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# Differential contributions of AF-1 and AF-2 activities to the developmental functions of RXRα

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## SUMMARY

**We have engineered a mouse mutation that specifically deletes most of the RXRα N-terminal A/B region, which includes the activation function AF-1 and several phosphorylation sites. The homozygous mutants (RXRα*af1$^o$*), as well as compound mutants that further lack RXRβ and RXRγ, are viable and display a subset of the abnormalities previously described in RXRα-null mutants. In contrast, RXRα*af1$^o$*/RAR$^{-/-}$(α, β or γ) compound mutants die in utero and exhibit a large array of malformations that nearly recapitulate the full spectrum of the defects that characterize the fetal vitamin A-deficiency (VAD) syndrome. Altogether, these observations indicate that the RXRα AF-1 region A/B is functionally important, although less so than the ligand-dependent activation function AF-2, for efficiently transducing the retinoid signal through RAR/RXRα heterodimers during embryonic development. Moreover, it has a unique role in retinoic acid-dependent involution of the interdigital mesenchyme. During early placentogenesis, both the AF-1 and AF-2 activities of RXRα, β and γ appear to be dispensable, suggesting that RXRs act as silent heterodimeric partners in this process. However, AF-2 of RXRα, but not AF-1, is required for differentiation of labyrinthine trophoblast cells, a late step in the formation of the placental barrier.**

Key words: Nuclear receptor, Retinoic acid, Gene knockout, Transcriptional activity, Activation function, Placenta, Limb, Mouse

## INTRODUCTION

Retinoids (vitamin A derivatives) are crucial for many aspects of vertebrate physiology and homeostasis (reviewed in Sporn et al., 1994). They also play essential roles in morphogenesis and organogenesis, as inferred from the large spectrum of developmental abnormalities displayed by vitamin A-deficient (VAD) fetuses (reviewed in Kastner et al., 1995; Dickman et al., 1997; White et al., 1998). Two families of nuclear receptors for retinoids, the RARs (α, β and γ isotypes and their isoforms; activated by all forms of retinoic acid, RA) and the RXRs (α, β and γ isotypes and their isoforms; activated by 9-*cis* RA only) transduce the retinoid signal. RARs and RXRs act in transfected cells in vitro as ligand-dependent transcriptional transregulators through binding as RAR/RXR heterodimers to *cis*-acting RA response elements present in cognate reporter genes (reviewed in Leid et al., 1992; Chambon, 1996; Mangelsdorf et al., 1995; Mangelsdorf and Evans, 1995). In addition, RXRs can act as heterodimerization partners with nuclear receptors other than RARs, such as thyroid hormone receptors, vitamin D3 receptor, peroxisome proliferator activated receptors and several orphan receptors (reviews: Mangelsdorf and Evans, 1995; Chambon, 1996; Perlmann and Evans, 1997). Importantly, RAR-specific and RXR-specific ligands synergize to activate target genes or to elicit biological responses in cell systems (Taneja et al., 1996; Taneja et al., 1997; Roy et al., 1995; Chen et al., 1996; Horn et al., 1996; Nagy et al., 1995; Vivat et al., 1997), as well as in whole embryos (Minucci et al., 1996; Minucci et al., 1997; Lu et al., 1997), indicating that both partners of the RAR/RXR heterodimer can be transcriptionally active. However, the liganded RXR is not active unless its RAR partner is itself liganded (Roy et al., 1995; Apfel et al., 1995; Chambon, 1996; Taneja et al., 1996; Taneja et al., 1997; Chen et al., 1996; Vivat et al., 1997; Botling et al., 1997).

To investigate the involvement of RARs and RXRs in the transduction of retinoid signals under physiological conditions in vivo, all RAR and RXR genes, and most of their isoforms have been knocked out in the mouse (for reviews see Kastner et al., 1995; Mark et al., 1999). Altogether, these studies have led to several important conclusions concerning the role of these receptors during morphogenesis and organogenesis: (1) RARs and RXRs mediate the developmental functions of RA; (2) functional redundancies exist among the various RARs and among the various RXRs; (3) RAR/RXRα heterodimers are most likely the main functional units that transduce the retinoid signal during development; (4) transcriptional activation of both partners in RAR/RXRα heterodimers are often required to activate target genes and to mediate under

physiological conditions the retinoid effects in morphogenesis and organogenesis.

The N-terminal A/B domain of RXRs contains an autonomous ligand-independent transcriptional activation function called AF-1, whereas the C-terminal, ligand-binding domain E, contains a ligand-dependent transcriptional activation function, AF-2 (reviewed in Leid et al., 1992; Chambon, 1996; Moras and Gronemeyer., 1998). For each RXR, at least two isoforms exist, which differ in their N-terminal region (Leid et al., 1992; Fleischhauer et al., 1992; Liu and Linney, 1993; Nagata et al., 1994). As to RXRα, the major isoform RXRα1 is widely expressed in embryos and adults, whereas RXRα2 and α3 are restricted to the adult testis (Brocard et al., 1996). Furthermore, RXRα can be phosphorylated at several serine and threonine residues in its A/B domain (Adam-Stitah et al., 1999). The AF-2 activity crucially depends upon a conserved amphipathic α helix (the AF-2 AD core; Bourguet et al., 1995; Chambon, 1996; Wurtz et al., 1996; and references therein), whose deletion in the mouse has revealed its requirement for a number of RA-dependent developmental events (Mascrez et al., 1998). However, little is known about the mechanisms through which AF-1 activates transcription or about the relevance of the A/B domain in the global activity of the receptor under physiological conditions in vivo. Depending on the promoter context, the AF-1 of a given RXR modulates the AF-2 activity in cultured cells (Nagpal et al., 1992; Nagpal et al., 1993; Dowhan and Muscat, 1996; Chambon, 1996; Taneja et al., 1997). Thus, the transcriptional activity of a given RXR isoform may ultimately be determined, not only by its AF-2 activity, but also by its isoform-specific A/B domain.

In order to determine the importance of RXRα AF-1 domain A/B during development in vivo, we have engineered a mouse mutant line that expresses a truncated RXRα protein (RXRαΔA/B). The phenotypic analysis of mice carrying this mutation indicate that RXRα AF-1 domain A/B is important for transducing RA signals in vivo. In addition, we have assessed the relative contribution of RXRα AF-1 and AF-2 activities in embryonic development and placentation.

## MATERIALS AND METHODS

### Homologous recombination

The RXRα targeting vector (pB48) was designed to delete exon 2 (with the exception of its splicing acceptor site), intron 2 and 102 bp of exon 3. On the one hand, a 4.6 kb *Spe*I-*Bam*HI DNA fragment (from a P19 teratocarcinoma cell genomic library; Clifford et al., 1996), containing a *Bgl*II restriction site introduced at the beginning of exon 2 through site-directed mutagenesis, was subcloned into pEMBL19+ (Dente et al., 1983), leading to plasmid pB41. On the other hand, a 3.0 kb *Bam*HI-*Xba*I DNA fragment (from a 129/sv mouse genomic library; Kastner et al., 1994), containing a *Bgl*II site introduced in exon 3 by site-directed mutagenesis, was cloned into pB41, reconstituting then the 7.6 kb *Spe*I-*Xba*I region of the RXRα locus (plasmid pB42). The 4.0 kb-long DNA located between the two *Bgl*II sites of pB42 was deleted and replaced by an oligonucleotide preserving the RXRα open reading frame, and containing a *Nhe*I restriction site (plasmid pB45). The 3.6 kb *Spe*I-*Xba*I fragment of pB45 was then cloned into a pBluescript plasmid (Stratagene) containing a 5.7 kb *Xba*I-*Hin*dIII genomic fragment (from a 129/sv mouse genomic library), leading to pB46. Finally, a loxP site-flanked TK-NEO fusion cassette (Metzger et al., 1995) was introduced at the *Xba*I site located in intron 3 (leading to construct pB48).

Embryonic stem (ES) cells were transfected with *Not*I-linearized pB48, as previously described (Lufkin et al., 1991). Out of 87 G418-resistant clones, two targeted clones (HS23 and BL19) were obtained. Homologous recombination was confirmed by Southern blot analysis of *Sca*I-, *Nhe*I- and *Spe*I-digested genomic DNA hybridized with probes A, B and Neo (see Fig. 1 and data not shown). In order to delete the ‘floxed’ TK-NEO cassette, HS23 ES cells were transiently transfected with pSG5-Cre (Gu et al., 1993). Excision was confirmed for clone HS23.26Cre by Southern-blot analysis (Fig. 1 and data not shown). To generate the first RXRα$af1^{o}$ mouse line, HS23.26Cre cells were injected into C57BL/6 blastocysts. To generate a second RXRα$af1^{o}$ mouse line, BL19 cells were used. In this line, the selectable marker was successfully excised by crossing heterozygotes with transgenic mice expressing Cre early during embryogenesis (CMV-Cre; Dupé et al., 1997). The phenotypes illustrated here are from line HS23.26Cre. However, identical observations were made using line BL19.

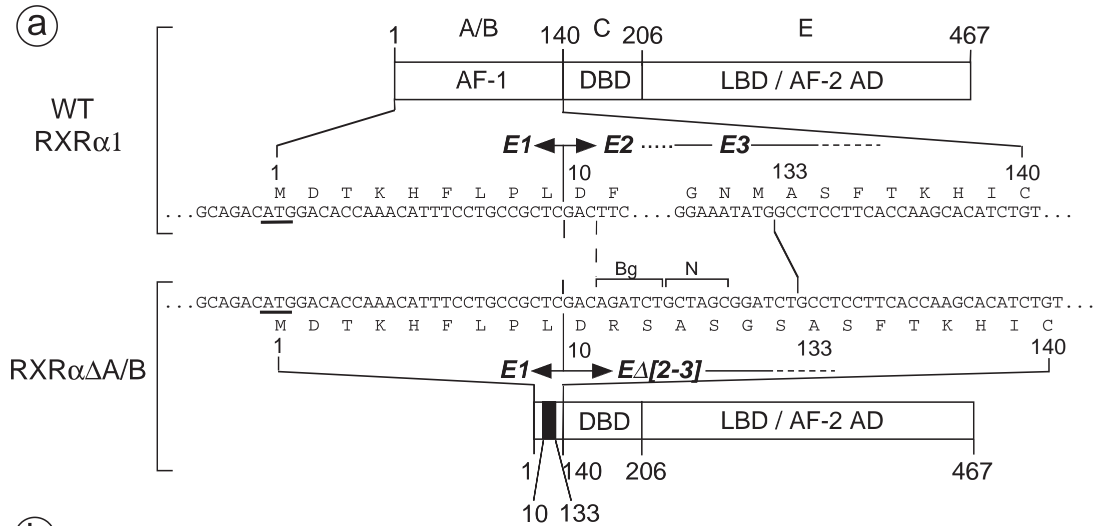
### Western blot analysis

Nuclear extracts were prepared from whole E12.5 embryos (Andrews and Faller, 1991). Proteins (15 μg) were separated on 10% gel by SDS-PAGE and transferred onto nitrocellulose membranes. RXRα and RXRαΔA/B proteins were detected using the 4RX3A2 anti-RXRα monoclonal antibody (1/500 dilution; Rochette-Egly et al., 1994) and revealed by chemiluminescence according to the manufacturer’s instructions (Amersham).

### Histology, immunohistochemistry and skeletal analyses

Embryos and fetuses were fixed in Bouin’s fluid, embedded in paraffin, serially sectioned and stained with Groat’s Hematoxylin and Mallory’s Trichrome (Mark et al., 1993). Skeletons of E18.5 fetuses were prepared as described (Lufkin et al., 1992). Nile Blue Sulfate staining and in situ hybridization for detection of stromelysin-3 transcripts were described previously (Dupé et al., 1999).

**Fig. 1.** Targeted deletion of the AF-1-containing A/B region of RXRα. (a) Representation of the wild-type RXRα1 isoform (WT RXRα1) and the mutant RXRα (RXRαΔA/B). Exons E1 to E3 are shown. The functional domains are depicted as followed: AF-1, activation function 1; AF-2 AD, activation function 2 activating domain; DBD, DNA-binding domain (region C); LBD, ligand-binding domain (region E). The RXRα*af1* mutation leads to the production of an RXRα protein truncated from amino acid 11 to 132. *Bgl*II(Bg) and *Nhe*I(N) restriction sites were introduced to allow detection of the mutant allele. EΔ[2-3] contains the first codon of exon 2 fused to the 3′ part of exon 3. The deletion is represented by a black box. (b) The WT RXRα locus, the targeting construct and the mutated loci obtained after replacement (I), and subsequent Cre-mediated excision of the ‘floxed’ TK-NEO cassette (II). The star represents the mutation EΔ[2-3] described in (a). LoxP sites are represented by black arrowheads. Probes A, B and C are 0.7 kb *Sca*I-*Spe*I, 0.6 kb *Eco*RI-*Bgl*II and 0.3 kb *Bam*HI-SpeI fragments, respectively. The size of the restriction fragment that allow identification of WT and targeted alleles (I) and (II) by Southern blot analysis using probes A, B, C and neo are indicated below (in kilobases). N, *Nhe*I; Bg, *Bgl*II; Sc, *Sca*I; S, *Spe*I; E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; X, *Xba*I; Xh, *Xho*I. (c) Southern blotting of ES cell DNA digested with *Bgl*II or *Spe*I and hybridized with probe A, B and neo, as indicated. WT (+/+) and RXRα*af1* mutant alleles before (+/(I)) or after (*af1*/+) excision of the ‘floxed’ TK-NEO cassette are indicated. (d) Detection of RXRαΔA/B protein. Nuclear extracts prepared from 12.5-day-old embryos WT (+/+), *af1* heterozygote (*af1*/+), *af1* homozygote ($af1^{o}$) and RXRα-null mutants (–/–) (Kastner et al., 1994) were analyzed by western blotting with the anti-RXRα monoclonal antibody 4RX3A2, directed against a C-terminal epitope.


a
WT
RXRα1
RXRαΔA/B
A/B
C
E
1
140
206
467
AF-1
DBD
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EΔ[2-3]
10
133
Bg
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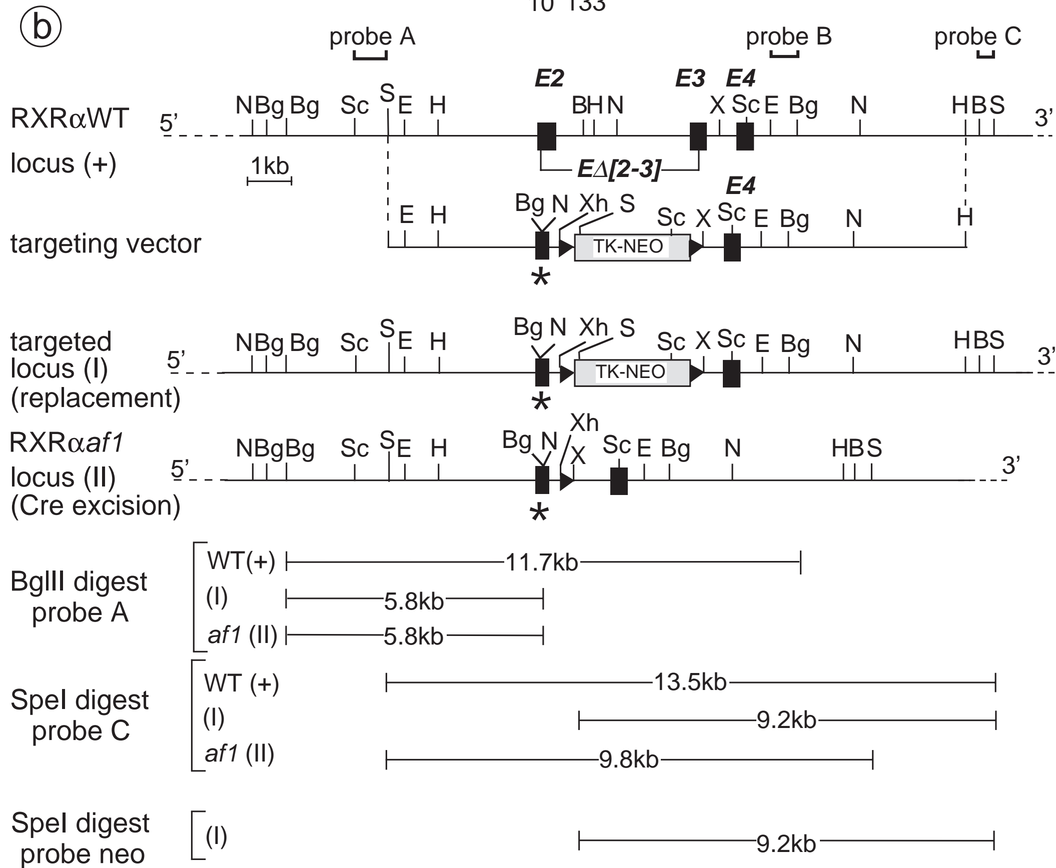
...GCAGACATGGACACCAAACATTTCCTGCCGCTCGACTTC....GGAAATATGGCCTCCTTCACCAAGCACATCTGT...
...GCAGACATGGACACCAAACATTTCCTGCCGCTCGACAGATCTGCTAGCGGATCTGCCTCCTTCACCAAGCACATCTGT...
M D T K H F L P L D R S A S G S A S F T K H I C


b
probe A
probe B
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RXRαWT
locus (+)
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targeting vector
targeted
locus (I)
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(Cre excision)
5'
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NBg Bg Sc S E H
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BHN
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X Sc E Bg N HBS
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Bg N Xh S
TK-NEO
Sc X Sc E Bg N H
*
BglII digest
probe A
WT(+)
11.7kb
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5.8kb
af1 (II)
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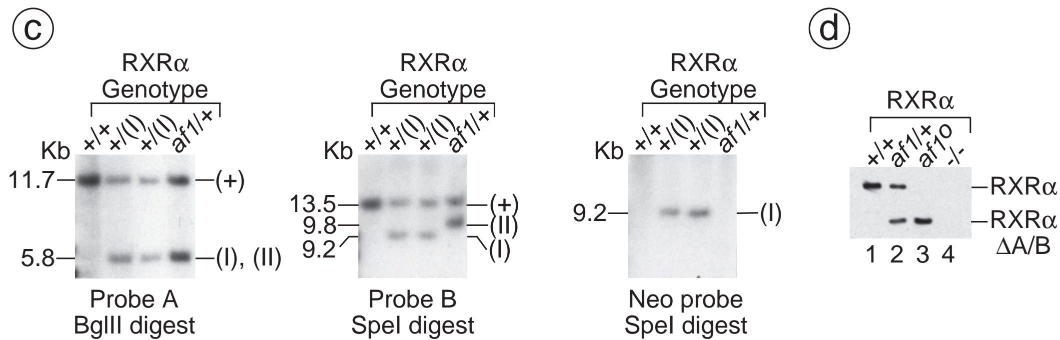
c
RXRα
Genotype
+/+
+/(I)
+/(I)
af1/+
Kb
11.7
5.8
(+)
(I), (II)
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BglII digest
13.5
9.8
9.2
(+)
(II)
(I)
Probe B
SpeI digest
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Neo probe
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d
RXRα
+/+
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af1o
-/-
RXRα
RXRα
ΔA/B
1 2 3 4

### Behavior studies

Xα*af1*$^o$ mutant mice were in a 129/Sv/C57BL/6 (25/75%) genetic background at the time of testing. All animals were housed in cohorts of three to five mice per cage in 12 hour light/dark cycle, with freely available food and water. Behavioral testing was conducted between 14:00 and 18:00. The open field test was performed as described (Krezel et al., 1998). For the elevated plus maze, mice were placed in the central part of the maze and the percentage of time spent in the open arm, and the number of times animal stretched its head to look down were recorded for 5 minutes. To measure fine locomotor skills, the rotarod, inclined plane and cord tests were employed (Krezel et al., 1998; Wolffgramm et al., 1990; Fehlings, 1995; Perry et al., 1995). The nociception was analyzed by evaluating response to acute thermal stimuli in the tail-flick and hot-plate tests, in which the latency to remove the tail from 50°C water and the time spent on the 54° plate before licking the hindpaws were measured, respectively. Finally in taste preference, mice were presented water and 15% sucrose in different parts of the test cage. The number and the time of visits were scored for 5 minutes over 4 consecutive days, indicating preference for sucrose. For each behavioral paradigm data were analyzed by analysis of variants, ANOVA and Tukey-Kramer post hoc tests.

## RESULTS

### Targeted deletion of the RXRα AF-1 domain A/B

A targeting vector was designed to alter the RXRα locus in such a way that it encodes a mutant protein lacking amino acids 11 to 132 of RXRα1. This region contains the autonomous AF-1 activation function, several phosphorylation sites (Adam-Stitah et al., 1999), as well as the initiator codon of RXRα2 and α3 isoforms (Brocard et al., 1996). Replacement in ES cells led to a targeted allele in which most of exon 2 (with the exception of the first 3 bp), intron 2 and 102 pb of exon 3 were deleted, and in which a 'floxed' TK-NEO cassette was inserted into intron 3. Cre-mediated excision of the selection marker led to the mutant allele (hereafter designated as RXRα*af1*), containing a single intronic loxP site (see Fig. 1b,c). Chimeric males derived from two independent ES cell clones (HS23.26Cre and BL19) transmitted the mutation through their germline, and the identity of the mutation was confirmed by sequencing RT-PCR products amplified from RNA of homozygous mutants (data not shown). In nuclear extracts prepared from whole E12.5 embryos, the truncated RXRαΔA/B protein was readily detected as a single species with an increased electrophoretic mobility, when compared with that of RXRα (Fig. 1d). Its overall level of expression in homozygous mutants was comparable with that of RXRα in wild type (WT) embryos (Fig. 1d, compare lanes 1 and 3). Moreover, the RXRαΔA/B and RXRα proteins were present in similar amounts in heterozygous embryos (Fig. 1d, lane 2). Therefore, the present mutation does not drastically alter the steady state level of the RXRα truncated protein. Consequently, its effects are likely to reflect only the lack of RXRα A/B domain.

We describe below the effects of deleting the RXRα A/B domain in mice as well as in mutants additionally null for RXRβ, RXRγ, RARα, RARβ or RARγ. To simplify nomenclature, homozygote mutant mice lacking the RXRα A/B domain are designated as Xα*af1*$^o$, and heterozygotes as Xα*af1*/+; RXRα, RXRβ, RXRγ, RARα, RARβ and RARγ homozygote null mutants are designated as Xα, Xβ, Xγ, Aα, Aβ and Aγ, respectively; for example, RXRα*af1*$^o$/RARα$^{-/-}$ mutants are referred to as Xα*af1*$^o$/Aα mutants.

### Xα*af1*$^o$ mutants are growth deficient and display congenital defects

Deletion of RXRα AF-1 domain A/B does not lead to lethal developmental defects, as viable and fertile Xα*af1*$^o$ mutants were obtained at the expected Mendelian ratio (out of 1103 littermates born from heterozygote crosses, 27% were wild type (*n*=299), 50% were heterozygote (*n*=553) and 23% were homozygote (*n*=251)). At embryonic day 18.5 (E18.5), the weight of Xα*af1*$^o$ mutants (1.19 g on average; *n*=20) was similar to that of wild type (1.22 g on average; *n*=18). In contrast, between 1 and 2 weeks of birth, Xα*af1*$^o$ mutants were on average 20% lighter than their wild-type littermates (Fig. 2). Several of the cachectic Xα*af1*$^o$ mutants (weight ratio < 0.5) died before weaning, whereas the others lived as long as their wild-type littermates (at least 1 year), but exhibited a weight deficit of about 10% during at least 6 months after birth (Fig. 3). This growth retardation was harmonious as (1) the ratios of adult tissue weights (liver, kidney, heart, lungs, visceral fat) to the total body weight and (2) ratios of the length of skeletal elements (femur and skull) to the total body-length did not reveal significant differences between Xα*af1*$^o$ mutants and wild-type animals. We previously showed that RXRα$^{+/-}$ mice are growth deficient (Kastner et al., 1994). The present data further indicate that the RXRα A/B domain is important for postnatal growth.

Soft tissue syndactyly of the hindlimbs was seen unilaterally (*n*=33) or bilaterally (*n*=83) in 70% of the Xα*af1*$^o$ adults. It affected mostly the base of the interdigital regions between digits 2-3 and 3-4 (data not shown). Hindlimb interdigital webbing was observed at a low frequency in RXRα$^{+/-}$ mice, but was absent in Xα*af2*$^o$ mutants (Kastner et al., 1997a;

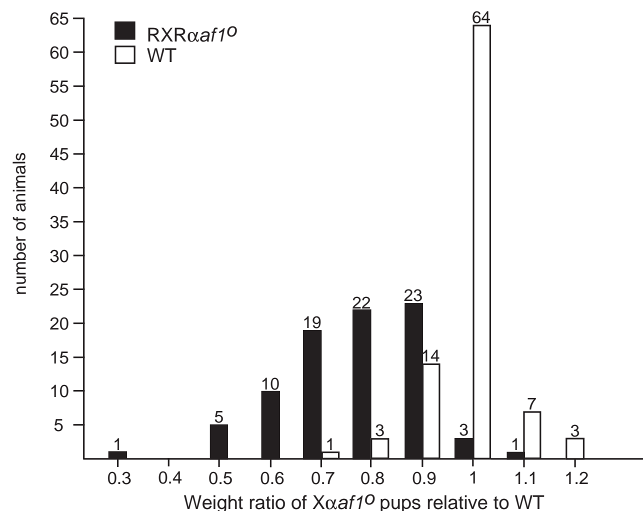


**Fig. 2.** Weight of wild type (WT) and Xα*af1*$^o$ mutants at 1-2 weeks of age. To standardize between litters, the weight of each pup obtained from Xα*af1*/+ intercrosses was expressed as the ratio of its weight relative to the average weight of the WT pups from the same litter. Ratios were then grouped within classes differing by 0.1 increment (*y*-axis). The number of Xα*af1*$^o$ and WT animals in each class is indicated on the top of the bars.

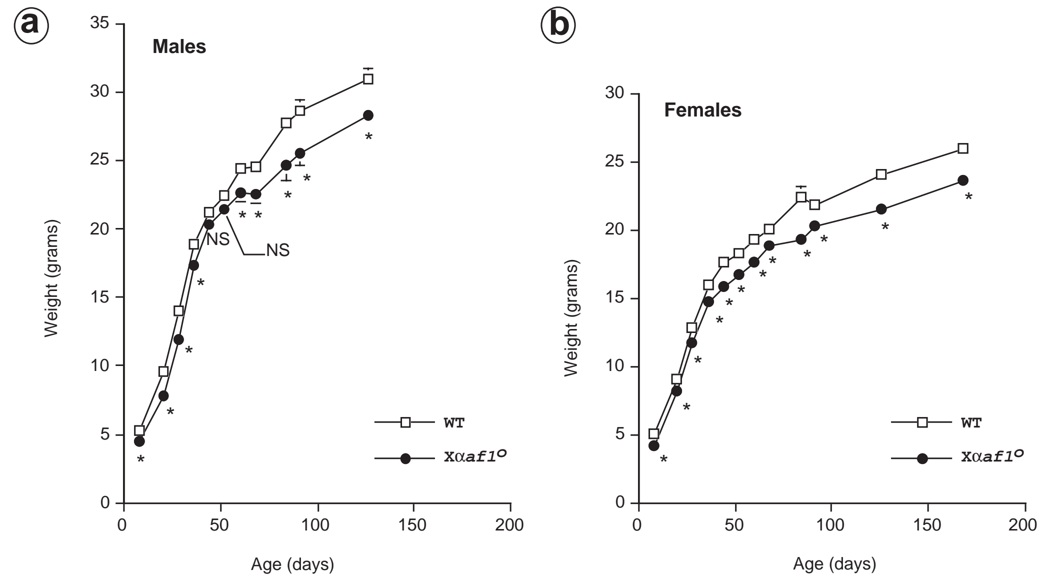


**Fig. 3.** Weight of wild-type (WT) and X$\alpha$*af1^o* mutant males (a) and females (b). Mean weights of offsprings ($n$=5 to 18) obtained from X$\alpha$*af1*/$^+$ intercrosses are presented with s.e.m. After testing for normality and variance homogeneity, values were subjected to Student's $t$ tests. Asterisks indicate the significance ($P$<0.05) for the differences observed between WT and X$\alpha$*af1^o* mutants. NS, not significant.

Mascrez et al., 1998). These data indicate that RXRα AF-1 domain A/B is involved in the involution of the interdigital mesenchyme (see below).

A persistent hyperplastic primary vitreous body (PHPV) was the only ocular defect observed in Xα*af1^o* mutants (two out of 18). In contrast, eye morphogenesis is severely altered in Xα and Xα*af2^o* fetuses, which display (in addition to a completely penetrant PHPV) corneal, retinal, lens and eyelid abnormalities (Kastner et al., 1994; Kastner et al., 1997a; Mascrez et al., 1998; see Table 2). Xα and Xα*af2^o* fetuses also exhibit cardiac abnormalities, namely a myocardial hypoplasia and an agenesis of the conotruncal septum (Kastner et al., 1994; Kastner et al., 1997a; Sucov et al., 1994). No heart defects were detected in E18.5 Xα*af1^o* mutants analyzed by histology ($n$=3). However at E9.5, electron microscopic analysis of Xα*af1^o* embryos ($n$=5) revealed a single case of precocious differentiation of the cardiomyocytes as in Xα and Xα*af2^o* mutants (Fig. 4; Kastner et al., 1994; Kastner et al., 1997b; Mascrez et al., 1998). Xα*af1^o* mutants exhibited skeletal abnormalities similar to those found in RARs and Xα*af2^o* mutants (Lohnes et al., 1993; Ghyselinck et al., 1997; Mascrez et al., 1998): (1) homeotic transformations and malformations of cervical vertebrae; (2) bilateral agenesis of the metoptic pilar (the posterior border of the optic nerve foramen); and (3) a misshapen cricoid cartilage (Table 1). Altogether, these results indicate that the RXRα AF-1 domain A/B is involved in the involution of the primary vitreous body, the differentiation of cardiomyocytes and the morphogenesis of some skeletal elements; but at first sight this domain also appears dispensable for the majority of the developmental events normally mediated by RXRα.

Retinoid receptors regulate brain functions (Krezel et al., 1998; Chiang et al., 1998). As RXRα is almost ubiquitously expressed in the central nervous system (Krezel et al., 1999), behavioral tests were performed on Xα*af1^o* mice. Sensory faculties, motor skills, locomotion activity, stereotypic behaviours, as well as stress responses, were not affected, as Xα*af1^o* mice performed as well as wild-type littermates in all these tests (see Materials and Methods for details). Thus, no obvious non-redundant function can be readily ascribed, in the central nervous system, to the RXRα AF-1 domain A/B.

### Deletion of the RXRα AF-1 domain A/B results in additional congenital defects in the absence of RXRβ and RXRγ

As Xα*af1^o* mutants displayed neither the prenatal lethality nor all the congenital defects previously observed in Xα and

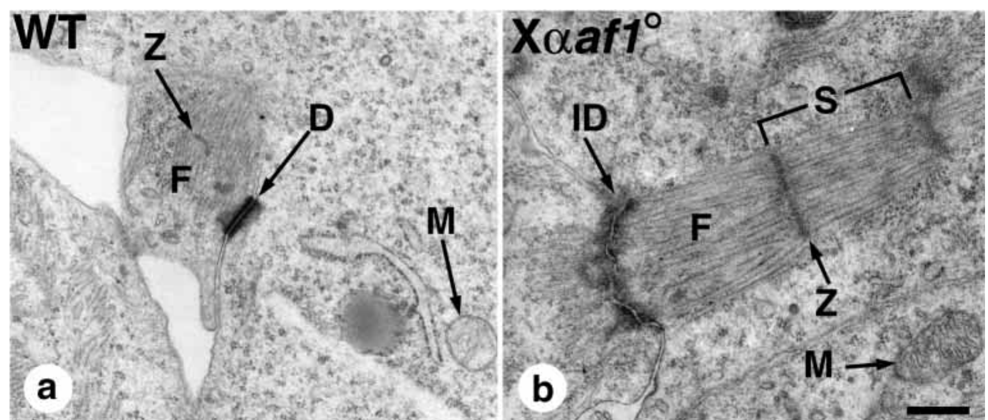


**Fig. 4.** Precocious differentiation of Xα*af1^o* ventricular cardiomyocytes. (a) The subepicardial myocytes of E9.5 wild-type (WT) embryos contain bundles of myofilaments, occasionally showing isolated Z lines and connected only by desmosomes. (b) In 70% of the subepicardial myocytes of this E9.5 Xα*af1^o* mutant the myofilaments are already organized into sarcomeres (S) that are connected between cells by a series of adherens junctions forming the intercalated discs (ID). D, desmosome; F, bundles of myofilaments; M, mitochondria; S, sarcomere; Z, Z line. Scale bar: 0.5 μm.

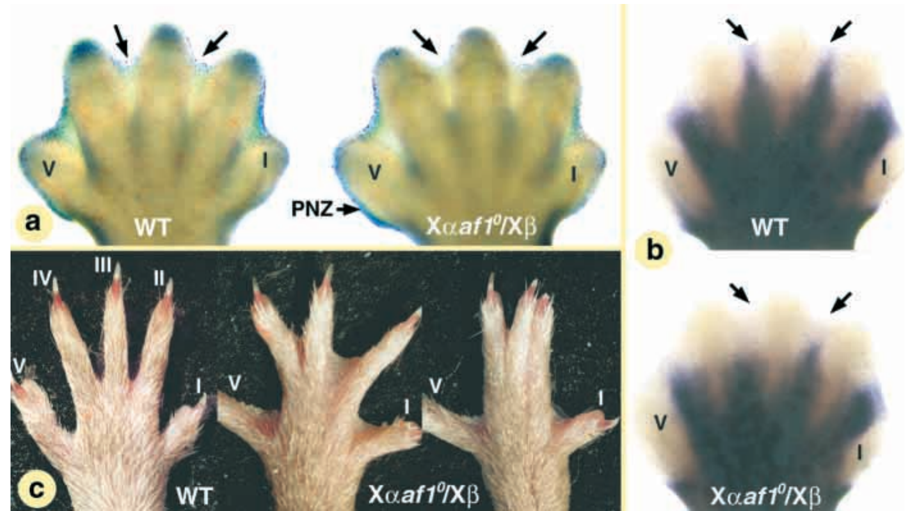


**Fig. 5.** Decreased interdigital cell death results in soft tissue syndactyly of the hindlimbs in Xα*af1^o*/Xβ mutants. Comparison of (a) cell death assessed by Nile Blue Sulfate staining, (b) expression of stromelysin-3 assessed by in situ hybridization and (c) morphology, between E14.5 (a,b) and adult (c) wild type (WT) and Xα*af1^o*/Xβ mutants. PNZ, posterior necrotic zone; I-V, digit one to five. The arrows point to the two interdigital regions, which are the most severely affected in adults.

Xα*af2^o* mutants, the RXRα AF-1 domain A/B might be largely dispensable during development. Alternatively, RXRβ and/or RXRγ may functionnally compensate for the absence of the RXRα AF-1 domain A/B. To investigate this possibility, RXRα*af1^o* mutation was introduced into RXRβ and RXRβ/RXRγ-null genetic backgrounds (Xβ/Xγ mutants develop normally; Krezel et al., 1996). Steady-state levels of RXRαΔA/B in Xα*af1^o*/Xβ and Xα*af1^o*/Xβ/Xγ E12.5 embryos were comparable with those of RXRα in Xβ and Xβ/Xγ embryos, respectively (data not shown).

Of the 362 mice genotyped after birth from Xα*af1*/+/Xβ+/− intercrosses, Xα*af1^o*/Xβ mutants were obtained at a Mendelian ratio (23 expected, 19 obtained). However, of the 261 mice born from Xα*af1*/+/Xβ+/−/Xγ intercrosses, only 10 Xα*af1^o*/Xβ/Xγ mutants were obtained (16 expected). But at E14.5, Xα*af1^o*/Xβ/Xγ fetuses were collected at a Mendelian ratio. This indicates that the absence of all RXR AF-1 domains A/B is not lethal at a developmental stage when organogenesis is almost completed, but results in impaired viability. Xα*af1^o*/Xβ and Xα*af1^o*/Xβ/Xγ mutant females were fertile, whereas males were sterile, owing to the loss of RXRβ (Kastner et al., 1996). In adults, the weight of Xα*af1^o*/Xβ mutants was about 10% less than that of Xα*af1^o* mutants (data not shown).

A similar bilateral and completely penetrant hindlimb interdigital webbing was observed in Xα*af1^o*/Xβ and in Xα*af1^o*/Xβ/Xγ mutants. It was more severe than in Xα*af1^o* mutants, as it often affected the full length of interdigital spaces (Fig. 5c and data not shown). E14.5 Xα*af1^o*/Xβ hindlimbs showed a marked decrease in the number of dying cells within interdigital spaces, as assessed by staining with Nile Blue Sulfate (Fig. 5a). Moreover, the expression of stromelysin 3, an RA-regulated matrix metalloproteinase involved in tissue remodeling processes that accompany apoptosis in developing limbs (Lefebvre et al., 1995; Dupé et al., 1999; Ludwig et al.,

**Table 1. Skeletal and cartilage abnormalities in E18.5 Xα*af1^o* mutants**

| Abnormalities | Genotypes and number of skeletons examined at E18.5: Wild type 14 5 (35%)* | Xα*af1^o* 20 19 (95%)* |
|---|---|---|
| Cranial skeletal abnormalities | | |
| Agenesis of metoptic pilar (Xα*af2^o*) (Aγ) | | |
| Unilateral | 2 (15%) | 8 (40%) |
| Bilateral | 0 | 3 (15%) |
| Axial skeletal abnormalities | | |
| Homeotic transformations | | |
| Posteriorizations | | |
| Posterior tubercle on basioccipital bone (Xα*af2^o*) (Aβ) (Aγ) | 0 | 1 (5%) |
| Transformation of C7 to T1 (Aα) (Aβ) | 0 | 4 (20%) |
| Anteriorizations | | |
| Eight instead of seven vertebrosternal ribs (Xα*af2^o*) (Aα) (Aγ) | 0 | U:2 (10%) |
| Malformations | | |
| Fusion of C1-AA with C2 dens (Xα*af2^o*) (Aα) (Aγ) | 3 (20%) | 8 (40%) |
| C2 bifid (Xα*af2^o*) (Aγ) | 0 | 3 (15%) |
| Abnormal cricoid cartilage‡ (Xα*af2^o*) | 1 (7%) | 7 (35%) |

Xα*af2^o*, Aα, Aβ, Aγ: these abnormalities are also observed in Xα*af2^o*, Aα, Aβ and Aγ fetuses.
C1-AA, anterior arch of the atlas; C1 to C7, first to seventh cervical vertebrae; T1 to T14, first to fourteenth thoracic vertebrae; U, unilateral.
*Number of animals exhibiting malformed skeletons.
‡Ventral extension of the cricoid cartilage. (For further details concerning these abnormalities, see Lohnes et al., 1994; Ghyselinck et al., 1997; Mascrez et al., 1998.)

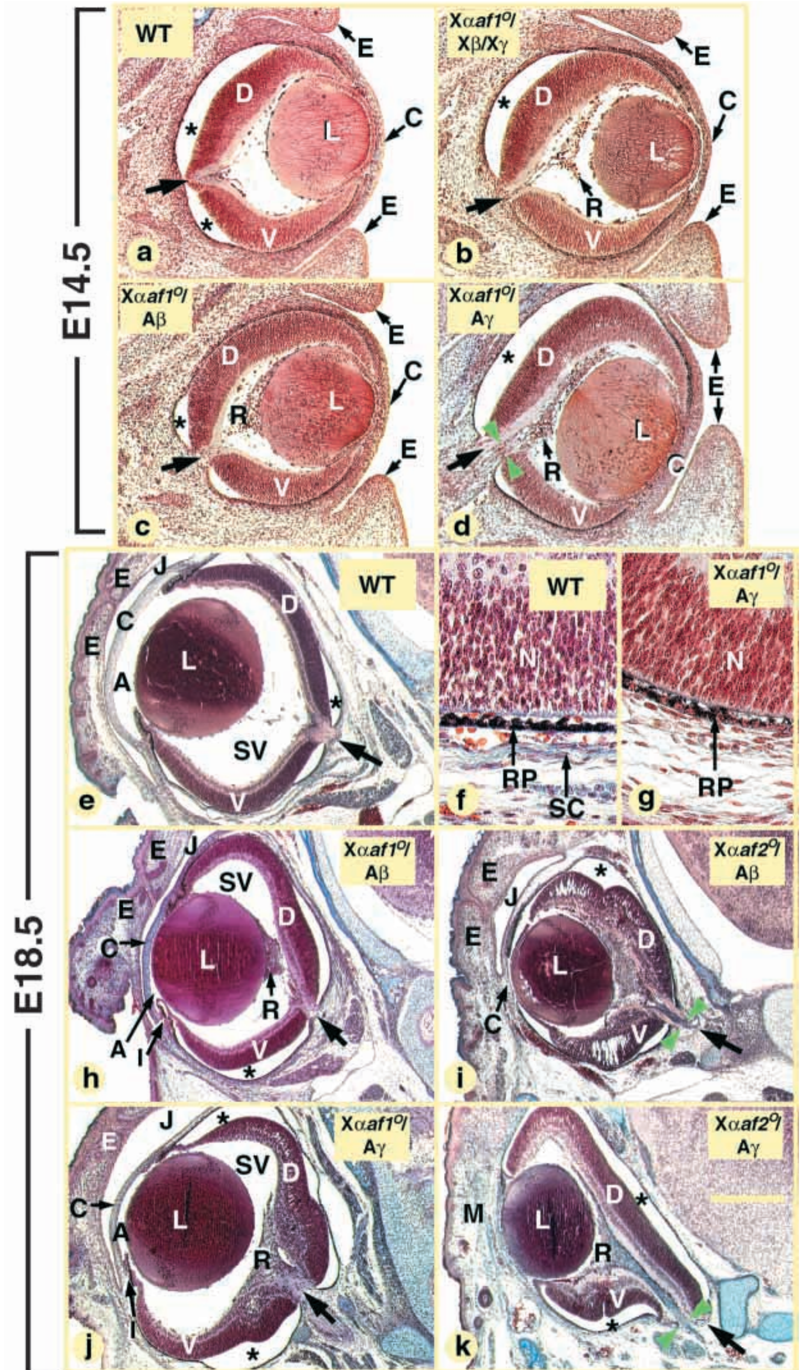


**Fig. 6.** Comparison of ocular malformations in Xα*af1*$^{o}$ and Xα*af2*$^{o}$ compound mutants. Frontal sections through the eye region of WT and mutant fetuses at E14.5 (a-d) and E18.5 (e-k). (h-k) Note that Xα*af1*$^{o}$/A(β or γ) and Xα*af2*$^{o}$/A(β or γ) mutants share the same spectrum of ocular defects, although these are systematically more severe in Xα*af2*$^{o}$ compound mutants. For example, the size of the conjunctival sac (J) and cornea (C) is only slightly reduced in Xα*af1*$^{o}$/Aβ and Xα*af1*$^{o}$/Aγ mutants (compare e,j,h) but markedly decreased in Xα*af2*$^{o}$/Aβ mutants (i), and absent in Xα*af2*$^{o}$/Aγ mutants (k). Likewise, the stroma of the iris (I), the anterior chamber (A) and the secondary vitreous body (SV) are present in Xα*af1*$^{o}$/Aβ and Xα*af1*$^{o}$/Aγ mutants, but not in their Xα*af2*$^{o}$ counterparts. Note also that in some mutants at E18.5, the relative sizes of the ventral and dorsal retina is not possible to assess due the existence of retinal folds. A, anterior chamber; C, cornea; D, dorsal retina; E, eyelids; I, iris stroma; J, conjuntival sac; L, lens; M, mesenchyme replacing the eyelids and cornea; N, neural retina; R, persistant hyperplastic primary vitreous; RP, retinal pigment epithelium; SC, sclera; SV, secondary vitreous body. The large arrow points to the optic nerve exit point. The green arrowheads delimit colobomas of the optic disc. The asterisks indicate artefactual detachment of the neural retina from the retinal pigment epithelium occurring during tissue processing. Scale bar in k: 200 μm (a-d); 300 μm (e,h-k); 40 μm (f,g).

2000), was markedly decreased in the interdigital spaces of E14.5 Xα*af1*$^{o}$/Xβ hindlimbs (Fig. 5b). These results indicate that the soft tissue syndactyly seen in a majority of Xα*af1*$^{o}$ adults, as well as in all Xα*af1*$^{o}$/Xβ, Xα*af1*$^{o}$/Xβ/Xγ adults, is caused by the persistence of the fetal interdigital mesenchyme.

All E18.5 Xα*af1*$^{o}$/Xβ (*n*=4) and E14.5 Xα*af1*$^{o}$/Xβ/Xγ (*n*=9) mutants analyzed histologically displayed a bilateral PHPV (compare Fig. 6a,b; Table 2). In addition, one Xα*af1*$^{o}$/Xβ/Xγ mutant displayed a ventricular myocardial hypoplasia, and another one an agenesis of the conotruncal septum comparable with that of Xα mutants (Table 3). These data confirm the requirement of A/B domain of RXR in the involution of the interdigital and primary vitreous body mesenchymes, and indicate that it is also involved in cardiac morphogenesis in some individuals.

The putative start codon of RXRα2 and α3, the two RXRα isoforms specifically expressed in the adult testis (Brocard et al., 1996), was removed in Xα*af1*$^{o}$ mutants. Therefore, the RXRα A/B domain deletion should also result in a null mutation for RXRα2 and α3. This was confirmed by western blot analysis (data not shown). Histological analysis of the testes of Xα*af1*$^{o}$ adults (*n*=4), which are fertile, did not reveal abnormalities, whereas Xα*af1*$^{o}$/Xβ/Xγ adults (*n*=2), which are sterile because of the RXRβ-null mutation, did not display any testis defect in addition to those exhibited by Xβ-null mutants (Kastner et al., 1996). Thus, RXRα2 and RXRα3 isoforms are not necessary for male fertility.

## In contrast to the RXRα AF-2 function, the RXRα AF-1 domain A/B is dispensable for placentation

The relative contributions of AF-1 and AF-2 activities to the functions of RXRα in placentation (Sapin et al., 1997; Wendling et al., 1999; Barak et al., 1999) were assessed by comparing wild-type, Xα*af1*$^{o}$, Xα*af2*$^{o}$, Xα*af1*$^{o}$/Xβ/Xγ and Xα*af2*$^{o}$/Xβ/Xγ placental morphologies. Placentas from E18.5 Xα*af1*$^{o}$ (*n*=3) and E14.5 Xα*af1*$^{o}$/Xβ/Xγ (*n*=6) mutants were macroscopically and histologically indistinguishable from wild-type placentas (data not shown). Histological defects observed at E14.5 in Xα*af2*$^{o}$ (*n*=5) and Xα*af2*$^{o}$/Xβ/Xγ (*n*=5) placentas consisted mainly in a thickening of labyrinthine

**Table 2. Abnormalities of the eye and of its adnexae in Xα*af1*$^o$ compound mutant fetuses**

| | Genotype, age and number of mutant fetuses | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Xα*af1*$^o$/Xβ | Xα*af1*$^o$/Xβ/Xγ | Xα*af1*$^o$/Aα | | Xα*af1*$^o$/Aβ | | Xα*af1*$^o$/Aγ | |
| Ocular abnormalities | E18.5<br>3 | E14.5<br>9 | E14.5<br>8 | E18.5<br>3 | E14.5<br>5 | E18.5<br>2 | E14.5<br>4 | E18.5<br>2 |
| **Lens abnormalities** | | | | | | | | |
| Ventral rotation of the lens<br>(Xα*) (Xα/Aγ*)‡ (Xα*af2*$^o$)(Xα*af2*$^o$/Aβ*; Xα*af2*$^o$/Aγ*)‡ | 0 | 0 | 0 | 0 | * | ND | * | ND |
| **Mesenchymal defects** | | | | | | | | |
| Agenesis of the eyelids and cornea<br>(Xα/Aγ*) (Xα*af2*$^o$/Aγ*) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Closer eyelid folds (E14.5)/small conjunctival sac (E18.5)<br>(Xα*) (Xα/Aβ*) (Xα*af2*$^o$) (Xα*af2*$^o$/Aβ*) | 0 | 0 | 0 | 0 | * | * | * | * |
| Thickened corneal stroma<br>(Xα*) (Xα/Aβ*) (Xα*af2*$^o$) (Xα*af2*$^o$/Aβ*) | 0 | 0 | 0 | 0 | B: 4/5 | ND | * | ND |
| Agenesis of the iris stroma<br>(Xα*) (Xα*af2*$^o$/Aβ*; Xαaf2$^o$/Aγ*) | 0 | NA | NA | 0 | NA | 0 | NA | 0 |
| Agenesis of the anterior chamber<br>(Xα*) (Xα*af2*$^o$/Aβ*; Xα*af2*$^o$/Aγ*) | 0 | NA | NA | 0 | NA | 0 | NA | 0 |
| Agenesis of the sclera<br>(Xα*) (Xα*af2*$^o$/Aβ*; Xα*af2*$^o$/Aγ*) | 0 | NA | NA | 0 | NA | * | NA | * |
| Retrolenticular membrane (PHPV)<br>(Xα*) (Xα$^{+/-}$/Aβ$^{+/-}$) (Xα*af2*$^o$*) | * | * | U: 1/8<br>B: 2/8 | B: 1/3 | * | * | * | * |
| **Retinal defects** | | | | | | | | |
| Shortening of ventral retina<br>(Xα*) (Xα/Aγ*)‡ (Xα*af2*$^o$) (Xα*af2*$^o$/Aβ*; Xα*af2*$^o$/Aγ*) | 0 | 0 | 0 | 0 | * | * | * | ND |
| Coloboma of the optic disc<br>(Xα*) (Xα*af2*$^o$/Aβ; Xα*af2*$^o$/Aγ*) | 0 | 0 | 0 | 0 | U: 1/5 | B: 1/2 | U: 1/4<br>B: 1/4 | B: 1/2 |
| **Agenesis of Harderian glands**<br>(Xα*af2*$^o$/Aβ; Xα*af2*$^o$/Aγ*) | 0 | NA | NA | 0 | NA | B: 1/2 | NA | * |
| **Agenesis of the naso-lacrimal duct**<br>(Xα*af2*$^o$/Aβ; Xαaf2$^o$/Aγ*) | 0 | NA | NA | 0 | NA | 0 | NA | * |

Xα and Xα*af2*$^o$, these abnormalities are observed in Xα and Xα*af2*$^o$ fetuses, respectively; Xα/Aβ and Xα/Aγ, these abnormalities are observed in Xα/Aβ or Xα/Aγ double-null mutants; Xα*af2*$^o$/Aβ and Xα*af2*$^o$/Aγ, these abnormalities are observed in Xα*af2*$^o$/Aβ or Xα*af2*$^o$/Aγ double-null mutants.

*These abnormalities are completely penetrant and bilateral.

‡These abnormalities are more severe than in Xα mutants.

B, bilateral; NA, not applicable, as the corresponding structure is not yet formed at E14.5; ND, not determined, as the relationships between the different components of the malformed eye are often difficult to evaluate at this developmental stage (see Fig. 6); U, unilateral. (For further details concerning these abnormalities, see Lohnes et al., 1994; Kastner et al., 1994; Kastner et al., 1997a; Ghyselinck et al., 1997; Mascrez et al., 1998.)

trabeculae, and a lack of clear frontier between the spongiotrophoblastic and the labyrinthine zones. They were similar to those previously detected in RXRα-null placentas (Sapin et al., 1997; data not shown).

The outcome of these placental abnormalities was assessed at E18.5: Xα*af2*$^o$ placentas were macroscopically distinguishable from wild-type and Xα*af2*/$^+$ placentas by their pale appearance, which reflects a deficiency in red blood cells within the labyrinthine zone (Fig. 7a,b; data not shown). Histological analysis of E18.5 Xα*af2*$^o$ placentas ($n$=4) showed (1) an abnormal, wavy aspect of the interface between spongiotrophoblast and labyrinth (Fig. 7c,d); and (2) a thickening of the labyrinthine trabeculae separating fetal capillaries and maternal blood sinuses (Fig. 7e-h). Labyrinthine trabeculae represent the placental barrier across which nutrient and gas exchanges between fetal and maternal blood occur (reviewed by Cross, 2000). Limitation of the rate of fetal-maternal exchanges caused by a thickened placental barrier is probably responsible for in utero growth retardation of Xα*af2*$^o$ mutants (Mascrez et al., 1998).

## In absence of a RAR partner, the RXRα AF-1 domain A/B becomes essential for embryonic development

The RXRα*af1*$^o$ mutation was introduced into RAR(α, β or γ)-null genetic backgrounds to assess the functional contribution of the RXRα AF-1 domain A/B in RAR/RXR heterodimers. Steady-state levels of RXRαΔA/B in E12.5 Xα*af1*$^o$/A(α, β or γ) mutant embryos were comparable with those of RXRα in Aα-, Aβ- and Aγ-null embryos, respectively (data not shown). Therefore, the abnormal phenotypes seen in Xα*af1*$^o$/A(α, β or γ) mutants most probably reflect the lack of the RXRα A/B domain.

Aα, Aβ and Aγ mutants are viable (Kastner et al., 1995; Ghyselinck et al., 1997). In contrast, no living Xα*af1*$^o$/Aα mutants were recovered from Xα*af1*/$^+$/Aα$^{+/-}$ intercrosses, even though they could be collected at Mendelian ratio at E18.5. Similarly, only few Xα*af1*$^o$/Aβ and Xα*af1*$^o$/Aγ adults were viable (two and one obtained versus ten and seven expected, respectively). The lethality of Xα*af1*$^o$/A(α, β or γ) mutants clearly show that the RXRα AF-1 domain A/B is essential, at least in some RAR genetic settings.

Xα*af1^o*/A(α, β or γ) compound mutants were analyzed by histology at E14.5 and E18.5. Xα*af1^o*/Aα mutants displayed, with a low penetrance, the majority of the cardiovascular, respiratory and urogenital defects previously observed in Xα/Aα mutants (Tables 3, 4; Kastner et al., 1994; Kastner et al., 1997a). Similarly, Xα*af1^o*/Aβ and Xα*af1^o*/Aγ mutants reproduced a milder form of the ocular defects observed in Xα/Aβ and Xα/Aγ fetuses (Table 2; Kastner et al., 1994; Kastner et al., 1997a), including closer eyelid folds (E in Fig. 6a,c,d), thickening of the ventral portion of the cornea (C), shortening of the ventral retina (V), ventral rotation of the lens (L), coloboma of the optic disc (green arrowheads in Fig. 6d) and absence of the sclera (SC, compare Fig. 6f with 6g). Thus, in a genetic background null for either RARα, RARβ or RARγ, the RXRα A/B domain becomes indispensable for a large subset of RA-dependent functions involved in morphogenesis.

It is interesting to note that the defects observed in Xα*af1^o*/A(α, β or γ) mutants were less penetrant or less severe than those observed in Xα*af2^o*/A(α, β or γ) mutants (Mascrez et al., 1998). For example, lung hypoplasia was present in about two thirds of Xα*af1^o*/Aα mutants, whereas it is completely penetrant in Xα*af2^o*/Aα mutants. One third of Xα*af1^o*/Aβ and Xα*af1^o*/Aγ mutants displayed a coloboma of the optic disc, an abnormality observed in all Xα*af2^o*/Aβ and Xα*af2^o*/Aγ mutants. Ocular abnormalities found with the same penetrance in Xα*af1^o*/RAR-null and in Xα*af2^o*/RAR-null mutants, were less severe in Xα*af1^o*/RAR-null mutants. For example, shortening of the ventral retina, ventral rotation of the lens, thickening of the corneal stroma and closer eyelid folds, are less severe in Xα*af1^o*/Aβ and Xα*af1^o*/Aγ mutants than in Xα*af2^o*/Aβ and Xα*af2^o*/Aγ mutants (Table 2; Fig. 6c,d; for additional examples, compare Fig. 6h and 6j with 6i and 6k). Altogether, these observations indicate that, for a large fraction of the RAR-dependent events, the functions of the RXRα AF-1 domain A/B are less crucial than those of the AF-2 activity.

### RARβ2 promoter activity in Xα*af1^o* mutant mice

We also investigated the possible involvement of the RXRα AF-1 domain A/B in the regulation of a transgene, whose expression is under the control of the RARβ2 promoter that contains a RA-reponse element (Mendelsohn et al., 1991). To this end, the RARβ2 promoter-*lacZ* reporter transgene was introduced into the RXRα*af1^o* genetic background. At E13.5, *lacZ* expression was similar in Xα*af1^o* (*n*=9) and wild-type (*n*=13) fetuses (data not shown). In order to eliminate a functional compensation by RXRβ (Mascrez et al., 1998; Wendling et al., 1999), *lacZ* expression was also studied in Xα*af1^o*/Xβ mutants. At E13.5, expression of *lacZ* was similar in wild-type (*n*=13) and Xα*af1^o*/Xβ (*n*=9) fetuses, including the interdigital regions (data not shown). Altogether, these data indicate that the RXRα AF-1 domain A/B, in contrast to RXRα AF-2 (Mascrez et al., 1998), is dispensable for in vivo transactivation by RXRα, at least in the context of the RARβ2 promoter.

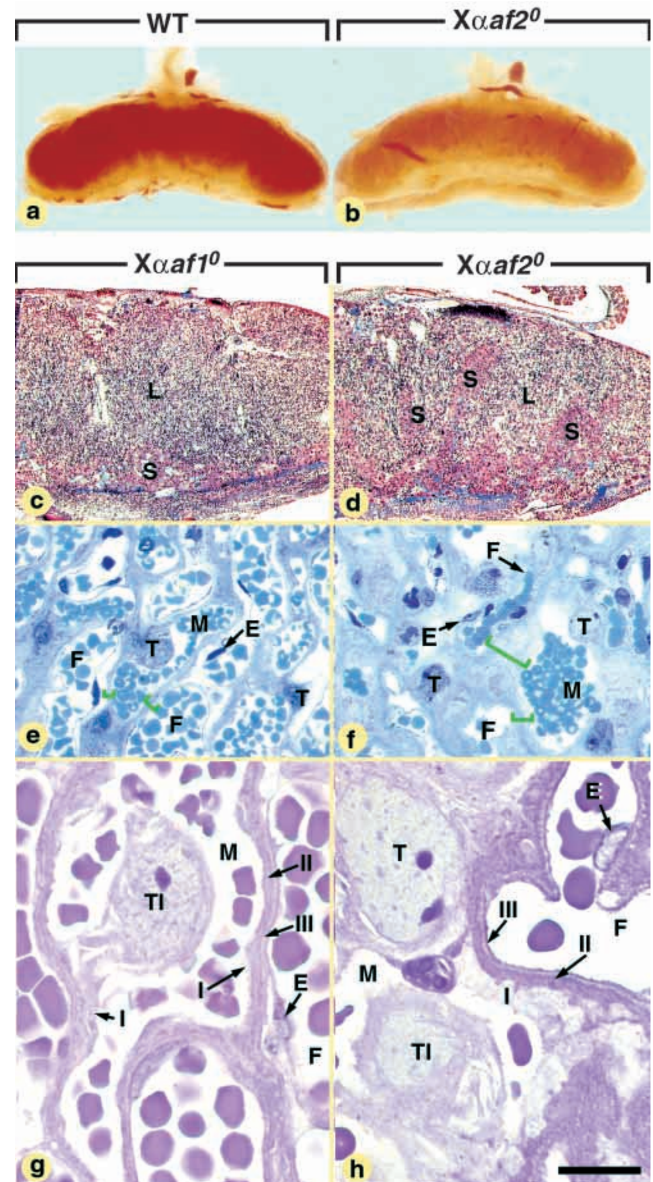


**Fig. 7.** Comparison of E18.5 wild-type (WT), Xα*af1^o* and Xα*af2^o* placentas. (a,b) Halved placentas from the same litter fixed in glutaraldehyde. (c-d) Histological sections from paraffin-embedded placentas. (e-h) Semithin sections: labyrinthine trabeculae consist of three layers of trophoblast cells (designated I, II and III counting from the maternal towards the fetal blood spaces) and of the endothelium lining fetal capillaries; the trabecular thickening in Xα*af2^o* placenta affects mainly layers I and II. E, nuclei of endothelial cells; F, fetal (allantoic) capillary; L, labyrinthine zone; M, maternal blood sinuses; S, spongiotrophoblast; T, nuclei of trophoblast cells; TI, nuclei of trophoblast cells from layer one; I, II and III, first, second and third layer of labyrinthine trabeculae. The green brackets delimit the placental barrier. Modified Mallory's trichrome (c,d), Toludine Blue (e,f) and periodic acid-schiff (g,h). (a) and (b) are displayed at the same magnification. Scale bar: 500 μm (c,d); 25 μm (e,f); 12 μm (g,h).

## DISCUSSION

The RXRα deletion created in the present study encompasses most of the N-terminal A/B domain. This region contains the autonomous ligand-independent transcriptional activation function, AF-1, which exhibits some specificity in transfected cells in vitro, depending upon the cell-type and promoter context (Nagpal et al., 1992; Nagpal et al., 1993). Little is

**Table 3. Abnormalities of the cardiovascular system, the respiratory system and certain glands in $X\alpha af1^{o}$ compound mutant fetuses**

| Abnormalities | Genotype, age and number of mutant fetuses | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $X\alpha af1^{o}$/Xβ | $X\alpha af1^{o}$/Xβ/Xγ | $X\alpha af1^{o}$/Aα | | $X\alpha af1^{o}$/Aβ | | $X\alpha af1^{o}$/Aγ | |
| | E18.5 3 | E14.5 9 | E14.5 8 | E18.5 3 | E14.5 5 | E18.5 2 | E14.5 4 | E18.5 2 |
| Cardiovascular defects | | | | | | | | |
| Ventricular myocardial hypoplasia (Xα*) ($X\alpha af2^{o}$) ($X\alpha af2^{o}$/Aα) | 0 | 1 | 4 | 1 | 0 | 0 | 0 | 0 |
| Agenesis of the conotruncal septum (14.5dpc)/high VSD (18.5dpc) (Xα) (Xα/Aα*; Xα/Aβ*; Xα/Aγ) ($X\alpha af2^{o}$) ($X\alpha af2^{o}$/Aα; $X\alpha af2^{o}$/Aβ; $X\alpha af2^{o}$/Aγ) | 0 | 1 | 2 | 1 | 0 | 0 | 0 | 0 |
| Persistent truncus arteriosus (PTA) (Xα/Aα*; Xα/Aβ; Xα/Aγ) ($X\alpha af2^{o}$/Aα) | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| Abnormal arteries derived from Ao.A.3-6 (Xα/Aα>Xα/Aβ and Xα/Aγ) ($X\alpha af2^{o}$/Aα; $X\alpha af2^{o}$/Aβ) | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Respiratory system abnormalities | | | | | | | | |
| Hypoplastic left lung (Xα/Aα) ($X\alpha af2^{o}$/Aα*; $X\alpha af2^{o}$/Aβ) | 0 | 0 | 6 | 1 | 0 | 0 | 0 | 0 |
| Hypoplastic right lung (Xα/Aα) ($X\alpha af2^{o}$/Aα*; $X\alpha af2^{o}$/Aβ) | 0 | 0 | 6 | 1 | 0 | 0 | 0 | 0 |
| Agenesis of oesophagotracheal septum (Xα/Aα>Xα/Aβ) ($X\alpha af2^{o}$/Aα; $X\alpha af2^{o}$/Aβ) | 0 | 0 | 1 (P) | 0 | 1 (P) | 0 | 0 | 0 |
| Glandular abnormalities | | | | | | | | |
| Shortening of the sublingual duct ($X\alpha af2^{o}$/Aβ*; $X\alpha af2^{o}$/Aγ*) | 0 | 0 | NA | 0 | NA | 0 | NA | * |

Xα and $X\alpha af2^{o}$, these abnormalities are observed in Xα and $X\alpha af2^{o}$ fetuses, respectively; Xα/Aα, Xα/Aβ and Xα/Aγ, these abnormalities are observed in Xα/Aα, Xα/Aβ or Xα/Aγ double-null mutants; $X\alpha af2^{o}$/Aα, $X\alpha af2^{o}$/Aβ and $X\alpha af2^{o}$/Aγ, these abnormalities are observed in $X\alpha af2^{o}$/Aα, $X\alpha af2^{o}$/Aβ or Xα*af2*/Aγ double-null mutants; AoA.3-6, third, fourth and sixth aortic arches – the abnormality consisted in an arch of the aorta on the right side.

*These abnormalities are completely penetrant and bilateral.

P, partial; NA, not applicable as the corresponding structure is not yet formed at E14.5. VSD, ventricular septal defect. (For further details concerning these abnormalities, see Mendelsohn et al., 1994; Kastner et al., 1994; Kastner et al., 1997a; Ghyselinck et al., 1997; Mascrez et al., 1998.)

known about the molecular mechanisms through which RXRα AF-1 domain A/B exerts its transcriptional activity. For other members of the nuclear receptor superfamily, e.g. PPARγ, the A/B domain has been shown to interact directly with co-activators, e.g. PGC-2 (Castillo et al., 1999). Along the same lines, phosphorylation of the A/B domain may modulate either the interaction of receptors with co-activators or their affinity for ligands. For example, phosphorylation of ERβ A/B domain promotes recruitment of the SRC-1 co-activator (Tremblay et al., 1999), whereas phosphorylation of the A/B domain reduces the ligand-binding affinity of PPARγ, thus negatively regulating the transcriptional activity of PPARγ (Shao et al., 1998). In F9 cells, phosphorylation of the RARγ A/B domain is required not only for induction of several RA-target genes (Taneja et al., 1997; Rochette-Egly et al., 1997; Bastien et al., 2000), but also for receptor degradation by the ubiquitin-proteasome pathway (Kopf et al., 2000). At least some of these features could probably be extended to RXRα, which is phosphorylated at several serine and threonine residues in the A/B domain (Adam-Stitah et al., 1999). The present investigation was designed to establish the developmental role of the RXRα A/B region, as a prerequisite to further genetic and molecular studies aimed at elucidating the molecular mechanisms that underlie physiological events in vivo.

### The AF-1 domain A/B of RXRα is required for the transduction of retinoic acid signals during development

Involution of the primary vitreous body and of the hindlimb interdigital mesenchyme, and cardiomyocyte differentiation and morphogenesis of some cranial skeletal elements and vertebrae are impaired in $X\alpha af1^{o}$ mutants (this study), in RAR(α, β and γ) mutants (Lohnes et al., 1993; Ghyselinck et al., 1997; Kastner et al., 1997b) and in animals with vitamin A and RA deficiency (Wilson et al., 1953; Lussier et al., 1993). It appears therefore that in RXRα/RAR heterodimers, the AF-1 domain A/B of RXRα participates in the transduction of retinoid signals normally required for the completion of these developmental events. The observation that the developmental defects exhibited by the $X\alpha af1^{o}$/Xβ/Xγ mutants can all be attributed to abnormalities in the RAR/RXR signaling pathway suggest that RXR partners other than RAR do not exert any developmental function that require the RXRα A/B region.

During development, RXRα is the most important RXR, as Xα fetuses die in utero and display severe cardiac and ocular defects, whereas Xβ/Xγ mutants are viable and do not exhibit any congenital defects (Kastner et al., 1994; Krezel et al., 1996). However, a lack of RXRα or its AF-2 activity can be functionally compensated by RXRβ and RXRγ in mouse embryos, as the spectrum of defects observed in Xα/Xβ and $X\alpha af2^{o}$/Xβ/Xγ mutants is much broader than that of Xα mutants (Mascrez et al., 1998; Wendling et al., 1999). RXRβ and RXRγ also compensate for the deletion of RXRα A/B domain, as the PHPV and the hindlimb interdigital webbing become completely penetrant and eventually more severe in $X\alpha af1^{o}$/Xβ/Xγ mutants; however, no additional abnormalities are generated in these latter mutants, except for single cases of myocardial hypoplasia and conotruncal septum agenesis. Thus,

**Table 4. Abnormalities of the urogenital tract in Xα*af1*$^o$ compound mutant fetusess**

| | Mutant genotype, age and number (male:female) of mutant fetuses | | | | | |
|---|---|---|---|---|---|---|
| | Xα*af1*$^o$/Aα | | Xα*af1*$^o$/Aβ | | Xα*af1*$^o$/Aγ | |
| Abnormalities | E14.5 4:4 | E18.5 1:2 | E14.5 3:2 | E18.5 1:1 | E14.5 1:3 | E18.5 0:2 |
| Kidney abnormalities | | | | | | |
| Agenesis (Xα/Aα) (Xαaf2$^o$/Aα) | U: 2/8 B: 1/8 | 0 | 0 | 0 | 0 | 0 |
| Hypoplasia (Xα/Aα) (Xα*af2*$^o$/Aα; *X*αaf2$^o$/Aγ*)* | U: 2/8 B: 2/8 | B: 1/3 | 0 | 0 | 0 | 0 |
| Hydronephrosis (Xα*af2*$^o$/Aβ*) | NA | NA | NA | 1/2 | NA | NA |
| Ureter abnormalities | | | | | | |
| Agenesis of ureter, partial or complete (Xα/Aα; Xα/Aβ) (Xα*af2*$^o$/Aβ; Xα*af2*$^o$/Aγ) | U: 2/8 B: 1/8 | 0 | 0 | 0 | 0 | 0 |
| Agenesis of the Müllerian duct (14.5 dpc) or of its derivatives (18.5 dpc females) | | | | | | |
| Complete (Xα/Aα*) | 4/8 | 0 | 0 | 0 | 0 | 0 |
| Partial (caudal portion missing) (Xα/Aβ; Xα/Aγ) (Xα*af2*$^o$/Aα*; Xα*af2*$^o$/Aβ) | U: 1/8 B: 3/8 | 0 | B: 1/5 | 0 | 0 | 0 |

Xα and Xα*af2*$^o$, these abnormalities are observed in Xα and Xα*af2*$^o$ fetuses, respectively; Xα/Aα, Xα/Aβ and Xα/Aγ, these abnormalities are observed in Xα/Aα, Xα/Aβ or Xα/Aγ double-null mutants; Xα*af2*$^o$/Aα, Xα*af2*$^o$/Aβ and Xα*af2*$^o$/Aγ, these abnormalities are observed in Xα*af2*$^o$/Aα, Xα*af2*$^o$/Aβ or Xα*af2*$^o$/Aγ double-null mutants.

*These abnormalities are completely penetrant and bilateral; B, bilateral; NA, not applicable: hydronephrosis was never observed in any of our previous mutants at this developmental stage; U, unilateral. (For details see Mendelsohn et al., 1994; Kastner et al., 1994; Kastner et al., 1997a; Ghyselinck et al., 1997; Mascrez et al., 1998.)

the functional compensation by RXRβ or RXRγ cannot account for the 'weak' phenotype of the Xα*af1*$^o$ mutants.

Two scenarios could account for the paucity of the developmental defects in Xα*af1*$^o$ and Xα*af1*$^o$/Xβ/Xγ mutants. First, the A/B domain might in fact be dispensable for the majority of the developmental events that require RXRα. Given the phenotypes of the various Xα*af1*$^o$/RAR(α, β or γ) mutants (see below and Tables 2-4), this possibilty is unlikely. Alternatively, the RXRα A/B domain might be necessary for optimal transactivation of several developmental target genes, through RXRα/RAR heterodimers. According to this second scenario, the contribution of the RXRα A/B domain to the transactivation potential of the heterodimers might not be detectable in the protected environment of the animal facility, unless the phenotyping techniques are sophisticated enough, or the examined sample size becomes very large (Brookfield, 1992; Thomas, 1993). Our findings that one out of nine Xα*af1*$^o$/Xβ/Xγ mutants displays an hypoplasia of the ventricular myocardium and that another one of these nine has an agenesis of the conotruncal septum, defects that are characteristic of the RXRα-null phenotype but have never been observed in the several dozens of wild-type embryos that we have analyzed over the past 10 years, support the second scenario (Kastner et al., 1994; Sucov et al., 1994). The requirement of RXRα A/B domain for myocardial growth or fusion of conotruncal ridges in some fetuses might then reflect stochastic inter-individual variations in the expression of other synergistic factors involved in RA-dependent cardiac morphogenesis. This hypothesis predicts that more VAD-related developmental abnormalities might be detected if a larger sample of Xα*af1*$^o$/Xβ/Xγ fetuses were examined.

Other evidence supporting the conclusion that the RXRα AF-1 domain A/B could be necessary for transactivation by the various RXRα/RAR heterodimers is suggested by the observation that the corresponding Xα*af1*$^o$/RAR(α, β or γ) mutants display most of the VAD-related defects observed in the cognate Xα/RAR(α, β or γ) double mutants (Kastner et al., 1994; Kastner et al., 1997a). In fact, in the absence of a given RAR, the RXRα A/B domain becomes essential for enabling the remaining RARs to functionally replace the missing one (discussed by Kastner et al., 1997a; Chiba et al., 1997a; Chiba et al., 1997b; Mascrez et al., 1998; see Tables 2-4). Altogether, these data favor the involvement of RXRα AF-1 domain A/B in most of the developmental events that are mediated through the various RAR/RXRα heterodimers, under conditions where either RAR and/or retinoid levels become limited.

### The RXRα AF-1, but not the AF-2 activity appears dispensable for placentation

Thickening of the labyrinthine trabeculae and lack of definite frontier between the labyrinth and spongiotrophoblast are hallmark features of RXRα$^{-/-}$ placentas, and probably reflect a failure of a late step of trophoblast cells differentiation (Sapin et al., 1997). Our data show that the RXR A/B domain appears largely unnecessary for the differentiation of trophoblast cells. In contrast, the AF-2 of RXRα is clearly essential for this differentiation process, as (1) Xα*af2*$^o$ placentas are histologically indistinguishable from placentas that lack RXRα; and (2) defects of Xα*af2*$^o$/Xβ/Xγ placentas are not more severe than in Xα*af2*$^o$. Furthermore, as Xα*af2*$^o$/RAR(α, β or γ) mutants are obtained at the expected Mendelian ratio at E18.5, and are not more growth deficient than Xα*af2*$^o$ mutants (B. M., M. M., N. B. G. and P. C., unpublished), it is likely that terminal differentiation of labyrinthine trophoblast cells involves heterodimers in which RXRα is transcriptionally active, but distinct from RXRα/RAR heterodimers. On the other hand, the formation of the labyrinthine zone of the placenta is totally inhibited in Xα/Xβ mutants, and severely impaired in PPARγ mutants, leading to embryonic death before E10.5 (Wendling et al., 1999; Barak et al., 1999). Thus, RXRα and RXRβ, probably in the form of PPARγ/RXR heterodimers, have an early role in placentogenesis. We have shown that

Xα*af1^o*/Xβ/Xγ and Xα*af2^o*/Xβ/Xγ mutants do not display these early defects. Thus, neither the RXRα AF-1 domain A/B nor the AF-2 activity is required on their own in early placentogenesis, suggesting that RXRs could act in this process as silent partners with PPARγ. Studies with RXRα mutants that lack both AF-1 and AF-2 are in progress to investigate this possibility further.

### The RXRα AF-1 domain A/B is specifically required for involution of the interdigital mesenchyme

Among the various RA-dependent morphogenetic events involving the RXRα AF-1 domain A/B, separation of the digits is the only one that specifically requires this region, as Xα*af1^o* and Xα*af1^o*/Xβ/Xγ mutants display soft tissue syndactyly of the hindlimbs, whereas Xα*af2^o* and Xα*af2^o*/Xβ/Xγ mutants never display this defect (Mascrez et al., 1998). Phosphorylation of RXRα at a specific serine residue located in the A/B domain is necessary for the antiproliferative response of F9 teratocarcinoma cells to RA (Rochette-Egly and Chambon, 2001). It is noteworthy that RA-dependent involution of the interdigital mesenchyme results from arrest of cell proliferation, as well as from apoptosis (Dupé et al., 1999). Therefore, the possibility exists that phosphorylation of the RXRα A/B domain might have important functions in the cascade of molecular events that, in vivo, leads to the normal disappearance of the interdigital mesenchyme.

### The AF-1 and AF-2 activities of RXRα are differentially required for transducing retinoic acid signals during development

AF-1 and AF-2 activities do not have the same importance in the transcriptional activity of RXRα during embryonic development: (1) Xα*af2^o*/Xβ/Xγ fetuses display a large array of congenital defects not found in Xα*af1^o*/Xβ/Xγ fetuses; and (2) Xα*af2^o*/RAR(α, β or γ) compound fetuses are more severely affected than Xα*af1^o*/RAR(α, β or γ) fetuses (Mascrez et al., 1998; see Tables 2-4). Moreover, the AF-2, but not the AF-1 of RXRα is crucial for the transcription of the RARβ2-*lacZ* transgene (Mascrez et al., 1998; this study). At the molecular level, little is known about the mechanism through which AF-1 activates transcription. In the case of ERα, AF-1 and AF-2 appear to interact simultaneously in vitro with distinct interfaces of the TIF2 co-activator, leading to synergistic activation of transcription (Benecke et al., 2000). Our present data showing that similar abnormalities are exhibited by Xα*af1^o*/RAR(α, β or γ) and Xα*af2^o*/RAR(α, β or γ) compound mutants, although more severe in the latter case, not only suggest that RXRα AF-1 and AF-2 may synergize in vivo as they do in vitro (Nagpal et al., 1993), but also that AF-2 is more important than AF-1 for most of the developmental events that require RXRα/RAR heterodimers.

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