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The RXRα ligand-dependent activation function 2 (AF-2) is important for mouse development

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SUMMARY

We have engineered a mouse mutation that specifically deletes the C-terminal 18 amino acid sequence of the RXRα protein. This deletion corresponds to the last helical α structure (H12) of the ligand-binding domain (LBD), and includes the core of the Activating Domain of the Activation Function 2 (AF-2 AD core) that is thought to be crucial in mediating ligand-dependent transactivation by RXRα. The homozygous mutants (RXRα*af2*^{o}), which die during the late fetal period or at birth, exhibit a subset of the abnormalities previously observed in RXRα$^{-/-}$ mutants, often with incomplete penetrance. In marked contrast, RXRα*af2*^{o}/RXRβ$^{-/-}$ and RXRα*af2*^{o}/RXRβ$^{-/-}$/RXRγ$^{-/-}$ compound mutants display a large array of malformations, which nearly recapitulate the full spectrum of the defects that characterize the fetal vitamin A-deficiency (VAD) syndrome and were previously found in RAR single and compound mutants, as well as in RXRα/RAR(α, β or γ) compound mutants. Analysis of RXRα*af2*^{o}/RAR(α, β or γ) compound mutants also revealed that they exhibit many of the defects observed in the corresponding RXRα/RAR compound mutants. Together, these results demonstrate the importance of the integrity of RXR AF-2 for the developmental functions mediated by RAR/RXR heterodimers, and hence suggest that RXR ligand-dependent transactivation is instrumental in retinoid signalling during development.

Key words: Retinoid receptor, RAR-RXR heterodimers, Compound mutants, Ligand-dependent transactivation, Functional redundancy, RARβ2 promoter, Mouse

INTRODUCTION

Vitamin A (retinol) is crucial for many aspects of vertebrate physiology (Wolbach and Howe, 1925; Sporn et al., 1994; Blomhoff, 1994). Retinoids [the active metabolites of vitamin A, most notably retinoic acid (RA)] have been shown to regulate complex gene networks involved in morphogenesis, organogenesis, growth, cellular differentiation and homeostasis (reviewed in Lotan, 1980; Chambon, 1994; Gudas et al., 1994; Kastner et al., 1995). Two families of receptors that belong to the superfamily of nuclear receptors, the retinoic acid receptors isotypes (RARα, RARβ and RARγ, and their isoforms) and the retinoid X receptor isotypes (RXRα, RXRβ, RXRγ, and their isoforms) are implicated in the transduction of the RA signal. Both RARs and RXRs act in transfected cells in vitro as ligand-dependent transcriptional transregulators through binding to specific *cis*-acting RA response elements (RAREs) of target genes. Either all-*trans* or 9-*cis* RA can bind and activate RARs, whereas RXRs bind and are activated by 9-*cis* RA only. Moreover, RXRs readily heterodimerize with RARs and these heterodimers bind in vitro to and transactivate in transfected cells from RAREs much more efficiently than RARs on their own. This suggested that RXR/RAR heterodimers might be the functional units transducing the retinoid signal in vivo (reviewed in Leid et al., 1992; Giguère, 1994; Chambon, 1994, 1996; Mangelsdorf et al., 1995; Mangelsdorf and Evans, 1995; Gronemeyer and Laudet, 1995; Perlmann and Evans, 1997). This possibility was supported by the synergistic effects of RXR- and RAR-selective synthetic retinoids on proliferation, apoptosis and/or differentiation of a variety of cultured cell lines (see below for references). Single and compound knock-outs of RXRα, RARα and RARγ genes established the functionality of RXR/RAR heterodimers in the RA-responsive F9 embryonal carcinoma (EC) cell line and furthermore demonstrated that different combination of RXR and RAR isotypes are differentially involved in the control of RA-induced cellular responses, as well as in the regulation of responsive gene expression (Boylan et al., 1993, 1995; Clifford et al., 1996; Chiba et al., 1997a,b).

To determine whether RARs and RXRs and their heterodimers are involved in the transduction of retinoid signals under physiological conditions, all RAR and RXR isotype genes were knocked-out in the mouse to yield single and compound mutants. This led to several important conclusions. Firstly, RARs mediate the developmental functions of retinoids in vivo, as, altogether, RARα/RARβ, RARα/RARγ and RARβ/RARγ double null

mutants recapitulate all the vitamin A deficiency (VAD)-induced defects characteristics of the fetal VAD syndrome (Lohnes et al., 1994; Mendelsohn et al., 1994; Luo et al., 1996; Ghyselinck et al., 1997 and references therein; for review see Kastner et al., 1995). Furthermore, most of these defects are not exhibited by single RAR isotype mutants, indicating that there is a marked functional redundancy between RARs that could possibly be artefactually generated by the knock-outs (see Kastner et al., 1995, 1997a; Taneja et al., 1996). Secondly, it was concluded that RXRs are also implicated in the retinoid signalling that involves RARs, as (i) RXRα null mutants display the cardiac and ocular defects characteristic of the fetal VAD syndrome (Kastner et al., 1994; Sucov et al., 1994; Dyson et al., 1995; Gruber et al., 1996), and (ii) compound mutants in which a RAR(α, β or γ) null mutation is associated with a RXRα null mutation, altogether recapitulate most of the abnormalities of the VAD syndrome, as well as those exhibited by RAR double mutants (Kastner et al., 1994, 1997a). These observation strongly supported the notion that RXR/RAR heterodimers are the functional units that transduce retinoid signals in vivo. Thirdly, RXRα appears to be the functionally most important RXR during development, as RXRβ/RXRγ double null mutants develop normally and the growth-deficient $RXR\alpha^{+/-}/RXR\beta^{-/-}/RXR\gamma^{-/-}$ mutants do not exhibit any overt developmental abnormalities, while severe developmental defects are synergistically generated in double mutants in which a RAR(α, β or γ) mutation is associated with a RXRα, but not a RXRβ or RXRγ mutation (Kastner et al., 1994, 1997a; Krezel et al., 1996, 1998).

There is therefore very little doubt that RXR/RAR heterodimers are crucial for the physiological transduction of the retinoid signal during mouse development. However, the physiological role of each of the two partners in ligand-dependent transactivation by RXR/RAR heterodimers remains unclear under physiological conditions for several reasons. Initially, some (Kurokawa et al., 1994; Valcarcel et al., 1994; Forman et al., 1995), but not all (Durand et al., 1994), studies in vitro and in transfected cells led to the conclusion that RXR cannot bind its ligand within RXR/RAR heterodimers. Therefore it was proposed that RXR might simply serves as an auxiliary DNA-binding factor for RAR, as its ligand-dependent activation function 2 (AF-2) located in the ligand-binding domain (LBD) (Nagpal et al., 1992) would remain inactive (for review see Leblanc and Stunnenberg, 1995). In contrast, subsequent studies demonstrated that an RXR ligand can bind to RXR/RAR heterodimers, irrespective of the ligand-binding status of the RAR partner (Apfel et al., 1995; Kersten et al., 1996; Chen et al., 1998). Moreover, synergistic transactivation by RAR- and RXR-selective ligands could be observed in a variety of cells cultured in vitro, particularly when the RAR ligand concentration was suboptimal (Apfel et al., 1995; Lotan et al., 1995; Roy et al., 1995; Chen et al., 1996; Clifford et al., 1996; Horn et al., 1996; La Vista-Picard et al., 1996; Nagy et al., 1996; Taneja et al., 1996; Botling et al., 1997; Chiba et al., 1997a,b; Defacque et al., 1997; Giannini et al., 1997; Minucci et al., 1997; Joseph et al., 1998). However, in all cases, the liganded RXR was transcriptionally inactive unless its RAR partner was liganded. It has been proposed that this intraheterodimeric subordination of the RXR AF-2 activity to the binding of the RAR ligand could be due to an allosteric effect of the unliganded RAR on its liganded RXR partner. Thus the formation of an 'active' RXR AF-2 interaction surface for co-activators would be prevented (Vivat et al., 1997), accounting for the observation that, within RXR-RAR heterodimers, RXR signalling can only operate through synergy with RAR ligands. A similar subordination within heterodimers between RXRs and either TR or VDR may prevent RXR ligands on their own promiscuously influencing thyroid hormone and vitamin D3 signalling (see Vivat et al., 1997). However, RXR signalling might be allowed in certain 'permissive' heterodimers in which RXR activity is apparently not subordinated to its partner (e.g. RXR/NGFI-B, RXR/PPAR, RXR/LXR; Forman et al., 1995; Perlmann and Jansson, 1995; Mukherjee et al., 1997). Thus, assuming that endogenous NGFI-B, PPAR and LXR ligands or weak constitutive activities of these receptors are ruled out in these latter studies, the repertoire of heterodimers transducing retinoid signals could extend well beyond RXR/RAR heterodimers (see Vivat et al., 1997).

The RXR ligand-activated AF-2 activity critically requires a conserved amphipathic α-helix (helix 12; Bourguet et al., 1995) that contains a motif highly conserved in all transactivating nuclear receptors (the core of the AF-2 activating domain or AF-2 AD core; see Chambon, 1996). Helix 12 is mandatory for interactions between 9-*cis* RA-liganded RXRα and putative co-activators, as it is involved in the receptor transconformation that is triggered upon ligand binding and creates the receptor surface required for these interactions, and therefore for AF-2 activity (Renaud et al., 1995; Chambon, 1996; Wurtz et al., 1996; Moras and Gronemeyer, 1998; and references therein). However, the deletion of RXR helix 12 does not significantly affect its ability to bind 9-*cis* RA, to form heterodimers with RARs, to bind to DNA response elements as either homodimers or heterodimers in vitro, and to transactivate through its N-terminal AF-1 activity (Nagpal et al., 1993; Durand et al., 1994; Zhang et al., 1994; Leng et al., 1995 and unpublished results from our laboratory).

To investigate the role played by RXR AF-2 activity in vivo, we have engineered here a mouse mutant line expressing a truncated RXRα (RXRαAF2^{o}) lacking the C-terminal helix 12. The study of these mice demonstrate that RXR AF-2 is of paramount importance for the developmental functions of RXR/RAR heterodimers. As the binding of a ligand (presumably 9-*cis* RA) is most probably required for RXR AF-2 activity, our study also suggests that a ligand activation of RXR is physiologically required for a number of RA-dependent developmental events.

MATERIALS AND METHODS

Targeting vector

Genomic clones containing the 3′ part of the mouse RXRα locus were obtained by screening a genomic library established in λEMBL3 from 129/sv mouse DNA with a mRXRα cDNA probe (Kastner at al., 1994). Before assembling the final targeting vector for homologous recombination (HR), an intermediary plasmid was generated (named pHR49b), in which two successive alterations were introduced by site-directed mutagenesis into a 2.5 kb RXRα genomic *Xba*I-*Kpn*I fragment containing exon 10 subcloned into Bluescript SK^{-}. The first mutagenesis, which resulted in a deletion within the sequences encoding the 18 C-terminal RXRα amino acids and introduced 2 stop codons, as well as *Spe*I, *Eco*RI and *Nco*I restriction sites (see Fig. 1), was performed with the oligonucleotide 5′-AGCTCA-TCGGGGACTAGTGAATTCCATGGAGGCACCACATCAAGC-3′. The second mutagenesis introducing *Bam*HI and *Nhe*I restriction sites

into the intron preceding exon 10 was performed with the oligonucleotide 5′-AGACCAGGTTCTGGTCTCGACGGATCC-AAAGCTAGCGAGGCACTGGGCTTGGC-3′ (original intronic sequences underlined; the 5′ 'A' is located 136 bp upstream of the beginning of exon 10). The targeting vector was constructed as follows. The 5.5 kb *Eco*RI-*Bam*HI RXRα genomic fragment containing exons 9 and 10 was inserted between *Eco*RI and *Bam*HI in pHR57 (modified from BSK$^-$, and containing a *Sac*I-*Not*I-*Eco*RI-*Bam*HI-*Sfi*I-*Pme*I polylinker), and the *Bam*HI site was destroyed by fill-in. The *Xba*I-*Kpn*I fragment was exchanged with the cognate fragment from pHR49b containing the modifications. The final vector was obtained by inserting the *Bam*HI-*Xba*I fragment from pHR56 (which contains a TK-NEO fusion gene flanked by two loxP sites, Metzger et al., 1995) into the engineered intronic *Bam*HI and *Nhe*I sites. This construct (pHR76) was linearized with *Not*I prior to electroporation into H1 ES cells (established in our laboratory) as previously described (Lufkin et al., 1991). After selection with G418, 113 resistant clones were expanded, their genomic DNA was prepared, restricted with *Nco*I and analyzed by Southern blotting with probe A (Fig. 1b,c). To demonstrate HR, the two positive clones (VG30 and VG106) were further analyzed after *Kpn*I digestion with probe A (not shown), *Bam*HI digestion with probes B, C and NEO, and after *Spe*I digestion with probes A, C and NEO (not shown). To delete specifically the floxed TK-NEO cassette, 4.10^6 VG30 ES cells were electroporated with 15 μg of a supercoiled Cre-encoding plasmid (Gu et al., 1991). Colonies were isolated at day 5-6 and expanded. Excision of the selectable marker was identified by Southern-blot analysis with probe A and *Nco*I digest (Fig. 1b,c). 9 out of 48 clones positive for excision were further analyzed by BamHI digest with probe C (not shown) and NEO (Fig. 1b,c). Cells from 3 positive clones were injected into C57BL/6 blastocysts and one clone (TS15) yielded a male chimera that transmitted the mutation through crosses with C57BL/6 females. Cells from the VG106 clone were directly injected into blastocysts to produce chimeras. Germline transmission was obtained from one chimeric male crossed with C57BL/6 females. The heterozygous mice still containing the floxed TK-NEO cassette were crossed with homozygous CMV-Cre transgenic mice ($Cre^{+/+}$, Dupé et al., 1997). The selectable marker was excised in all littermates obtained from these crosses (VG106.1 mice). Heterozygous offspring of both TS15 and VG106.1 mouse lines were subsequently back-crossed with 129/SV mice.

Western blot analysis

Nuclear extracts from whole E12.5 embryos were prepared according to Andrews and Faller (1991), 15 μg of protein were separated on 10% gel by SDS-PAGE and transferred onto nitrocellulose membranes. RXRα and RXRαAF2^o proteins were detected with the anti-mRXRα polyclonal antibody RPRXα(A) (used at a 1/1000 dilution, Rochette-Egly et al., 1994) and horseradish peroxidase-linked goat anti-rabbit immunoglobulin (Jackson Research Laboratories) that was revealed by chemoluminescence according to the manufacturer's (Amersham) protocol.

Histological and skeletal analyses

Mouse embryos and fetuses were fixed in Bouin's fluid, processed for Paraplast embedding, serially sectioned (7 μm) and stained with Groat's hematoxylin and Mallory's trichrome (Mark et al., 1993). For whole-mount skeletal analysis, fetuses were collected at E18.5 and stored at –20°C. Skeletons were prepared as described (Lufkin et al., 1992).

RESULTS

Targeted mutation of RXRα AF-2 and generation of mutant mice

A targeting vector was designed to generate a RXRα mutant allele *(af2)* encoding a protein lacking the 18 last amino acids (position 450 to 467) which include helix 12 and the AF-2 AD core (hereafter designated RXRαAF2^o). Upon homologous recombination in ES cells, a stop codon was introduced within exon 10 at amino acid position 450, while a floxed TK-NEO cassette was inserted into intron 9. Cre-mediated excision of the selection marker yielded the mutant allele RXRα*af2* (see Fig. 1a-c, and Materials and Methods). Two lines of mutant mice derived from independent ES cell clones were obtained (TS15 and VG106.1). The mutation was confirmed by sequencing RT-PCR products amplified from RNA of homozygous embryos (data not shown). Using nuclear extracts from whole 12.5 days post-coitum (12.5 dpc or E12.5) embryos, the RXRαAF2^o protein was detected in homozygotes as a single species migrating slightly faster than wild-type (WT, +/+) RXRα, while expressed at a similar level (Fig. 1d, compare lanes 1 and 3). Accordingly, mutant RXRαAF2^o and WT proteins were present at identical levels in heterozygotes (Fig. 1d, lane 2, and data not shown).

We describe below the effects of the RXRα*af2* mutation in single mutant mice, as well as in compound mutants bearing an additional mutation in the RXRβ and/or RXRγ genes, or in either the RARα, RARβ or RARγ genes. For the sake of simplicity, homozygote and heterozygote mutants bearing the RXRα mutation are designated as Xα*af2^o* (for RXRα*af2^o*) and Xα*af2*/+, while RXR (α, β and γ) and RAR (α, β and γ) homozygote null mutants are designated as Xα, Xβ and Xγ, and Aα, Aβ and Aγ, respectively, the '$^{-/-}$' sign indicating homozygotes being omitted. For example, RXRα*af2^o*/RARα$^{-/-}$ mutants are referred to as Xα*af2^o*/Aα. All data presented here correspond to mouse mutants derived from the TS15 ES cell line, but the phenotypes of mutants derived from the VG106.1 line were identical.

Lethality of the RXRα*af2^o* mutation

Among 506 mice born from Xα*af2*/+ intercrosses, a single Xα*af2^o* homozygote mutant was alive at postnatal day 8 and reached adulthood (Table 1; two additional adult Xα*af2^o* mutants were recovered from more complex crosses, e.g. Xα*af2*/+/Aα$^{+/-}$ x Xα*af2*/+/Aα$^{+/-}$). Caesarean delivery at E18.5 yielded Xα*af2^o* mutants from Xα*af2*/+ intercrosses. However, their number was lower than predicted from Mendelian distribution (Table 1), indicating that a fraction of RXRα*af2^o* mutants died earlier. Moreover, their weight was reduced by ~30% when compared with their WT littermates (Fig. 2). When left on the bench, some of these E18.5 Xα*af2^o* mutants died within minutes, despite visible efforts to breathe, whereas the others died within 12 hours, in contrast to WT pups who lived for at least 24 hours. Histological analysis of four E18.5 Xα*af2^o* fetuses did not reveal any obvious lethal abnormalities. No apparent deficit of RXRα*af2^o* fetuses was noticed at E12.5-E14.5 (Table 1). However, some of the E14.5 homozygote mutants examined appeared oedematous and were probably undergoing heart failure (see below and Table 2). Thus, the RXRα*af2^o* mutation is lethal, but with great individual variations, as approximately one third of the mutants died in utero between E14.5 and E18.5, while the others died at birth or shortly afterwards. In contrast, all RXRα null mutants die in utero between E11.5 and E16.5 (Kastner at al., 1994; Sucov et al., 1994).

RXRα*af2^o* mutants exhibit, with a lower penetrance, most of the abnormalities observed in RXRα null mutants, together with some additional defects

Only 1 out of 12 Xα*af2^o* mutants analysed histologically at

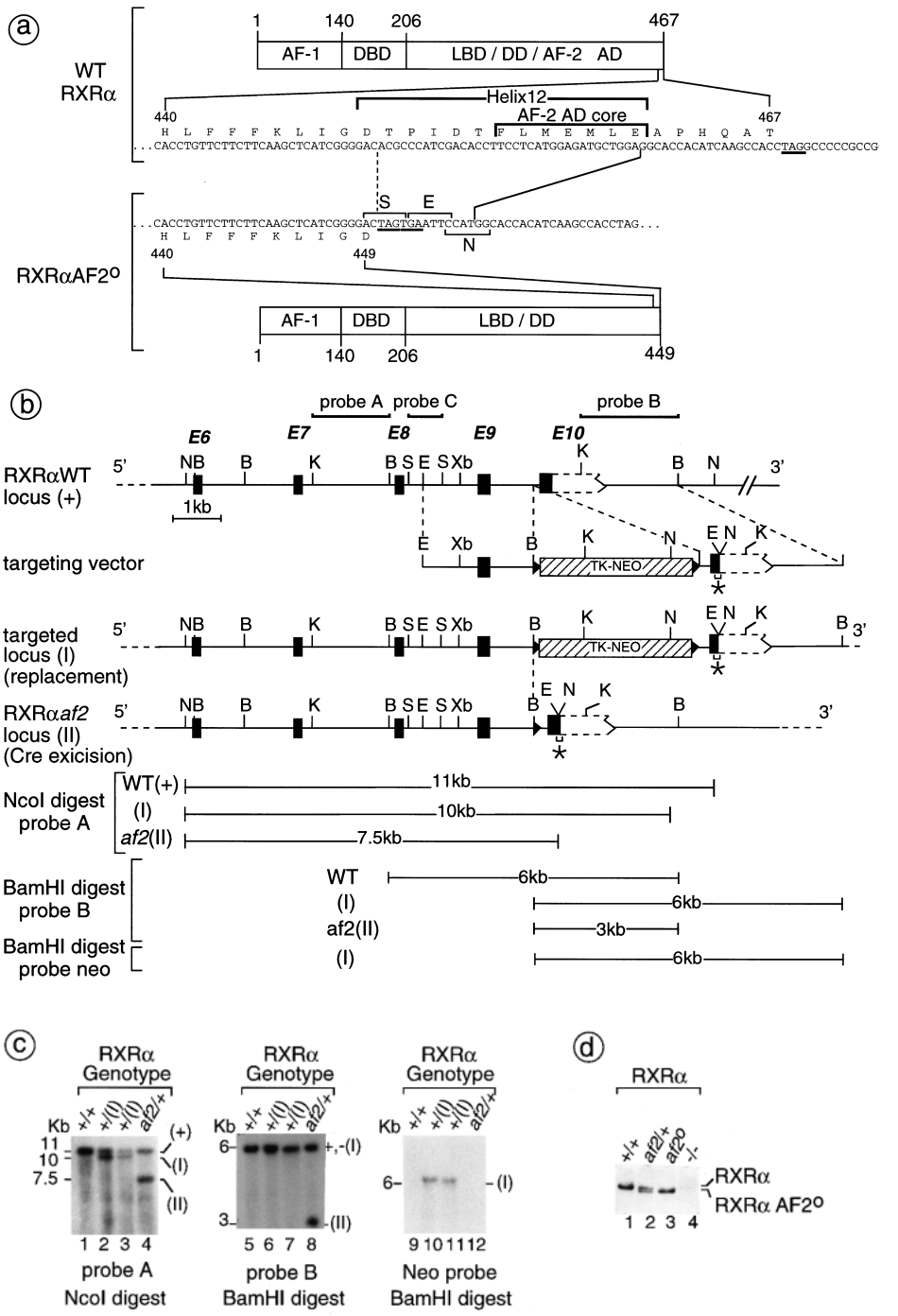

Fig. 1. Targeted deletion of sequences encoding helix 12. (a) Representations of wild type (WT) and mutant RXRα cDNA and protein. The sequence of the RXRα cDNA region of interest is shown. The functional domains are indicated: AF-1, activation function 1; AF-2 AD, activation function 2 activating domain; DBD, DNA-binding domain; LBD, ligand-binding domain; DD, dimerisation domain. The RXRα*af2* mutation corresponds to the deletion of 35 nucleotides encoding helix 12 which contains the AF-2 AD core and to the introduction of two underlined stop codons after codon 449, thus leading to the production of a RXRα protein, C-terminally truncated at aa 449 (RXRαAF2^o). *Spe*I(S), *Eco*RI(E) and *Nco*I(N) restriction sites were introduced to allow detection of the mutant locus. (b) Diagram showing the WT RXRα locus, the targeting construct, and the mutated loci obtained after replacement targeting [(I)], and subsequent CRE-mediated excision [(II)] of the floxed TK-NEO cassette. The star corresponds to the mutation described in (a). Note that the genomic organisation of RXRα gene 3′UTR sequences is unknown; thus exon 10 is depicted as a broken box on its 3′ side. Boxes indicate exons (E6 to E10; our unpublished results). LoxP sites flanking the TK-NEO cassette are represented by arrowheads. Probe A, probe B and probe C are 1.7 kb *Kpn*I-*Bam*HI fragment, 1.9 kb *Kpn*I-*Bam*HI fragment and 0.7 kb *Sac*I-*Sac*I fragment, respectively. The size of the restriction fragments that allow separation of the WT and targeted loci (I) and (II) using Southern blot analysis with probes A, B and neo, are indicated below (in kilobases). B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; N, *Nco*I; S, *Sac*I; Xb, *Xba*I. (c) Southern blotting of WT (+/+) (lanes 1, 5, 9) and RXRα*af2* mutant alleles before (+/(I))(lanes 2, 6, 10 for VG106 clone; lanes 3, 7, 11 for VG30 clone) and after (*af2*/+) (lanes 4, 8, 12 for TS15 clone) excision of the loxP-flanked TK-NEO cassette (see Materials and Methods). ES cell DNA was digested with *Nco*I or *Bam*HI and analyzed by Southern blotting with probe A, probe B and a neomycin probe (neo) as indicated (see b). (d) Detection of the RXRαAF2^o protein. Nuclear extracts prepared from WT (+/+, lane 1), *af2* heterozygote (*af2*/+, lane2), *af2* homozygote (*af2^o*, lane 3) and RXRα total null mutant (–/–, lane 4) 12.5-day-old embryos were analyzed by western blotting with the anti-RXRα polyclonal antibody RPRXα(A), directed against an N-terminal epitope.

E14.5 exhibited a ventricular myocardium hypoplasia similar to that observed in almost all RXRα null (Xα) mutants (Kastner et al., 1994, 1997b; see Table 2). Only 1 out of 6 Xα*af2^o* embryos examined at E9.5 displayed a Xα mutant-like ventricular myocardium consisting of a single layer of elongated and loosely associated subepicardial myocytes, of which a high proportion (70%) was precociously differentiated (data not shown; see Kastner et al., 1997b). However, ~20% of the subepicardial myocytes of the five other E9.5 Xα*af2^o* embryos exhibited a premature differentiation and contained sarcomeres never seen in their WT littermates (data not shown). Agenesis of the conotruncal septum, seen in ~30% of E14.5 Xα mutants (Kastner et al., 1994, 1997a), was also detected in 3 out of 12 E14.5 Xα*af2^o* mutants; however, as 2 of these mutants were selected on the basis of their oedematous appearance (see legend to Table 2), the real incidence of this

Table 1. Viability of RXRα*af2* mutants

	Genotype and number of animals			
	+/+	*af2*^{0}/+	*af2*^{0}	Total
E9.5	6 (26%)	10 (44%)	7 (30%)	23
E12.5	19	61	26	106
E13.5	14 (26%)*	24 (48%)*	20 (26%)*	58
E14.5	55	73	40	168
E18.5	96 (26%)	212 (58%)	62 (17%)	366
Adult	192 (38%)	313 (62%)	1 (0.2%)	506

*Average distribution for E12.5, E13.5 and E14.5 animals.

defect was probably lower. Thus, Xα*af2*^{o} mutants exhibit Xα-like cardiac defects, albeit with a reduced penetrance and severity.

All E14.5 (*n*=12), as well as E18.5 (*n*=4) and adult (*n*=3) Xα*af2*^{o} mutants displayed a fully penetrant and bilateral persistent hyperplastic primary vitreous body (PHPV, retrolenticular membrane, R in Fig. 3b; Table 3; and data not shown). This defect, which is the most frequent abnormality in VAD fetuses (Wilson et al., 1953), was previously found in all Xα mutants (Kastner et al., 1994) and in a majority of Aβ (Ghyselinck et al., 1997) and Xα$^{+/-}$/Aβ$^{+/-}$ (Kastner et al., 1997a) mice, suggesting that RXRα/RARβ heterodimers mediate a retinoid-dependent function normally required for involution of the primary vitreous body. Our present data indicate that the AF-2 of RXRα is involved in this function.

Two amongst the 12 E14.5 Xα*af2*^{o} mutants also exhibited bilateral ocular abnormalities (closer eyelid folds, thickened ventral portion of the corneal stroma, shorter ventral retina and ventral rotation of the lens; compare E, C, V, D and L in Fig. 3a and b; Table 3), identical to those observed in all Xα mutants (Kastner et al., 1994). Thus, with the exception of the coloboma of the optic disc, the ocular abnormalities seen in E14.5 Xα*af2*^{o} mutants are those previously found in Xα mutants, but their penetrance is reduced (Kastner et al., 1994; see Table 3). However, with the exception of a retrolenticular membrane, no abnormalities of the eye and its adnexae (e.g. Harderian gland and nasolacrimal duct agenesis) were observed in four E18.5 Xα*af2*^{o} mutants.

Skeletal abnormalities, which affected mostly the cervical region of the vertebral column and included some homeotic transformations, as well as bilateral agenesis of the metoptic pilar and abnormalities of the cricoid cartilage, were found with a variable penetrance and expressivity in all E18.5 Xα*af2*^{o} mutants examined (Table 4). The in utero death of RXRα null mutants at E14.5-E16.5 has precluded the analysis of their skeletal and cartilaginous elements (Kastner et al., 1994; Sucov et al., 1994). However, similar skeletal abnormalities have been found in Xα$^{+/-}$/Aα$^{+/-}$ or Xα$^{+/-}$/Aγ$^{+/-}$ compound heterozygotes (Kastner et al., 1997a), as well as in Aγ and Aα mutants (Lohnes et al., 1993; Ghyselinck et al., 1997), indicating that RXRα/RARα and RXRα/RARγ heterodimers are most probably involved in the corresponding skeletal morphogenetic events (Kastner et al., 1997a). Thus, the AF-2 of RXRα appears to be involved in the function of these heterodimers during skeletal and cartilaginous morphogenesis.

Additionally, Xα*af2*^{o} mutants displayed, with a low penetrance, defects that were not observed in RXRα null mutants, including partial agenesis of the oesophagotracheal septum, hypoplastic lungs, one case of diaphragmatic hernia and one case of ectopic openings of the ureters into the caudal urogenital sinus (Tables 2, 5). These abnormalities that belong to the fetal VAD syndrome (Wilson and Warkany, 1948, 1949) were previously observed in RAR/RAR and/or RXRα/RAR compound null mutants (Kastner et al., 1997a; Ghyselinck et al., 1997; for additional references see Kastner et al., 1995; Tables 2, 5). They may, therefore, reflect dominant negative properties of the RXRαAF2^{o} protein.

The RXRα*af2*^{o} mutation causes severe abnormalities in the absence of RXRβ and RXRγ

The observation that the defects exhibited by Xα*af2*^{o} mutants are in general less penetrant and/or less severe than those previously observed in RXRα null mutants suggests that, in many instances, the RXRα AF-2 activity may not be required. Alternatively, the remaining RXRβ and/or RXRγ may compensate for the loss of the RXRα AF-2 in Xα*af2*^{o} mutants, but not for the loss of RXRα in Xα mutants. To investigate the above possibilities, the RXRα*af2*^{o} mutation was introduced into RXRβ and/or RXRγ null mutants, which are known to develop normally (Kastner et al., 1996; Krezel et al., 1996). Double Xα*af2*^{o}/Xβ and Xα*af2*^{o}/Xγ mutants, as well as triple Xα*af2*^{o}/Xβ/Xγ mutants, were generated and their phenotype analyzed. Importantly, the level of expression of the RXRαAF2^{o} protein in the triple mutants was similar to that of the RXRα protein in Xβ/Xγ or WT embryos, and the level of

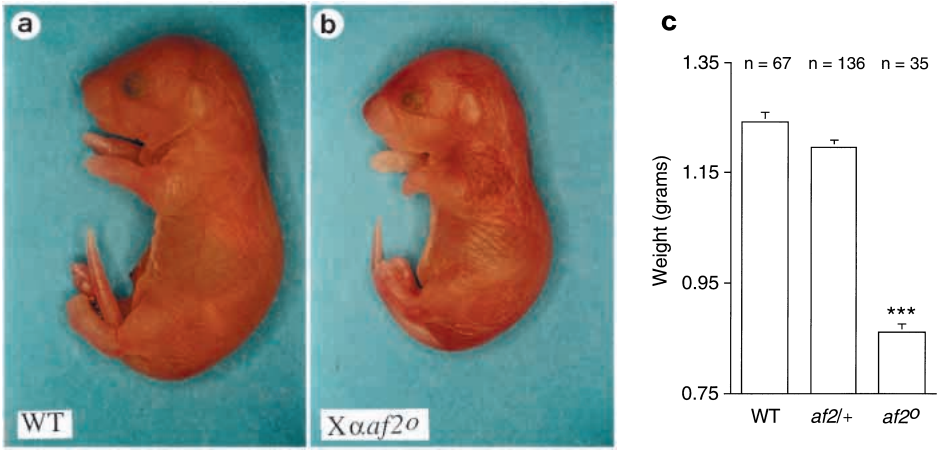

Fig. 2. (a,b) External appearance of representative wild-type (a) and RXRα*af2*^{o} mutant mice (b) at E18.5. (c) Mean weights of WT and Xα*af2*^{o} mutants are presented with standard errors. The data was subjected to a Bartlett's test for equal variances (P=0.56) followed by a one-way ANOVA test and Tukey's multiple comparison post-test. Xα*af2*^{o} mutants weighed significantly less than both Xα*af2*/+ and WT (P<0.001 for both), whereas no significant difference was observed between WT and Xα*af2*/+. Asterisks indicate groups which differ significantly (***P<0.001).

Table 2. Abnormalities of the cardiovascular system, the respiratory system and certain glands in RXRα*af2⁰* single and compound mutants

	Genotype, age (dpc) and number of mutant fetuses									
	Xα*af2⁰*		Xα*af2⁰* Xβ	Xα*af2⁰* Xβ/Xγ	Xα*af2⁰*/Aα		Xα*af2⁰*/Aβ		Xα*af2⁰*/Aγ	
Abnormalities	14.5 10	14.5* 2	14.5 6	14.5 5	14.5 3	18.5 3	14.5 4	18.5 4	14.5 3	18.5 3
Cardiovascular defects										
Ventricular myocardial hypoplasia (Xα#) (Aα/Aγ)	0	1/2	3/6	2/5	#	2/3	0	2/4	0	1/3
Agenesis of the conotruncal septum (E14.5)/high ventricular septal defects (E18.5) (Xα) (Xα/Aα#;Xα/Aβ#;Xα/Aγ)(Aα/Aβ#;Aα/Aγ#)	1/10	2/2	2/6	#	1/3	2/3	3/4	#	#	2/3
Persistent truncus arteriosus (PTA) (NCC) (Xα/Aα#;Xα/Aβ;Xα/Aγ) (Aα/Aβ#;Aα/Aγ#)	0	0	0	2/5	1/3	2/3	0	0	0	0
Abnormal arteries derived from Ao.A. 3-6 (NCC) (Xα/Aα>Xα/Aβ and XαAγ) (Aα/Aβ#;Aα/Aγ#; Aβ/Aγ)	0	0	1/6 (a)	# (b,c,d)	0	2/3 (d,e)	2/4 (c)	1/4 (d)	0	0
Respiratory system abnormalities										
Agenesis of left lung (Aα/Aβ)	0	0	2/6	0	0	0	0	0	0	0
Hypoplastic left lung (Xα/Aα) (Aα/Aβ)	2/10	1/2	4/6	#	#	#	2/4	0	0	0
Hypoplastic right lung (Xα/Aα) (Aα/Aβ#)	2/10	1/2	#	#	#	#	2/4	0	0	0
Agenesis of oesophagotracheal septum (Xα/Aα>Xα/Aβ•) (Aα/Aβ#)	1/10	1/2	#	#	2/3	1/3	2/4	2/4	0	0
Diaphragmatic hernia	0	1/2	4/6	1/5	0	0	0	0	0	0
Glandular abnormalities										
Salivary gland hypoplasia (Xα/Aγ#) (Aα/Aγ#)	0	0	0	0	0	0	0	0	#	#
Shortening of the sublingual duct (Aα/Aγ#;Aβ/Aγ#; Aα/Aβ)	NA	NA	NA	NA	NA	0	NA	#	NA	#
Shortening of the submandibular duct (Aα/Aγ#)	NA	NA	NA	NA	NA	0	NA	0	NA	#
Persistent cervical thymus (NCC) (Aα/Aβ; Aα/Aγ#)	NA	NA	NA	NA	NA	0	NA	1/4	NA	#

*These two fetuses were selected on the basis of a generalized, externally visible oedema.
(Xα): these abnormalities are also observed in RXRα null fetuses.
(Xα/Aα), (Xα/Aβ) and (Xα/Aγ): these abnormalities are also observed in RXRα/RAR(α,β or γ) double null mutants.
(Aα/Aβ), (Aα/Aγ) and (Aβ/Aγ): these abnormalities were also observed in RARα/RARβ, RARα/RARγ and RARβ/RARγ double mutants.
This abnormality is fully penetrant (and bilateral).
• Small oesophagotracheal fistula present in half of the Xα/Aβ mutants were overlooked in Kastner et al. (1997a).
NA, not applicable, as these ducts are not fully formed at E14.5.
The high ventricular septal defect represents a manifestation at E18.5 of the lack of formation of the conotruncal septum observed in E14.5 mutants.
Ao.A. 3-6, third, fourth and sixth aortic arches; the abnormalities included: (a) double arch of the aorta; (b) arch of the aorta on the right side; (c) retrooesophageal right subclavian artery; (d) right pulmonary artery arising from the inominate artery; (e) double arch of the aorta.
NCC: these defects are likely to be caused by abnormal migration, proliferation, death or differentiation of cardiac neural crest cells.
See Kastner et al., 1994 and 1997(a and b); Mendelsohn et al., 1994; Lohnes et al., 1994 and Ghyselinck et al., 1997 for further details concerning these abnormalities, and Table 3 for additional glandular abnormalities in the eye region.

RXRβ transcripts was similar in WT and Xα*af2ᵒ* mutants (data not shown).

Xα*af2ᵒ*/Xγ fetuses were indistinguishable from Xα*af2ᵒ* mutants (data not shown), but Xα*af2ᵒ*/Xβ and Xα*af2ᵒ*/Xβ/Xγ mutants were consistently much more severely affected, certain defects being more severe or more penetrant in the triple mutants (see Tables 2, 3, 5). All triple and Xα*af2ᵒ*/Xβ mutant fetuses died in utero before E17.5 [data not shown; note, however, that triple mutant fetuses were obtained at almost Mendelian frequency at E14.5 (obtained: 7; expected: 9)]. Externally, all of these double and triple E14.5 mutants appeared oedematous. They displayed very narrow palpebral fissures (compare Fig. 4d and e-g), obvious forelimb deficiencies (see below), and all Xα*af2ᵒ*/Xβ/Xγ mutants exhibited deficiencies of median craniofacial structures. The medial nasal processes of E14.5 fetuses were markedly hypoplastic (MN in Fig. 4h,i), resulting in a median facial cleft (open arrow in Fig. 4i). The maxillary processes (M in Fig. 4h,i) displayed abnormal extensions towards the midline (asterisks in Fig. 4i), which may reflect an attempt to compensate for the lack of midfacial tissue, whereas the lateral nasal processes (LN in Fig. 4h,i) were apparently normal.

Table 3. Abnormalities of the eye and of its adnexae in RXRα*af2^0* single and compound mutant fetuses

	Genotype, age (dpc) and number of mutant fetuses									
	Xα*af2^0*		Xα*af2^0* Xβ	Xα*af2^0* Xβ/Xγ	Xα*af2^0*/Aα		Xα*af2^0*/Aβ		Xα*af2^0*/Aγ	
Ocular abnormalities	14.5 10	14.5* 2	14.5 6	14.5 5	14.5 3	18.5 3	14.5 4	18.5 4	14.5 3	18.5 3
Lens abnormalities										
Ventral rotation of the lens (Xα#) (Xα/Aγ#)++ (Aβ/Aγ#)	B:2/10	0	#(+)	#(++)	B:1/3	0	#	#	#(++)	#
Corneal-lenticular stalk (Xα/Aγ#) (Aα/Aγ; Aβ/Aγ)	0	0	0	B:2/5	0	0	0	0	#	#
Lens degeneration (Aα/Aγ; Aβ/Aγ#)	0	0	0	0	0	0	0	0	0	#
Mesenchymal defects										
Agenesis of the eyelids and cornea (Xα/Aγ#)	0	0	0	0	0	0	0	0	#	#
Closer eyelid folds (E14.5)/small conjunctival sac (E18.5) (Xα#) (Xα/Aβ#) (Aα/Aγ#; Aβ/Aγ#)	B:2/10	0	#(+)	#(++)	0	0	#	#	NA	NA
Thickened corneal stroma (Xα#) (Xα/Aβ) (Aα/Aγ#; Aβ/Aγ#)	B:2/10	0	#(+)	#(++)	0	0	#	#	NA	NA
Agenesis of the iris stroma (Xα#) (Aα/Aγ; Aβ/Aγ#)	NA(1)	NA(1)	NA(1)	NA(1)	NA(1)	0	NA(1)	#	NA(1)	#
Agenesis of the anterior chamber (Xα#) (Aα/Aγ#; Aβ/Aγ#)	NA(1)	NA(1)	NA(1)	NA(1)	NA(1)	0	NA(1)	#	NA(1)	#
Agenesis of the sclera (Xα#) (Aα/Aγ#; Aβ/Aγ#)	NA(1)	NA(1)	NA(1)	NA(1)	NA(1)	0	NA(1)	#	NA(1)	#
Retrolenticular membrane (PHPV) (Xα#) (Xα+/–/Aβ+/–) (Aβ) (Aα/Aβ#; Aβ/Aγ#)	#	#	#	#	B:2/3	#	#	#	#	#
Retinal defects										
Shortening of ventral retina (Xα#) (Xα/Aγ#)++ (Aβ/Aγ#)	B:2/10	0	#(+)	#(++)	B:1/3	0	#	NA(2)	#(++)	NA(2)
Extensive coloboma of the retina (Xα/Aγ) (Aα/Aγ#)	0	0	0	B:2/5	0	0	0	0	U:1/3 B:1/3	B:1/3
Coloboma of the optic disc (Xα#) (Aα/Aγ#; Aβ/Aγ)	0	0	U:1/6 B:4/6	#	0	0	U:2/4 B:1/4	#	#	#
Coloboma of the iris (Xα/Aγ#) (Aβ/Aγ)	0	0	U:1/3	B:4/5	0	0	0	0	#	#
Agenesis of Harderian glands (Aγ) (Aα/Aγ#; Aβ/Aγ#)	NA(1)	NA(1)	NA(1)	NA(1)	NA(1)	0	NA(1)	3/4	NA(1)	#
Agenesis of naso-lacrimal duct (Aα/Aγ#; Aβ/Aγ#)	NA(1)	NA(1)	NA(1)	NA(1)	NA(1)	0	NA(1)	1/4	NA(1)	#

*These 2 fetuses were selected on the basis of a generalized, externally visible oedema.
(Xα), (Aβ) and (Aγ): these abnormalities are also seen in RXRα, RARβ and RARγ null fetuses.
(Xα/Aα), (Xα/Aβ) and (Xα/Aγ): these abnormalities are also observed in RXRα/RAR(α, β or γ) double null mutants.
(Aα/Aβ), (Aα/Aγ) and (Aβ/Aγ): these abnormalities are also observed in RARα/RARβ, RARα/RARγ and RARβ/RARγ double mutants.
(+) and (++): these abnormalities are more severe as compared with the RXRα null phenotype. # These abnormalities are fully penetrant (and bilateral, when applicable). U, unilateral; B, bilateral; NA, not applicable. (1) The corresponding structure is not yet formed at E14.5; (2) the relative lengths of the ventral and dorsal portions of the retina cannot be evaluated at this stage due to extensive foldings. ND, not determined.
For further details concerning these abnormalities see Lohnes et al., 1994; Kastner et al., 1994, 1997a and Ghyselinck et al., 1997.

In marked contrast to the mild abnormal phenotype of Xα*af2^o* single mutants, histological analysis of serial sections of double and triple mutants revealed a large array of malformations, affecting a wide variety of tissues (Tables 2, 3, 5). Defects not observed in Xα*af2^o* single mutants included abnormal patterning of aortic arch-derived arteries, persistent truncus arteriosus (PTA), agenesis of the left lung, various forms of retinal colobomas (see Fig. 3d), presence of a corneal-lenticular stalk, Müllerian duct agenesis or renal hypoplasia (Tables 2, 3, 5; and data not shown). Interestingly, the frequency and/or the severity of the defects already seen in single Xα*af2^o* mutants were greatly increased (see Tables 2, 3, 5, and Fig. 3b and d for a comparison of ocular defects).

All triple mutants (but only one out of six double mutants) analyzed at E14.5 also exhibited selective deficiencies of the bones forming the median portion of the cranial base: the lamina cribriform of the ethmoid bone and the presphenoid bone were consistently disrupted, while the nasal septum was

duplicated (data not shown). In addition, all triple, but not double mutants exhibited an atavistic supernumerary cartilaginous element between the brain and the trigeminal ganglion (the pila antotica; data not shown, see Lohnes et al., 1994), and 1 (out of 7) triple mutant displayed an exencephaly (Fig. 4g).

Forelimbs abnormalities were observed in all E14.5 triple mutants (analysed by scanning electron microscopy or histology; Fig. 4j-l; and data not shown), as well as in a number

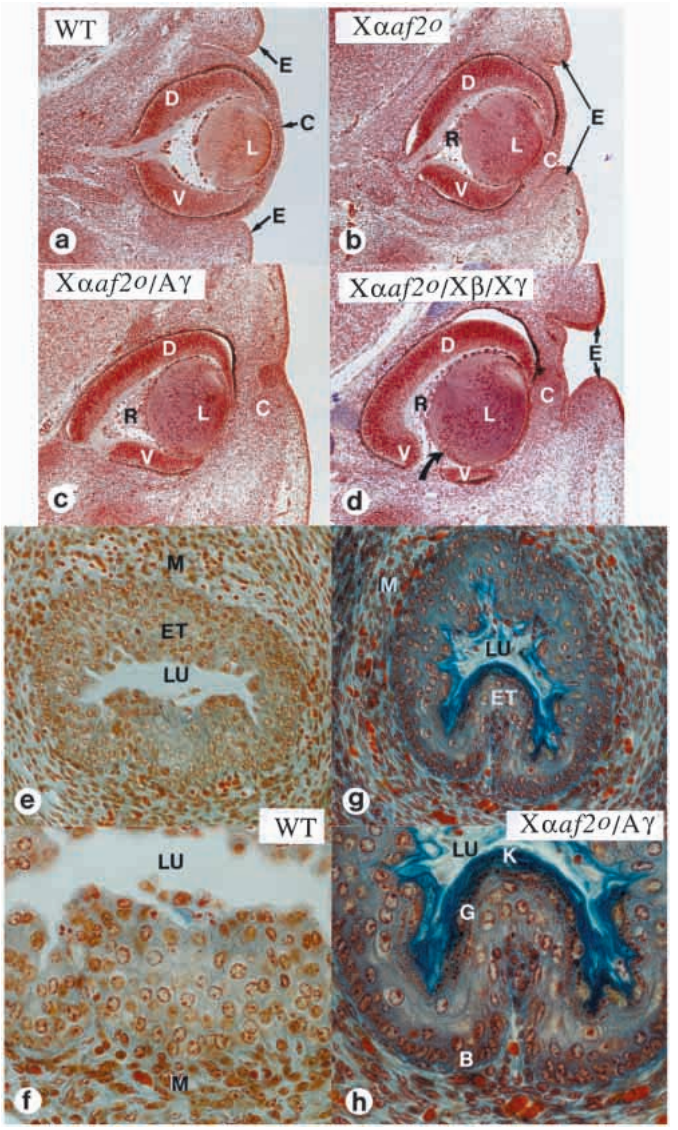

Fig. 3. Histological abnormalities observed in Xα*af2^o*, single and compound mutants (genotypes as indicated). (a-d) Comparison of frontal sections through the eye region of E14.5 wild-type (WT) and mutant fetuses. Note, in all mutants, the short ventral retina (V), the thickening of the presumptive corneal stroma (C) and closer or absent eyelid folds (E). (e-h) Cross sections of proximal urethra at E18.5. In the WT fetus (e,f), the urethral epithelium (ET) is stratified, but not keratinized. In contrast, the mutant epithelium (g,h) shows an organisation resembling that of normal adult skin, with well-defined basal (B), granular (G) and cornified (K) layers. B, basal layer; C, presumptive corneal stroma; D, dorsal retina; E, eyelids; ET, urethral epithelium; G, granular layer; K, cornified layer; L, lens; Lu, lumen of the urethra; M, mesenchyme of the urethra; R, persistent hyperplastic primary vitreous (retrolenticular membrane); V, ventral retina. The curved arrow in d crosses an abnormal ventral opening (i.e. a typical coloboma of the retina). Magnifications ×40 (a-d), ×200 (e,g) and ×400 (f,h).

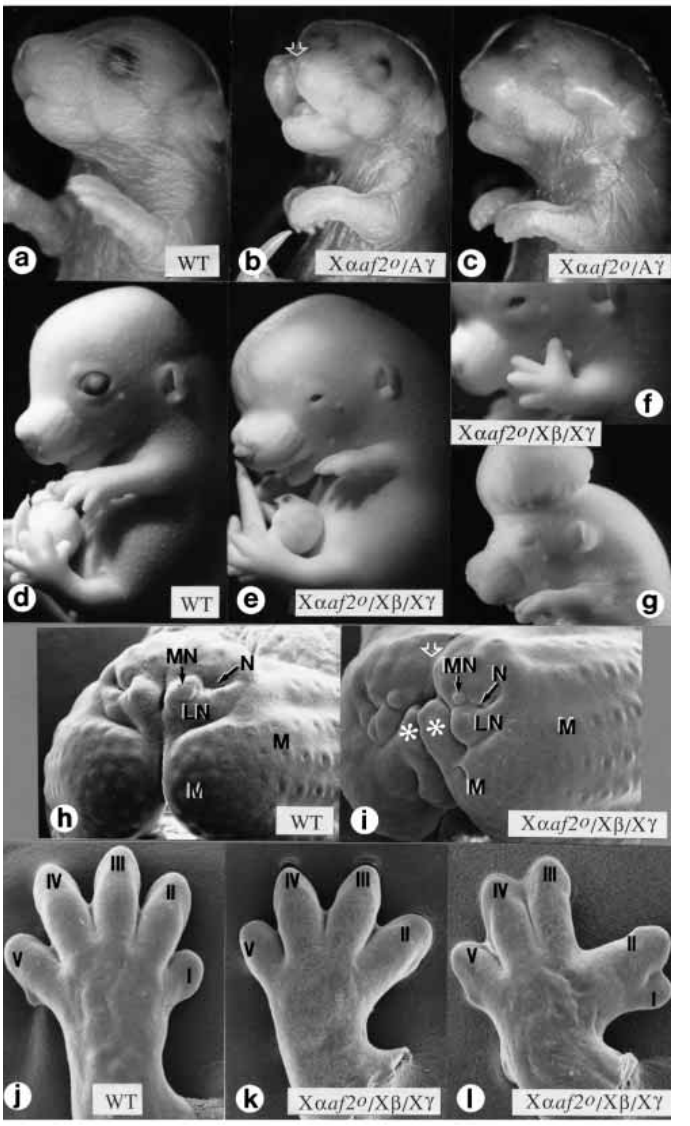

Fig. 4. External features of wild-type (WT) and Xα*af2^o* compound mutants (genotypes as indicated) at E18.5 (a-c) and E14.5 (d-l). (h,i) Scanning electron micrographs of the snout: note in the triple mutants (i) the marked hypoplasia of medial nasal processes (MN), which results in a median facial cleft (open arrow), the abnormal extensions towards the midline of the maxillary processes (asterisk), which likely reflects an attempt to compensate for the lack of midfacial tissue, and the apparent normality of lateral nasal processes (LN). (j-l) Dorsal views of the left forearm and handplate; the shortening and abnormal curvature of the forearm (observed in 8 out of 10 limbs) reflects radial agenesis (see also Fig. 5); the handplate displays a loss of digit **I** (k) (6 out of 10 limbs) or a fusion between a rudimentary digit **I** and the second digit (2 out of 10 limbs). Digits are numbered in roman numerals; LN and MN, lateral and medial nasal processes, respectively. M, maxillary process; N, nostril. Open arrows point to midfacial clefts. Same magnifications in a-c, in d and e, in h and i and in j-l.

of E16.5 Xα*af2*^{o}/Xβ mutants (analysed by whole-mount skeletal preparation; Fig. 5a-f; and data not shown). In contrast, the hindlimbs of all double and triple mutants were normal. The forearms appeared in most of the cases shortened and curved (8 out of 10 triple mutants; see Fig. 4d-g and j-l), while the handplate was either normal (2 out of 10 triple mutants), displayed fusion of digits I and II (2 out of 10 triple mutants, Fig. 4f,l) or loss of digit I (6 out of 10 triple mutants, Fig. 4k). Histological analysis of 6 triple mutant forelimbs revealed partial or complete radius agenesis (5 out of 6, data not shown; see also the Xα*af2*^{o}/Xβ mutants in Fig. 5b,c,f) and agenesis of the digit I (data not shown; see also the Xα*af2*^{o}/Xβ mutants in Fig. 5e,f) or the existence of a soft tissue syndactyly between a small digit I and digit II (data not shown). In one Xα*af2*^{o}/Xβ case, the humerus, as well as digits I and II, were lacking (Fig. 5b), and the acromial process of the scapula was absent in another case (Fig. 5c). Anterior carpal bones were also lacking in a number of cases [e.g. the scapho-lunatum (SL), central bone (C) and the distal carpal bone (D1) in Fig. 5f].

Thus, abnormalities that result from the introduction of the RXRα*af2*^{o} mutation in a RXRβ or a RXRβ/RXRγ null mutant background (apparently phenotypically normal, Kastner et al., 1996; Krezel et al., 1996) recapitulate many of the developmental abnormalities (including those of the forelimbs) which have been previously observed in RAR compound mutants (Lohnes et al., 1994; Mendelsohn et al., 1994; Ghyselinck et al., 1997; and see Tables 2, 3, 5). Therefore, in many instances RXR AF-2s appear to play a critical role in the transduction of the RA signal during mouse development (note in this respect that the phenotype of Xα*af2*/+/Xβ/Xγ triple mutants was identical to that of Xβ/Xγ mutants, with the exception of an additional retrolenticular membrane). However, some defects, which are fully penetrant in certain RAR compound mutants (e.g. PTA

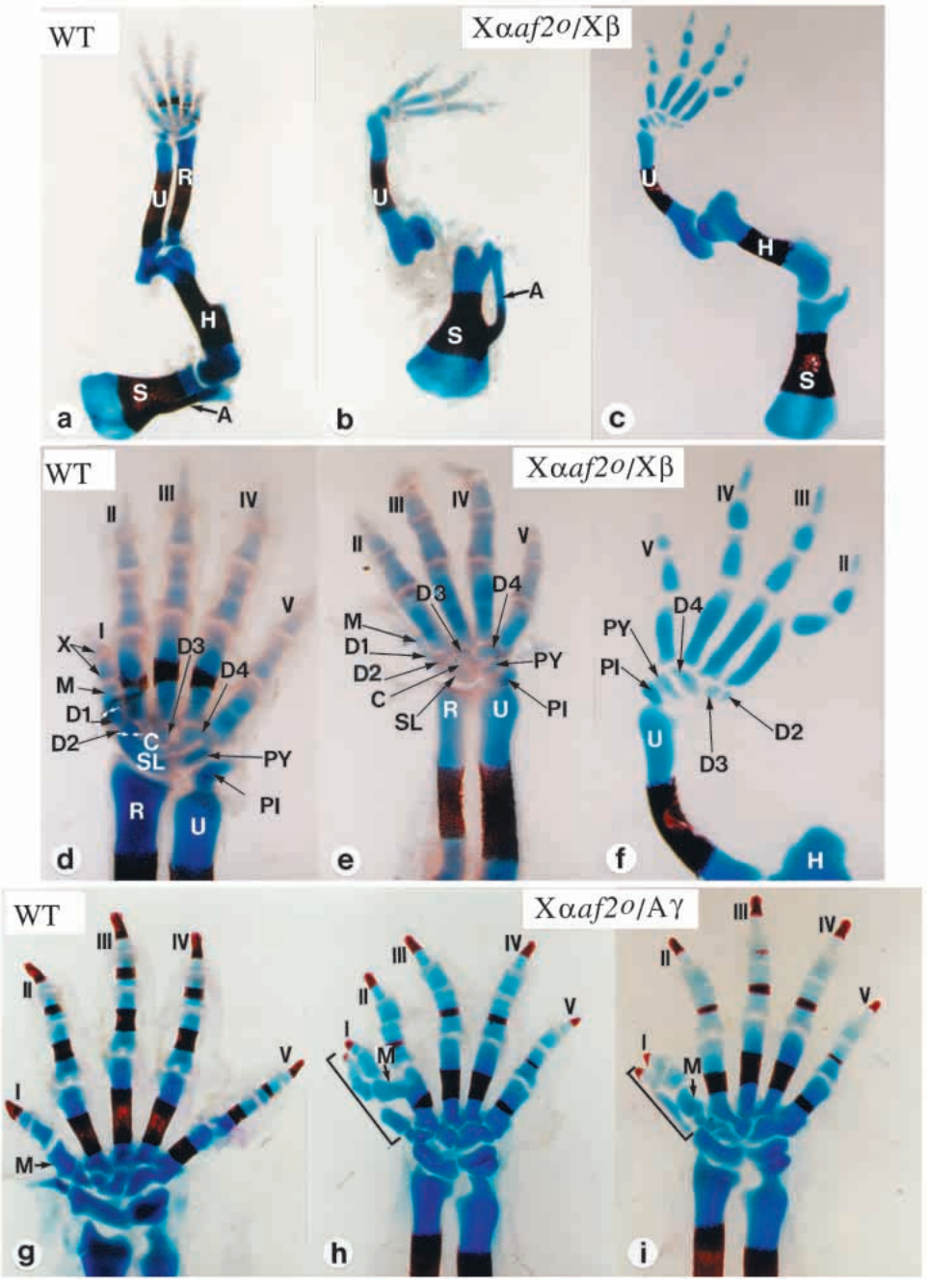

Fig. 5. Comparison of E16.5 (a-f) and E18.5 (g-i) forelimb skeletal preparation of wild-type (WT) and Xα*af2*^{o} compound mutants (genotypes as indicated). (a-f) Forelimb skeletal deficiencies were observed in all Xα*af2*^{o}/Xβ mutants (*n*=3). The right forelimb was either normal (*n*=2) or lacked the two phalanges of the first digit (e); the left forelimb was always abnormal showing agenesis of the acromial process of the scapula (*n*=1, c), hypoplasia (*n*=1) or absence of the humerus (*n*=1, b), absence of the radius (*n*=3, b,c,f), absence of all skeletal elements of digit I (*n*=1, c,f), or both digits I and II (*n*=2, b), and lack of anterior carpal bones (e.g. the scapholunatum (SL), central bone (C) and distal carpal bone (D1), f). (g-i) Xα*af2*^{o}/Aγ forelimbs with a supernumerary preaxial digit characteristic of digit I; note that the orthotopic digit I is fused to the metacarpal bone of digit II in h. The digits are numbered in roman numerals, digit I (thumb) being the most anterior and digit V the most posterior. A, acromial process of the scapula; C, central carpal bone; D1-D4, distal carpal bones; H, humerus; M, metacarpal bone of digit one; PI, pisiform carpal bone; PY, pyramidal carpal bone; R, radius; S, scapula; SL, scapholunatum carpal bone; U, ulna; X, phalanges of digit one. In h and i, the bracket encompasses the skeletal elements of the supernumerary digit. Same magnifications in a-c and in d-i.

Table 4. Skeletal and cartilage abnormalities in RXRα*af2⁰* mutants

	Genotype and number of skeletons examined at E18.5	
	RXRα$^{+/+}$	RXRα*af2⁰*
	22	19
Abnormalities	7 (32%)[a]	19 (100%)[a]
Cranial skeletal abnormalities		
Agenesis of metoptic pilar (posterior border of the optic nerve foramen)		
Partial	0	4
Complete	0	9
Axial skeletal abnormalities		
Homeotic transformations		
Posteriorizations		
Posterior tubercule on basioccipital bone	1 (5%)	3 (16%)
Fusion of basioccipital bone with anterior arch of the atlas	0	5 (26%)
Anteriorizations		
Ectopic anterior arch on C2 (C2 → C1)	1 (5%)	3 (16%)
Tuberculum anterior on C7 (C7 → C6)	0	5 (26%)
Eight instead of seven vertebrosternal ribs (T8 → T7)	U:1 (5%)	U:4 (21%) B:2 (11%)
Ribs on L1 (L1 → T14)	0	U:1 (5%) B:2 (11%)
Malformations		
C1 bifid	0	U:8 (42%) B:2 (11%)
C2 bifid	0	U:8 (42%) B:2 (11%)
C3 bifid	0	U:2 (11%)
Fusion of neural arch of C2 and C3	0	U:2 (11%)
C1 anterior arch fused with C2	6 (27%)	13 (68%)
Abnormal cricoid cartilage[b]	5 (23%)	16 (84%)

C1 to C7, first to seventh cervical vertebrae; T1 to T14, first to fourteenth thoracic vertebrae; L1, first lumbar vertebra; U, unilateral; B, bilateral.

[a]Number of animals exhibiting malformed skeletons.

[b]Ventral extension of the cricoid cartilage which was often fused to the first tracheal ring.

or aortic arch defects; Mendelsohn et al., 1994; Lohnes et al., 1994; Ghyselinck et al., 1997) were only partially penetrant in Xα*af2⁰*/Xβ/Xγ triple mutants, indicating that, in some instances, RA signalling can be performed in the absence of an RXR AF-2 activity (see Discussion).

Xα*af2⁰*/RAR compound mutants

Concomitant mutations of RXRα and either RARα, RARβ or RARγ lead to the appearance of specific defects not found or much milder in single mutants, thus reflecting the critical involvement of the corresponding heterodimers (Kastner et al., 1994, 1997a). The RXRα*af2⁰* mutation was therefore introduced in either RAR(α, β or γ) null genetic backgrounds to assess the contribution of RXRα AF-2 in these heterodimers. The level of the RXRαAF2⁰ protein in compound Xα*af2⁰*/RAR (α, β or γ) mutants was similar to that of RXRα in the corresponding RAR single mutants (data not shown).

The various Xα*af2⁰*/RAR(α, β or γ) double mutants displayed most of the malformations found in the corresponding RXRα/RAR double null mutants (Tables 2, 3, 5; see also Kastner et al., 1994, 1997a). For instance, both Xα/Aα and Xα*af2⁰*/Aα mutants exhibited hypoplastic lungs, agenesis of the oesophagotracheal septum and kidney hypoplasia (or agenesis) with a high degree of penetrance, while both Xα/Aβ and Xα*af2⁰*/Aβ mutants presented similar set of ocular defects, and both Xα/Aγ and Xα*af2⁰*/Aγ mutants displayed the same severe ocular abnormalities, as well as salivary gland hypoplasia (see Tables 2, 3, 5). Interestingly, a high frequency of ventricular myocardial hypoplasia was seen in Xα*af2⁰*/Aα mutants (Table 2), suggesting that RXRα/RARα heterodimers are preferentially involved in ventricular development. This RXRα/RARα synergy could not be previously observed in Xα/Aα mutants, as Xα single null mutants have a penetrant ventricular myocardial hypoplasia on their own (Kastner et al., 1994, 1997b). However, a preferential role of the RARα isotype in the control of ventricular myocyte differentiation was suggested by the observation of a high frequency of differentiated subepicardial myocytes in E9.5 RARα null mutants (Kastner et al., 1997b).

Thus, in the absence of a given RAR isotype, the RXRα AF-2 is often essential for the realization of the function preferentially exerted by the corresponding RXRα/RAR heterodimer. This conclusion is further supported by the observation that several defects were more severe or more penetrant in certain Xα*af2⁰*/RAR double mutants than in Xα*af2⁰*/Xβ/Xγ triple mutants (see Tables 2, 3, 5). These defects include complete absence of eyelids (Fig. 3c,d), the presence of a corneal-lenticular stalk, midfacial defects (compare in Fig. 4b,c with e,g), salivary gland hypoplasia, kidney agenesis, agenesis of the caudal ureter and ventricular myocardial hypoplasia (Tables 2, 3, 5). Interestingly, the ocular abnormalities found in E14.5 RXRα$^{-/-}$/RARγ$^{+/-}$ mutants (Kastner et al., 1994, 1997a) were also found in Xα*af2⁰*/RARγ$^{+/-}$ mutants (*n*=8, data not shown), strongly supporting the conclusion that RXRα AF-2 critically contributes to the activity of RXRα/RARγ heterodimers.

Several abnormalities, which could not be observed in Xα/RAR mutant fetuses because of their early death in utero, were found in E18.5 Xα*af2⁰*/RAR mutants (Tables 2, 3, 5). These included agenesis of Harderian glands and nasolacrimal ducts (Table 3), partial persistence of thymus tissue in the neck (in both Xα*af2⁰*/Aβ and Xα*af2⁰*/Aγ mutants, Table 2), hydronephrosis (in Xα*af2⁰*/Aβ mutants, Table 5), lens degeneration (in Xα*af2⁰*/Aγ mutants, Table 3), and keratinization of the entire urethra (in Xα*af2⁰*/Aγ mutants, Table 5; Fig. 3e-h). Note that a keratinization of the distal urethra was also observed in Aα/Aγ mutants (our unpublished results). All E18.5 Xα*af2⁰*/Aγ mutants displayed malformed upper incisors, truncated snouts, mid-facial clefts and deficiencies of median-anterior skeletal elements, which were always associated with an agenesis of the corpus callosum, an association characteristic of human prosencephaly (Fig. 4b,c; and data not shown; see Hunter, 1993). Interestingly, these various defects have been previously observed in RAR compound mutants (e.g. holoprosencephaly-like malformations in Aα/Aγ double mutants; see Mendelsohn et al., 1994; Lohnes et al., 1994; Ghyselinck et al., 1997; see also Tables 2, 3, 5). These observations further link RXRα and RAR functions and, in addition, indicate which RAR heterodimeric partner is critically involved in certain developmental events.

Comparison of the phenotypes of Xα*af2⁰*/RAR and Xα/RAR compound mutants also revealed a set of defects

Table 5. Abnormalities of the urogenital tracts in RXRα*af2⁰* single and compound mutants

	Mutant genotype, age (dpc) and number (male:female) of mutant fetuses									
	Xα*af2⁰*		Xα*af2⁰* Xβ	Xα*af2⁰* Xβ/Xγ	Xα*af2⁰*/Aα		Xα*af2⁰*/Aβ		Xα*af2⁰*/Aγ	
Abnormalities	14.5 5:5	14.5* 0:2	14.5 3:3	14.5 4:1	14.5 2:1	18.5 0:3	14.5 3:1	18.5 3:1	14.5 0:3	18.5 2:1
Kidney abnormalities										
Agenesis (a) (Xα/Aα) (Aα/Aγ)	0	0	0	0	U:1/3	U:1/3	0	0	0	0
Hypoplasia (Xα/Aα#) (Aα/Aβ#) (Aα/Aγ)	0	0	3/6	1/5	U:1/3 B:1/3	U:1/3 B:2/3	0	0	0	B:1/3
Hydronephrosis (Aα/Aβ; Aβ/Aγ)	0	0	0	0	0	0	0	#	0	0
Ureter abnormalities										
Agenesis of caudal ureter (Xα/Aα; Xα/Aβ) (Aα/Aγ#; Aα/Aβ; Aβ/Aγ)	0	0	0	0	0	0	0	B:1/4	0	U:1/3
Ectopic ureteral openings (b) (Xα/Aα; Xα/Aβ; Xα/Aγ) (Aα/Aβ; Aβ/Aγ)	B:1/10	0	U:2/6 B:3/6	#	U:1/3 B:1/3	U:1/3	B:3/4	B:3/4	2/3	0
Keratinisation of urethra (Aα/Aγ)	0	0	0	0	0	0	0	0	0	#
Agenesis of the Müllerian duct (E14.5) or of its derivatives (E18.5 females) (c)										
Complete (Xα/Aα#) (Aα/Aβ#)	0	0	0	U:2/5	0	0	0	0	0	0
Partial (caudal portion missing) (Xα/Aβ; Xα/Aγ) (Aα/Aγ#; Aβ/Aγ)	0	0	U:3/6 B:3/6	U:2/5 B:3/5	#	#	B:2/4	0	0	0

*These fetuses were selected on the basis of a generalized, externally visible oedema.
(Xα/Aα), (Xα/Aβ) and (Xα/Aγ): these abnormalities are also observed in RXRα/RAR(α, β or γ) double null mutants.
(Aα/Aβ), (Aα/Aγ) and (Aβ/Aγ): these abnormalities are also observed in RARα/RARβ, RARα/RARγ and RARβ/RARγ double null mutants.
(a) Accompanied by a complete absence of the ureter. (b) Opening of the ureter in the terminal portion of the Wolffian duct and/or commun openings of the Wolffian duct and ureters in the urogenital sinus (E14.5) or opening of the ureters in the urethra (E18.5). (c) Absence of the oviducts, uterus and cranial vagina.
U, Unilateral; B, bilateral; # these abnormalities are completely penetrant (and bilateral).
For further details see Kastner et al. (1994, 1997a); Mendelsohn et al. (1994) and Ghyselinck et al. (1997).

occurring in a milder form in Xα*af2⁰*/RAR mutants. Agenesis of the Müllerian duct (Table 5), which was always complete in Xα/Aα mutants, affected only the caudal portion of this structure in Xα*af2⁰*/Aα mutants. Furthermore, the frequency of caudal Müllerian duct agenesis was also reduced in Xα*af2⁰*/Aβ and Xα*af2⁰*/Aγ when compared to Xα/Aβ and Xα/Aγ mutants (Table 5; Kastner et al., 1997a). The effect of the Xα*af2⁰* mutation was also milder than that of the RXRα null mutation on morphogenetic events involving cardiac neural crest cells (NCC): a lack of aorticopulmonary septation (leading to a persistence of truncus arteriosus, PTA) occurred in all Xα/Aα mice, but only in three out of six Xα*af2⁰*/Aα mutants (Table 2). Similarly, patterning of the great cephalic arteries, perturbed in most Xα/Aα mutants, was normal in 4 out of 6 Xα*af2⁰*/Aα mutants (Table 2). Interestingly, the lower sensitivity of aorticopulmonary septation to the lack of RXRα AF-2 was also reflected by the absence of PTA in 3 out of 5 Xα*af2⁰*/Xβ/Xγ mutants (Table 2, see above). These observations indicate that RXRα AF-2 is not required, or less critical, for a subset of RA-dependent functions.

Finally, the penetrance of some defects was higher in certain Xα*af2⁰*/RAR mutants than in the corresponding Xα/RAR mutants, suggesting that the RXRαAF2⁰ protein may exert dominant negative effects in certain RAR isotype null background. All E14.5 and E18.5 Xα*af2⁰*/Aγ mutants had severe deficiencies in frontonasal mesectoderm-derived skeletal elements, including partial agenesis of the elements forming the anterior portion of the cranial base (lamina cribriform of the ethmoid, presphenoid and basisphenoid bones, partial agenesis of frontal bones, absence or duplication of nasal septum). None of these defects (with the exception of one case of duplication of the nasal septum) were seen in E14.5 Xα/Aγ mutants (Kastner et al., 1997a). Similarly, two skeletons out of three Xα*af2⁰*/Aγ mutants examined, showed a unilateral preaxial polydactyly with a supernumerary digit **I** (bracketed in Fig. 5g-i) which was not observed in Xα/Aγ mutants (Kastner et al., 1997a).

RARβ2 promoter activity in RXRα*af2⁰* mutants

As the integrity of the RXR AF-2 function appears to be important for the RA-induced activity of the RARβ2 promoter in P19 EC cells (Blanco et al., 1996; Minucci et al., 1997; Dey and Ozato, 1997; see Discussion for additional references), we investigated its possible involvement in the mouse by introducing a RARβ2 promoter-*lacZ* reporter transgene in the Xα*af2⁰* mutant genetic backgrounds, through crosses with RARβ2-*lacZ* transgenic mice (Mendelsohn et al., 1991). The pattern of expression of RARβ2-*lacZ* at E12.5 (not shown) and

E13.5 was almost identical in Xα*af2^o*/RARβ2-*lacZ* transgenic mice, with the exception of the interdigital region where the β-galactosidase staining was decreased in the mutants (compare Fig. 6a and c). In marked contrast, all E12.5 (not shown) and E13.5 Xα*af2^o*/RXR$\beta^{-/-}$/RARβ2-*lacZ* mutants (n=10) exhibited a drastic reduction in *lacZ* expression throughout the embryos (Fig. 6d), whereas no reduction was seen in RXR$\beta^{-/-}$ mutants (Fig. 6b). Thus, RXR AF-2 appears to be indispensable for the transcriptional activity of the RARβ2 promoter, and some functional cooperation may occur between RXRαAF2^o and RXRβ. However, in contrast to what was observed in P19 EC cells overexpressing a truncated RXRβ lacking the AF-2 AD core (Blanco et al., 1996; see Discussion), the RXRα*af2^o* mutation (as well as the Xα*af2*/+ heterozygotic mutation – data not shown) did not exert any general dominant negative effect on RARβ2 promoter activity.

Previous studies (see Discussion for references) have shown that RAR- and RXR-selective ligands can act synergistically in various cell systems, particularly when the RAR-selective ligand is present in limiting amounts. Therefore, we hypothesized that increasing the RA level in Xα*af2^o*/RXR$\beta^{-/-}$ mutants might restore the RXRα*af2^o*/RAR heterodimer-mediated induction of the RARβ2 promoter-*lacZ* transgene. Pregnant dams were gavage-fed with all-*trans* RA at E13.5 and fetuses collected 6 hours later were stained for β-galactosidase activity. RA-treated WT/RARβ2-*lacZ*, RXR$\beta^{-/-}$/RARβ2-*lacZ*, Xα*af2^o*/RARβ2-*lacZ* and Xα*af2^o*/RXR$\beta^{-/-}$/RARβ2-*lacZ* fetuses, all exhibited an increase in RARβ2 promoter activity (Fig. 6e-h, respectively). It is noteworthy that the RA treatment restored the expression of *lacZ* in the interdigital region of Xα*af2^o* mutants, which suggests that higher levels of liganded RAR may compensate for the absence of RXRα AF-2. Most interestingly, although Xα*af2^o*/RXR$\beta^{-/-}$/RARβ2-*lacZ* transgenic fetuses (n=5) exhibited, upon RA treatment, a much lower *lacZ* expression than their WT, RXR$\beta^{-/-}$ or Xα*af2^o* transgenic counterparts (Fig. 6h compared with Fig. 6e-g), their β-galactosidase staining was consistently similar to that of untreated Xα*af2^o* transgenics, including a lack of β-galactosidase staining in interdigital areas. Thus, as expected, a further activation of RARs by RA excess in Xα*af2^o*/RXR$\beta^{-/-}$/RARβ2-*lacZ* transgenics resulted in an increase of RARβ2 promoter activity, supporting the conclusion that RXR/RAR heterodimers mediate the RA signal on the RARβ2 RARE in the mouse. Moreover, the observation that the RA-

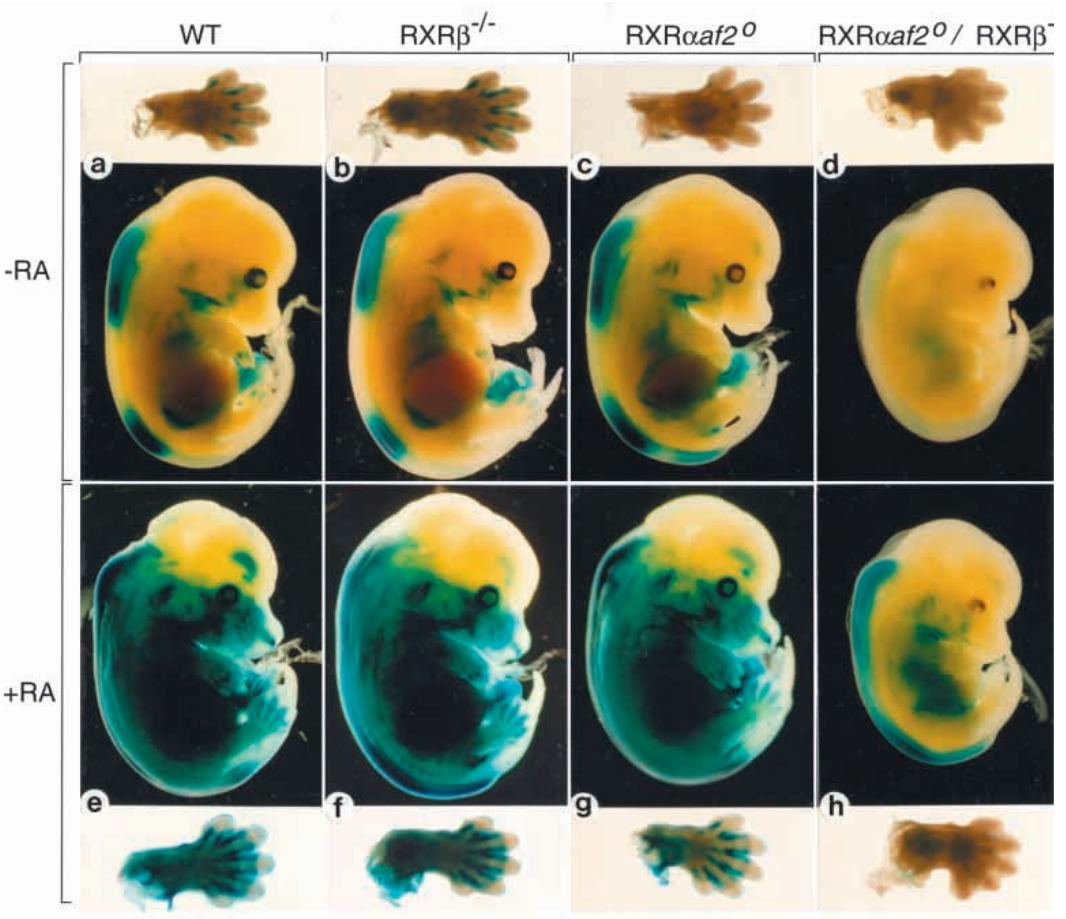

Fig. 6. mRARβ2-*lacZ* transgene expression in E13.5 fetuses. Lateral view (dark-field illumination) of X-gal-stained (Mendelsohn et al., 1991) whole-mount fetuses and dorsal view of their corresponding left forelimb. (a-d) β-gal staining comparison of untreated (–RA) wild-type (WT) (a), RXR$\beta^{-/-}$(b), Xα*af2^o* (c) and Xα*af2^o*/RXR$\beta^{-/-}$ (d) fetuses. *lacZ* expression is visible in the spinal cord with a gap located between the limbs (a-c), whereas it is severely reduced in Xα*af2^o*/RXR$\beta^{-/-}$ mutants (d). Staining is also seen at the level of sensory organs (ears, eyes and nostrils), as well as around the eyes (a-c), but not in Xα*af2^o*/RXR$\beta^{-/-}$ mutants (d). The β-gal staining that is observed in the proximal region of the forelimbs and hindlimbs, where it is associated with interdigital mesenchyme of WT and RXR$\beta^{-/-}$ mutants (a,b) is strongly weakened in Xα*af2^o* mutants (c) and absent in Xα*af2^o*/RXR$\beta^{-/-}$ mutants (d). (e-h) Comparison of the mRARβ2 promoter activity upon RA treatment (+RA) in wild-type (WT)(e), RXR$\beta^{-/-}$ (f), Xα*af2^o* (g) and Xα*af2^o*/RXR$\beta^{-/-}$ (h) mutants. RA administration to dams [80 mg/kg all-*trans* RA for 6 hours at E13.5, as described in Mendelsohn et al. (1991)] markedly stimulated the mRARβ2 promoter activity in all fetuses (compare a with e, b with f, c with g and d with h). Note that the patterns of enhancer *lacZ* expression are very similar in WT, RXR$\beta^{-/-}$ and RXRα*af2^o* fetuses. Note also that β-gal staining is markedly stimulated in the interdigital region of RA-treated RXRα*af2^o* mutants (g), but not in RXRα*af2^o*/RXR$\beta^{-/-}$ mutants (h). The staining of RXRα*af2^o*/RXR$\beta^{-/-}$ mutants is much weaker resembling that of untreated WT fetuses with the exception of the interdigital regions, which remain unstained in the mutant.

induced increase in *lacZ* expression in Xα*af2^o*/RXR$\beta^{-/-}$ mutants was much lower than that occurring in WT mice and Xα*af2^o* mutants, clearly indicates that AF-2s of RAR and RXR can strongly synergize upon RA treatment to maximally induce the activity of the RARβ2 promoter.

We conclude from these results that, as established above for the generation of developmental defects, RXR AF-2 is required for the activity of the RARβ2 promoter. Moreover, the observation that the absence of RXR AF-2 can be compensated to some extent by an increase in RA levels, provides strong support to the suggestion that RXR/RAR heterodimers mediate the RA signal on the RARβ2 promoter RARE, and furthermore that AF-2s of RXR and RAR can act synergistically.

DISCUSSION

Previous genetic studies have established that RXR/RAR heterodimers act as functional units transducing the retinoid signals during mouse development and, furthermore, that RXRα is the main heterodimerization partner of the RARs (see Introduction). The present study of mice carrying a mutation that abrogates RXRα AF2 activity, either alone or in combination with additional RXR(β and/or γ) or RAR(α, β or γ) mutations, demonstrates the physiological importance of RXR AF-2 in the functions of RXR/RAR heterodimers during development.

The AF-2 of RXRα is instrumental and RXRs are indispensable for developmental events controlled by retinoids

Two lines of evidence support the conclusion that RXRα AF-2 is implicated in the mediation of retinoid signals through RXR/RAR heterodimers. The first one is provided by the study of Xα*af2^o*, Xα*af2^o*/Xβ and Xα*af2^o*/Xβ/Xγ mutants. Xα*af2^o* mutant fetuses die before or at birth, and exhibit all of the heart and many of the ocular abnormalities found in RXRα null mutants, albeit with a reduced penetrance and/or severity. The development of Xβ/Xγ double null mutants is apparently normal (Krezel et al., 1996). In marked contrast, although Xα*af2^o*/Xγ mutant fetuses are indistinguishable from Xα*af2^o* mutants, Xα*af2^o*/Xβ and Xα*af2^o*/Xβ/Xγ mutants are severely and almost similarly affected. Thus, the mildness of Xα*af2^o* defects, when compared with those of RXRα null mutants, may reflect some functional cooperation between RXRαAF2^o and RXRβ and, to a much lesser extent, RXRγ (see below). This developmental RXR AF-2 requirement is substantiated at the level of a target gene by the large decrease in activity of the RA-dependent RARβ2 promoter in RXRα*af2^o*/Xβ mutants.

The second line of evidence implicating RXRα AF-2 in the mediation of the retinoid signal through RXR/RAR heterodimers is provided by the study of Xα*af2^o*/RAR(α, β or γ) double mutants. Their defects mostly recapitulate those found in the corresponding RXRα/RAR double null mutants, indicating that, in the genetic background of a given RAR mutation, RXRα AF-2 becomes often essential to enable the remaining RAR(s) to functionally replace the knocked-out RAR (see below and Kastner et al., 1995, 1997a for further discussion of this point). Interestingly, in contrast to Xα/RAR double null mutants, Xα*af2^o*/RAR double mutant fetuses do not die in utero, thus allowing the identification of which RXRα/RAR isotype heterodimers are critically involved in developmental events occurring at late stages of the fetal life. For instances, RXRα/RARβ and RXRα/RARγ heterodimers appear to play important roles in the development of the kidney excretory system and in the maintenance of the differentiated state of the urethral epithelium, respectively.

It must be stressed that the present study is the first one to show that mutations affecting RXRs only (Xα*af2^o*/Xβ and Xα*af2^o*/Xβ/Xγ) generate on their own most of the abnormalities found in the fetal VAD syndrome, as well as in RAR/RAR and RXR/RAR compound mutants. Importantly, no additional abnormalities were detected. This definitely demonstrates that RXRs together with RARs are required for retinoid signalling in vivo and makes it unlikely that RXR homodimers are critically involved in the transduction of the retinoid signal during mouse development. RXRs also form heterodimers with a number of additional nuclear receptor partners (Blumberg et al., 1998; Kliewer et al., 1998; for reviews see Mangelsdorf and Evans, 1995; Chambon, 1996; Perlmann and Evans, 1997). The observation that all of the defects exhibited by the Xα*af2^o*/Xβ/Xγ mutants can be attributed to abnormalities in the RAR/RXR signalling pathway suggests that these partners do not exert any developmental functions that require RXR AF-2 and can be readily detected before birth. In this respect, we note that no developmental defects have been reported to be associated with TRα (Fraichard et al., 1997), TRβ (Forrest et al., 1996), VDR (Li et al., 1997; Yoshizawa et al., 1997), NGFI-B (Lee et al., 1995a) and PPARα (Lee et al., 1995b) knock-outs. Alternatively, with some partners, the RXR AF-2 activity could be less critical for the function of the heterodimeric units than in the case of RXR-RAR heterodimers. PPARγ might be such a partner, as PPARγ null embryos die at 9.5-10.5 dpc from placental defects (Y. Barak and R. Evans, personal communication) not exhibited by our Xα*af2^o*/Xβ/Xγ triple mutants, but present in Xα/Xβ compound null mutants (O. Wendling, P. C. and M. M., unpublished results; see below).

Marked variability in the requirement of RXR AF-2 for retinoid-dependent developmental processes

Retinoid-dependent developmental events can be classified into three categories, based on differential susceptibility to genetic deficiency in retinoid receptors (Kastner et al., 1997a). Events of the first class are very sensitive to a reduced RAR or RXRα gene dosage, being impaired in single RAR isotype or RXRα mutants, and often in RXR$^{+/-}$/RAR$^{+/-}$ mutant mice [e.g. patterning of cervical vertebrae, involution of the primary vitreous body, ventricular cardiomyocyte differentiation; for details, see Kastner et al. 1997a,b, and Ghyselinck et al., 1997]. As most of these processes are impaired in Xα*af2^o* mutants, this first class of events stringently requires RXRα AF-2. The impairment of the second class of events (by far the most frequent), which are much harder to impair by genetically decreasing the amount of RAR and/or RXR, requires the concomitant inactivation of two specific RAR isotypes or of a specific RXRα/RAR pair (Kastner et al., 1997a). With few exceptions (e.g. failure of aorticopulmonary septation), these events are not affected in single Xα*af2^o* mutants, but fully impaired in the corresponding specific Xα*af2^o*/RAR double mutants, as well as in Xα*af2^o*/Xβ and Xα*af2^o*/Xβ/Xγ double and triple mutants. Interestingly, there are notable exceptions, the abnormalities being either less penetrant (myocardial

hypoplasia, corneal lenticular stalk) or even lacking (salivary gland hypoplasia, agenesis of the eyelids and cornea, kidney agenesis) (see Tables), indicating that the RXR AF-2 is not always indispensable. A third class of retinoid-dependent events may correspond to events implicated in early embryonic VAD defects (Heine et al., 1985; Marsh-Armstrong et al., 1995; Twal et al., 1995; Bavik et al., 1996; Costaridis et al., 1996; Maden et al., 1996, 1997; Dickman et al., 1997), but not impaired in RAR double mutants. These events may not require RXR AF-2 under any circumstances as, for instance, the early embryonic lethal defects that are exhibited by RXRα/RXRβ double null mutants (Kastner et al., 1996; Krezel et al., 1996; O. Wendling, P. C. and M. M., unpublished results) are not observed in the present Xα*af2^o*/Xβ/Xγ triple mutants.

At the molecular level, these differential requirements for RXR AF-2 may be best accounted for by our previous proposal (Kastner et al., 1997a) that (i) the retinoid signal for a given developmental process is most efficiently mediated by a 'preferential' RXR/RAR heterodimer, whose transcriptional activity is above a critical threshold level, and (ii) in some receptor knock-out mutants, the remaining RAR and/or RXR isotypes may still be functionally close enough to be able to replace the missing partner(s) of the 'preferential' heterodimer, in providing the critical level of activity. Defects caused by the RXRα*af2^o* mutation on its own will then correspond to events in which the cooperation between RXRα AF-2 and the 'preferential' RAR is indispensable for reaching the critical level of activity. On the contrary, defects whose generation requires both the RXRα*af2^o* mutation and the preferential RAR knockout (e.g. RARα in the case of a RXRα/RARα preferential heterodimer) will correspond to cases where the activity of the substituting heterodimer (e.g. RXRα/RARγ) still provides the critical level of activity. The additional RXRα*af2^o* mutation will then be required to generate the defects by bringing this level below the threshold. Alternatively, most of these 'second class' defects will also be generated by Xα*af2^o*/Xβ and Xα*af2^o*/Xβ/Xγ mutations which, by preventing RXRβ (and RXRγ) functionally substituting for RXRα AF-2 (see below), will decrease the level of activity of RXR/RAR heterodimers below the threshold. This threshold concept is well illustrated by the large decrease in the RARβ2 promoter activity of the RARβ2-*lacZ* transgene in Xα*af2^o*/Xβ mutants when compared with Xα*af2^o* mutants. The exceptions noted above (defects observed in Xα*af2^o*/RAR, but not in Xα*af2^o*/Xβ/Xγ mutants) will correspond to cases where the RXR AF-2 activity is not required to reach the threshold level, provided that the 'preferential' RAR is present.

In any event, an RXR AF-2 appears to be required in many instances, as a number of developmental events that are not affected by the single RXRα*af2^o* mutation are impaired upon further mutation of RXRβ and RXRγ. We have previously argued that much of the functional redundancy seen in knockout experiments reflects potentialities that are artefactually revealed in the mutants (Taneja et al., 1996; Kastner, 1997a). Therefore, if we assume that RXRβ (and RXRγ) do not perform the RXRα AF-2 functions under wild-type situations, all developmental events that are impaired in Xα*af2^o*/Xβ/Xγ mutants might require RXRα AF-2. As these mutants exhibit many of the abnormalities of the fetal VAD syndrome, most of the retinoid-dependent developmental events could involve the RXRα AF-2 function, in agreement with our previous conclusion that RXRα may assume most of the RXR developmental functions (see Introduction).

Is 9-*cis* RA required for RXR AF-2 activity during development?

It was initially controversial from in vitro and transfection studies whether RXR was transcriptionally active within RXR-RAR heterodimers, or rather serves as a silent RAR partner unable to bind its cognate ligand (reviewed in Leblanc and Stunnenberg, 1995; Mangelsdorf and Evans, 1995; Minucci and Ozato, 1996; Chambon, 1996; see Introduction). Subsequent studies using RAR- and/or RXR-selective synthetic retinoids and cultured cell lines definitely established that RXR can be a synergistic transcriptionally active partner (see Introduction for references). However, in all instances, RXR was transcriptionally inactive in the presence of its cognate ligand, unless its RAR partner was liganded (the so-called RXR subordination; Vivat et al., 1997; see Introduction). A similar synergy between RAR- and RXR-selective ligands was recently observed in vivo in chicken (Lu et al., 1997) and mouse (Elmazar et al., 1997; O. Wendling, P. C. and M. M., unpublished results) embryos.

Paralleling the cell differentiation and transcriptional activation data, the addition of a RAR ligand, but not of a RXR ligand, was shown to induce occupancy of the RARE and promoter elements, as well as chromatin structure alteration, at the RARβ2 promoter of P19 and NB4 cells (Minucci et al., 1997; Bhattacharyya et al., 1997; Chen et al., 1996; and references therein). However, while a suboptimal concentration of a RAR-selective ligand generated a very weak footprinting at the RARE, addition of the RXR-selective ligand strongly enhanced promoter occupancy (Minucci et al., 1997). Thus, the further liganding of RXR appears to increase the stability of heterodimer binding to an RARE, although on its own this RXR liganding has no effect. Interestingly, overexpression of a C-terminally truncated RXRβ, similar to RXRαAF2^o, significantly decreased the occupancy of the RARβ2 RARE and promoter elements in P19 cells, and had a dominant negative effect on the activity of a RARE reporter gene (Blanco et al., 1996). These studies indicate that both the integrity of the RXR AF-2 domain and a RXR ligand are important for increasing the stability of RARβ2 RARE occupancy, particularly at suboptimal concentrations of RAR ligand. Similarly, expressing physiological levels of RXRαAF2^o in RXRα$^{-/-}$ F9 cells (Clifford et al., 1996) did not restore primitive endodermal differentiation (nor expression of target RA responsive genes), when the RARγ AF-2 activity was lowered by using limiting concentrations of a RARγ-selective ligand. The concomitant addition of a RXR-selective ligand had no effect, even though RXRαAF2^o efficiently binds RXR ligands. In contrast, the expression of RXRαAF2^o restored both induction of differentiation and target gene expression in the presence of saturating amount of RARγ-selective ligand (J. Clifford, D. Metzger and P. C., unpublished results). Thus, Ozato's group and our own studies in EC cells indicate that, in the presence of both RXR and RAR ligands, RXRαAF2^o/RAR heterodimers are less efficient than wild-type heterodimers at promoting stable occupancy of RA response elements. As RXRαAF2^o/RAR heterodimers bind RARE in vitro as efficiently as WT heterodimers irrespective of ligand presence (Durand et al., 1994 and our unpublished results), this RXR AF-2- and ligand-dependent enhancement of promoter occupancy

most probably involves events occurring at the chromatin level (Bhatacharyya et al., 1997).

Our present study provides several lines of evidence suggesting that the role of RXR AF-2 within RXR/RAR heterodimers could be similar in EC cells and in mouse embryonic and fetal tissues. Firstly, RXRα AF-2 is obviously required for optimal function of RXRα/RAR heterodimers, but the X*αaf2^o* mutant phenotype is less dramatic than that of Xα mutants, indicating that RXRαAF2^o retains some RXRα function(s). Secondly, many of the X*αaf2^o* mutation-linked defects are generated only in a RXRβ$^{-/-}$ background, which on its own does not result in any developmental defect. This is most easily accounted for by assuming that RXRβ and RXRαAF2^o can functionally cooperate. If the stability of occupancy of RAREs by RXRαAF2^o/RAR heterodimers is indeed lower than that of wild-type heterodimers (see above), it is easy to imagine how the additional binding of RXRβ/RAR heterodimers could improve, at least partially, the occupancy of RA response elements and thus compensate for the lower efficiency of RXRαAF2^o/RAR heterodimer binding. Only concomitant RXR*αaf2^o* and RXRβ mutations would drastically reduce the occupancy of RA response elements, thus resulting in defects similar to those generated by RAR/RAR double knock-outs. Note, in this respect, that RXRβ appears to be ubiquitously expressed at many developmental stages (Dollé et al., 1994; Mangelsdorf et al., 1992). Thirdly, a decrease in the efficiency of binding of RXRαAF2^o/RAR heterodimers to RA response elements, would also explain why the RXR*αaf2^o* mutation is not a strong dominant negative one (see Results section), as indicated by the absence of defects in X*αaf2*/+ heterozygotes and the relative mildness of the RXR*αaf2^o* phenotype. Such a cooperation between RXRαAF2^o/RAR and RXRβ/RAR heterodimers would also account for the observation that the expression of the RARβ2-*lacZ* transgene is mostly unaffected in X*αaf2^o* mutants, but drastically decreased in X*αaf2^o*/RXRβ$^{-/-}$ double mutants, whereas it is somewhat restored in these double mutants upon administration of RA.

Finally, as the synergistic effects of RAR- and RXR-selective ligands in a number of cell systems and in chicken and mouse embryos suggest that RXR AF-2 activity is ligand-dependent (see above for references), it is tempting to speculate that the developmental abnormalities generated by the present RXR*αaf2^o* mutation reflect a key role of RXR ligands (9-*cis* RA or RXR-specific ligands not yet uncovered) in transactivation by retinoids during development. Supporting this possibility, Solomin et al. (1998), using a RXR-specific reporter transgene, have recently provided evidence that RXR ligands are likely to be present in mouse embryos. Alternatively, the requirement for RXR AF-2 may not be correlated with a RXR ligand-dependent function. Rather the integrity of RXR AF-2 would be required for transactivation events triggered by RXR post-translational modification (e.g. phosphorylation) or by ligand binding to the RAR partner (see Botling et al., 1997). The generation of mice bearing a RXR LBD mutation specifically preventing 9-*cis* RA binding may help in discriminating between these possibilities.

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