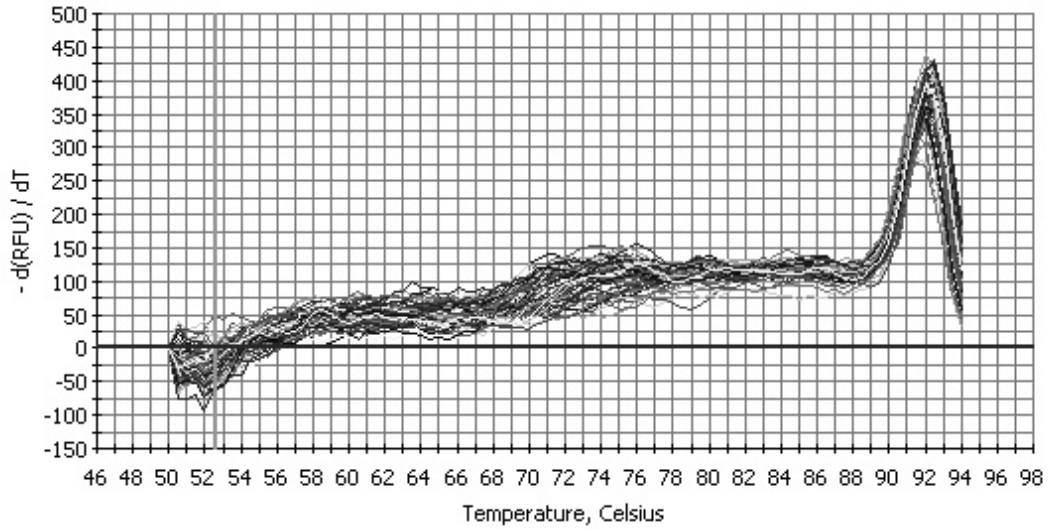

Fig.7.10: Melt curve graphs from the qRT-PCR products of bmp-2 transcript amplification.

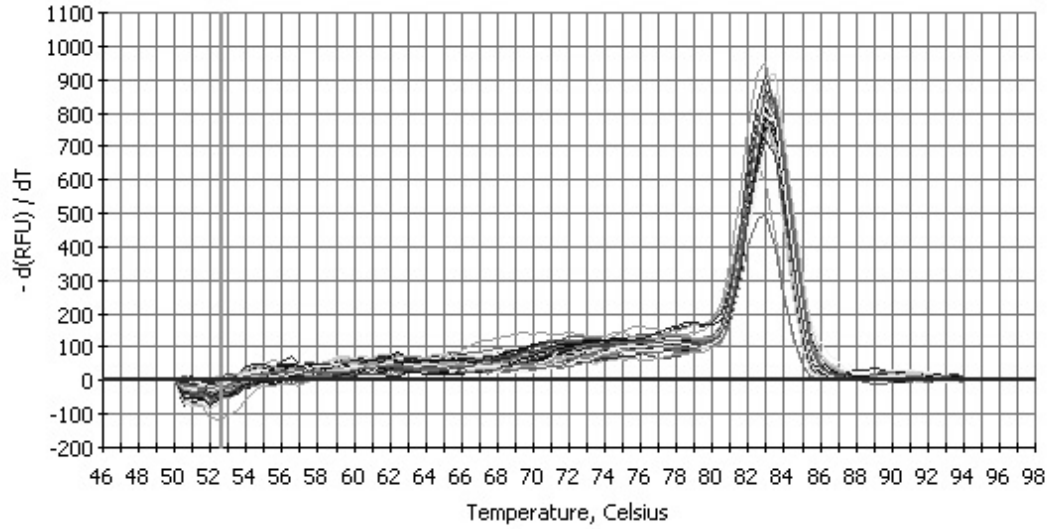

Fig.7.11: Melt curve graphs from the qRT-PCR products of shh transcript amplification.

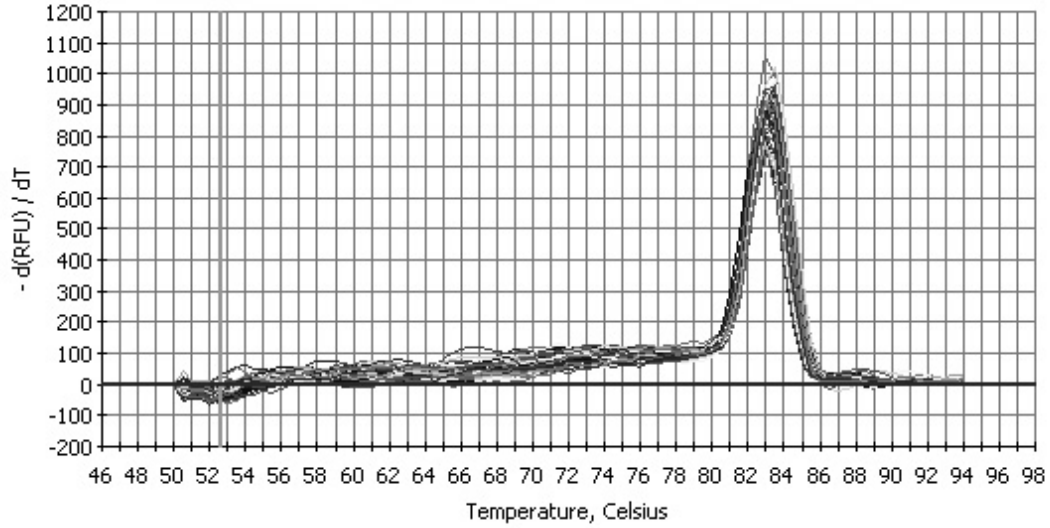

Fig.7.12: Melt curve graphs from the qRT-PCR products of TBP transcript amplification.

Erklärung zur Dissertation

Hierdurch erkläre ich, dass ich die hier vorliegende Dissertation mit dem Titel:

**„Biological activity of a novel retinoic acid metabolite,
S-4-oxo-9-cis-13,14-dihydro-retinoic acid“**

selbstständig verfasst habe und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Ort, Datum Unterschrift

Publikationsliste

Tagungsbeiträge

- Madalina Stefan, Henning Hopf, Jan Philipp Schuchardt, Gregor Eichele, Heinz Nau, 2005. Synthesis and Characterization of a New Vitamin A Metabolite. Spring symposium 2005, Japan chemical society.

- Heinz Nau, Jan Philipp Schuchardt, Norbert Giese, Gerd Hamscher, 2006. Sind Dioxine in tierischen Lebensmitteln wirklich gesundheitsschädlich? Beitrag in „Forschung fürs Leben“, Schwerpunkt Lebensmittelwissenschaften, Herausgeber Stiftung Tierärztliche Hochschule Hannover, 2006.

- Norbert Giese, Jan Philipp Schuchardt, Matti Viluksela, Helen Hakansson, Heinz Nau, 2007. S-9-cis-4-oxo-13,14-dihydro-retinoic acid, a new sensitive biomarker for TCDD toxicity. 46th annual meeting of the society of toxicology, 25-29 march 2007, Charlotte, North Carolina, USA.

Eingereichtes Manuskript

- Jan Philipp Schuchardt, David Wahlström, Norbert Giese, Malin Hedengren-Faulds, Madalina Stefan, Henning Hopf, Helen Håkansson, Gregor Eichele, Katarina Pettersson, and Heinz Nau, 2007. Biological activity in vitro and in vivo by the endogenous retinoid metabolite S-4-oxo-9-cis-13,14-dihydro-retinoic acid. Eingereicht bei: *Endocrinology.*

Manuskript in Endfassung

- Madalina Stefan, Jan Philipp Schuchardt, Norbert Giese, Heinz Nau, and Henning Hopf, 2007. Enantioselective synthesis and full characterization of a new chiral vitamin A metabolite. Wird eingereicht bei: *European Journal of Organic Chemistry.*

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