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Genetic evidence that the retinoid signal is transduced by heterodimeric RXR/RAR functional units during mouse development

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SUMMARY

We describe here the analysis of congenital malformations in compound mutant fetuses bearing null alleles in one RXR (α, β or γ) and one RAR (α, β or γ) isotype gene. A marked synergy was observed between the effects of mutations in RXRα and RARs, as a large number of developmental defects previously found mainly in RAR single and compound mutants were recapitulated in specific RXRα/RAR compound mutants. Several malformations were seen only in one type of RXRα/RAR mutant combination, whereas others were seen in several types of RXRα/RAR double mutants. No synergy was observed between the effects of mutations of either RXRβ or RXRγ mutations and those of any of the RAR mutations. These genetic data suggest that RXR/RAR heterodimers are the functional units transducing the retinoid signal for a large number of RA-dependent processes, and furthermore, that RXRα is the main RXR implicated in the developmental functions of RARs. The significance of these observations is discussed with respect to the problem of functional specificity and redundancy among retinoid receptors in vivo.

Key words: retinoic acid receptors, morphogenesis, gene knockout, mouse, embryonic development, genetic redundancy

INTRODUCTION

Vitamin A is important for many aspects of vertebrate physiology (Wolbach and Howe, 1925; see Sporn et al., 1994 and Blomhoff, 1994 for reviews and references). Most notably, retinoids (the active metabolites of vitamin A) appear to play an essential role during mammalian development, as studies of rat fetuses from vitamin A-deficient (VAD) dams revealed a large spectrum of abnormalities, known collectively as the fetal vitamin A deficiency syndrome (Wilson and Warkany, 1948, 1949; Warkany and Schraffenberger, 1946; Wilson et al., 1953). During the last decade, the characterization of two families of nuclear receptors for retinoids, the RARs (RARα, β, and γ; activated by all forms of physiologically occurring retinoic acids) and the RXRs (RXRα, β and γ; activated only by 9-cis RA), has revealed the complexity of the molecular machinery transducing the retinoid signal (for reviews, see Mangelsdorf and Evans, 1995; Chambon, 1996). Additional complexity was brought to light by the discovery that RXRs can not only form homodimers, but also heterodimers with a number of other nuclear receptors. Most notably, it was shown that RXRs are the nuclear factors required by RARs to bind tightly to a variety of cognate response elements in vitro (Leid et al., 1992; for additional references and a review, see Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995; Gronemeyer and Laudet, 1996; Chambon, 1996). This multiplicity of receptors and retinoid-responsive heterodimeric configurations raises a number of questions concerning their actual roles in the transduction of the retinoid signal in vivo.

Genetic analysis of the functions of the various RARs in the mouse, in both single and compound mutants, has clearly shown that RARs are involved in the mediation of the developmental functions of retinoids as, taken all together, these mutants recapitulate the complete spectrum of defects previously associated with the fetal VAD syndrome (Lohnes et al., 1994; Mendelsohn et al., 1994a; Luo et al., 1996; Grondona et al., 1996; Ghyselinck et al., unpublished observations). In addition to establishing the involvement of RARs in the known developmental role of vitamin A, the various RAR-deficient mice have also revealed many abnormalities that had not previously been associated with an impaired vitamin A function, most notably cranio-facial, axial and limb skeletal abnormalities (Lohnes et al., 1994; Mendelsohn et al., 1994a; Grondona et al., 1996; for a review see Kastner et al., 1995). Interestingly, most of these defects were observed only in double RAR mutants, indicating that in the absence of a given RAR, the remaining RARs can still perform many of the RAR developmental functions.

The role of RXRs in the mediation of the developmental retinoid signal is less clear. RXRβ and RXRγ null mutant mice are viable and do not display any abnormality obviously related to a known function of vitamin A (Kastner et al., 1996; Krezel

et al., 1996). In contrast, RXRα null fetuses die in utero and exhibit a hypoplasic ventricular myocardium and ocular abnormalities, which are similar to defects found in the fetal VAD syndrome (Sucov et al., 1994; Kastner et al., 1994). Furthermore, a preliminary analysis of a few RXRα/RARα and RXRα/RARγ compound mutants revealed a synergy between the effects of RXRα and RAR mutations: the anterior eye segment defects exhibited by RXRα$^{-/-}$ mutants appeared markedly enhanced upon additional inactivation of one or two alleles of RARγ; several RXRα/RAR compound mutants exhibited aortic arch abnormalities and/or partial or total lack of septation of the aortic sac, whereas these defects were not present in the corresponding single mutants (see Kastner et al., 1994). Thus, RXR/RAR heterodimers could actually be the functional units transducing the retinoid signal in vivo. However, as this synergy between RXR and RAR mutations was observed only for a rather restricted set of abnormalities, the question remains as to whether the RXRs are generally involved in the transduction of the RA signal by RARs. We report here a phenotypic characterization of all combinations of RXR (either α, β or γ)/ RAR (either α, β or γ) compound mutants. Taken all together, these various combinations of RXR/RAR mutations synergistically recapitulate the majority of the defects seen in RAR double mutants. These observations provide strong genetic evidence for an essential role for functional interactions between RXRs and RARs and, furthermore, indicate that RXRα is the RXR that is predominantly implicated in RAR action during ontogenesis.

MATERIALS AND METHODS

Mice

The RARα, RARβ, RARβ2, RARγ, RXRα, RXRβ and RXRγ single mutant mice lines have been respectively described in Lufkin et al. (1993), Ghyselinck et al. (manuscript submitted), Mendelsohn et al. (1994b), Lohnes et al. (1993), Kastner et al. (1994, 1996) and Krezel et al. (1996). All genotypings were performed by Southern blotting on tail DNA (for mice) or placental DNA (for fetuses), as described in these publications. To generate the double mutant fetuses, compound mutant mice of the appropriate genotype were mated and noon of the day of the vaginal plug was taken as day 0.5 of gestation. All the mice used in the present study were from a mixed 129/Sv/C57BL/6 genetic background.

Histological and skeletal analyses

Skeletons were prepared as described previously (Lufkin et al., 1992). For histological analyses, embryos or skinned fetuses were fixed in Bouin's solution. Paraffin sections, 7 μm thick, were stained with Groat's hematoxylin and Mallory's trichrome (Mark et al., 1993).

RESULTS

Generation of RXRα/RAR, RXRβ/RAR and RXRγ/RAR compound mutants

Mouse lines carrying null alleles for both a RXR gene (RXRα, RXRβ or RXRγ) and a RAR gene (RARα, RARβ, RARβ2 or RARγ) were generated by crossing the corresponding single mutant mice. To simplify nomenclature, RXR and RAR mutant alleles will be designated hereafter as Xα, Xβ, Aα, Aβ, etc., and the $^{-/-}$ sign indicative of homozygocity will be omitted. For example, RXRα$^{-/-}$/RARα$^{-/-}$ and RXRα$^{-/-}$/RARα$^{+/-}$ mutants will be referred to as Xα/Aα and Xα/Aα$^{+/-}$ mutants, respectively. In some of these compound mutants, we describe the occurrence of many defects that do not occur in the corresponding RXR or RAR single mutants.

(A) Ocular malformations in RXRα/RAR mutants

(1) Eyelid defects and anterior segment dysgenesis

As previously reported (Kastner et al., 1994), the closeness of the origins of the eyelids (which results in a hypoplastic conjunctival sac after eyelid closure; compare Fig. 1a with b), the thickening of the corneal stroma (compare C in Fig. 2a,b), and agenesis of the eye anterior chamber are constant features of the RXRα null phenotype. Similar ocular abnormalities were also observed in Aβ2/Aγ and Aβ/Aγ mutants (Lohnes et al., 1994; Ghyselinck et al., unpublished observations; C and small arrows in Fig. 2f). In the RXRα null genetic background, the severity of the eyelid and anterior segment defects increased in a graded manner upon inactivation of one and both alleles of the RARγ gene (Kastner et al., 1994); in Xα/Aγ mutants, the eyelids (small arrows in Fig. 2) and cornea (C) were replaced by a thick layer of undifferentiated mesenchyme (M, Fig. 2d and e) filling the space between the lens (L) and the surface ectoderm (E), these two latter structures always being connected through an epithelial stalk originating from a small pit at the surface (P, Fig. 1d). These observations strongly argue for the existence of a cooperation between RXRα and RARγ for eyelid and anterior segment formation. A synergy was also observed between the effects of the RXRα and the RARβ or RARβ2 mutations, as all Xα/Aβ and Xα/Aβ2 mutants exhibited a marked increase in the thickening of their corneal stroma and had a smaller palpebral fissure than Xα mutants (compare Fig. 1b with c, and small arrows in Fig. 2b and c; Table 1; and data not shown). However, the ocular abnormalities were less severe in these Xα/Aβ and Xα/Aβ2 mutants than in Xα/Aγ mutants (i.e. agenesis of the eyelids and cornea was never observed) and were comparable to those observed in Xα/Aγ$^{+/-}$ mutants (see Kastner et al., 1994). No synergy for eyelid and anterior segment malformations was observed between RXRα and RARα null mutations, as Xα/Aα mutants were not more affected than Xα single mutants (Kastner et al., 1994; and Table 1).

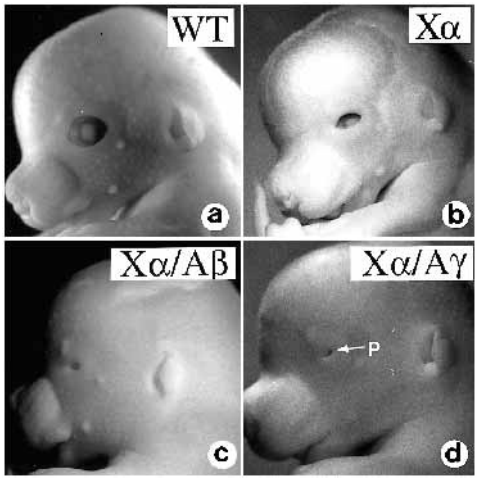


Fig. 1. External aspect of the eye region of 14.5 dpc wild type (WT) and mutant fetuses (genotypes as indicated). P, epithelial pit.

Table 1. Ocular defects in 14.5 dpc RXRα$^{-/-}$/RAR compound mutants

	RXRα$^{-/-}$/RAR (X/A) mutant genotypes and number of 14.5 dpc fetuses analysed					
Abnormalities	Xα/Aα$^{+/-}$ 6	Xα/Aα 6	Xα/Aβ$^{+/-}$ 5	Xα/Aβ 8	Xα/Aγ$^{+/-}$ 6	Xα/Aγ 6
Shorter ventral retina	(–)	++ (1/6) – (5/6)	(–)	(–)	+	++
Agenesis of the ventral retina (a)	0	0	0	0	0	U:1/6 B:1/6
Thicker corneal stroma	(–)	(–)	(–)	+	+	++
Smaller eyelids	(–)	(–)	(–)	+	+	NA
Eyelid agenesis	0	0	0	0	0	B:6/6

+ and ++ indicate increasing degrees in the severity of the defects when compared with RXRα single null mutants and (–) the absence of such increases; 0, indicates the absence of the defect. Note that the ocular phenotypes of Xα/Aβ and Xα/Aβ2 fetuses were apparently identical.

(a) Typical complete coloboma of the retina; B, bilateral; U, unilateral; NA, not applicable. For further details see the text and Kastner et al. (1994).

(2) Ventral retina defects

A shortening of the ventral portion of the retina has been previously reported in all Xα mutant fetuses (Kastner et al., 1994; compare D and V in Fig. 2a and b) and in some VAD fetuses (Warkany and Schraffenberger, 1946). Furthermore, we have previously reported that some Aα/Aγ mutant mice displayed a typical complete coloboma of the retina, an abnormality manifested by a complete absence of the ventralmost portion of the retina, which results from the lack of closure of the optic fissure (Lohnes et al., 1994).

A short ventral retina was not detected in any of the 18.5 dpc RAR compound mutants that were analysed (see Lohnes et al., 1994), but this abnormality might have been missed due to the presence of extensive retinal foldings. In fact, when analysed at 14.5 dpc, all Aβ/Aγ mutant retinas showed an obvious shortening of their ventral portion (Ghyselinck et al., unpublished observations; compare D and V in Fig. 2a and f). Altogether these data implicate RARs in both ventral retina formation and growth. The ventral retina was shorter in Xα/Aγ than in Xα fetuses (Fig. 2d), and in two cases such compound mutants showed an absence of the ventral retina in the anterior region of the optic cup, corresponding to a unilateral or bilateral typical complete coloboma of the retina (Fig. 2e). The occurrence of both types of ventral retinal defects in Xα/Aγ mutants suggests that the processes of formation and growth of the ventral retinal field may involve a common, RA-dependent, genetic pathway. In any event, our data indicate synergistic effects of the RXRα and RARγ null mutations to impair the development of the ventral retina. In contrast, no obvious synergy for generating ventral retinal defects was observed between RXRα and either RARα or RARβ mutations (Table 1).

(3) Retrolenticular membrane

A retrolenticular membrane, which results from the persistence and hyperplasia of the primary vitreous body, was observed in all Xα mutants (R in Fig. 2b; Kastner et al., 1994), as well as in most Aβ or Aβ2 mutants (Grondona et al., 1996; Ghyselinck et al., unpublished observation). Interestingly, a synergy between the effects of RXRα and RARβ mutations was frequently observed, a retrolenticular membrane being present in the eyes of 66% of Xα$^{+/-}$/Aβ$^{+/-}$ mutants, whereas this abnormality was observed much less frequently in single heterozygotes of either genotype (Table 2).

(B) Abnormalities of the cardio-vascular system in RXRα/RAR mutants

(1) Abnormalities of the aorticopulmonary septum and great arteries located near the heart

Persistent truncus arteriosus (PTA) results from a failure of complete aortic sac division by the aorticopulmonary septum (AP, Fig. 3a,d,e), which is derived from the cardiac neural crest, a subpopulation of neural crest cells (NCC) originating from the caudal rhombencephalon. A PTA has been observed in all Aα/Aβ2, Aα/Aβ and Aα/Aγ mutants (Mendelsohn et al., 1994a; Ghyselinck et al., unpublished observations). A complete PTA was present in all Xα/Aα mutants and in some Xα/Aβ and Xα/Aγ mutants (Table 3; TA, Fig. 3c), and a partial PTA (i.e. an aortico-pulmonary window; white arrows in Fig. 3b) was observed in one Xα/Aα$^{+/-}$ and one Xα/Aγ fetus (Table 3). In addition, a single case of transposition of the great arteries was observed in a Xα/Aα$^{+/-}$ mutant (Fig. 3d-g). In this abnormality, the aorta (AS, Fig. 3d,e) arises ventrally from the right ventricle (rV, Fig. 3g) and the pulmonary trunk (PT, Fig. 3d,e)

Table 2. Persistent hyperplastic primary vitreous (retrolenticular membrane) in adult (i.e. 3 to 8 month old) RARβ and RXRα$^{+/-}$ single and RARβ/RXRα double mutants

	RAR(A) and RXR(X) mutant genotypes (number of animals analysed)					
Abnormalities	Aβ$^{+/-}$ (16)	Aβ (36)	Xα$^{+/-}$ (10)	Aβ$^{+/-}$ Xα$^{+/-}$ (18)	Aβ Xα$^{+/-}$ (16)	WT (16)
Persistent hyperplastic primary vitreous (PHPV) (retrolenticular membrane)	B:1/16	U:2/36 B:30/36	B:1/10	U:9/18 B:6/18	U: 1/16 B:13/16	U:1/16
Percentage of eyes with a PHPV	6%	86%	10%	66%	84%	3%

B, bilateral; U, unilateral; WT, wild type.

arises dorsally from the left ventricle (fV, Fig. 3g). Thus, the relative positions within the heart of the infundibula of these two vessels are inverted (compare AF and PF in Fig. 3f and h). It is noteworthy that, although not observed in RAR compound mutants analysed so far, transposition of the great arteries is caused by a failure of the aorticopulmonary septum to spiral (compare AP in Fig. 3a and d), and therefore corresponds, like PTA, to a developmental defect of cardiac neural crest cells.

In addition to the aorticopulmonary septum, the cardiac neural crest gives rise to the tunica media of the systemic arch (arch of the aorta), subclavian and commun carotid arteries (Kirby and Waldo, 1990, and references therein). We have shown that the patterning of these vessels was always altered in Aα/Aβ, Aα/Aβ2 and Aα/Aγ double null mutants (Mendelsohn et al., 1994a; Ghyselinck et al., unpublished observations). Similar defects in arterial patterning were observed in the vast majority of the Xα/Aα mutants, and in some Xα/Aβ and Xα/Aγ mutants (e.g. right-sided arch of the aorta; rAA in Figs 3c, 4f; compare with the normal arch AA in Figs 3a, 4e; see also the comment of Table 3). Amongst RXRα homozygote/RAR heterozygote compound mutants, both Xα/Aα$^{+/-}$ and Xα/Aγ$^{+/-}$ mutants occasionally displayed defects in the structures derived from cardiac NCC (Table 3).

(2) Abnormalities of the conotruncal and atrio-ventricular septa

The conotruncal septum (CT, Fig. 3h), although continuous with its aorticopulmonary counterpart, has a distinct embryological origin, since it is derived from the fusion of two local outgrowths of the endocardium (Noden, 1991). A conotruncal septal defect (high ventricular septal defect) was frequently observed in RAR compound mutants and occurred generally as a defect secondary to PTA (discussed in Mendelsohn et al., 1994a). As expected, the compulsory association 'conotruncal septum agenesis-PTA' was found in RXRα$^{-/-}$/RAR compound mutants (Table 3). Primary conotruncal septal defects (i.e. without a PTA) were observed in approx. 35% of the RXRα single null mutants and in some Xα/Aα$^{+/-}$, Xα/Aγ$^{+/-}$, Xα/Aγ and Xα/Aβ2 mutants (Table 3; IV in Fig. 3g,i; compare with CT in Fig. 3h). The penetrance of this abnormality became complete upon the removal of only one allele of the RARβ gene from the RXRα null background. It is noteworthy that in this case, and in contrast to most other phenotypes described here, the synergy was observed only with the RARβ null allele and not the RARβ2 mutant allele, which suggests that the RARβ1/β3 isoforms could be preferentially involved in conotruncal septation.

The two atrioventricular cushions (see E, Fig. 3k), which are also derived from the endocardium, fuse during embryogenesis to form the septum intermedium (or atrioventricular septum; AVS in Fig. 3j). Absence of this fusion results in a common atrioventricular canal (curved arrow in Fig. 3k). In RXRα/RAR compound mutants, as in RAR double mutants (Mendelsohn et al., 1994a), this defect was always associated with, and thus might be secondary to, severe defects of the heart outflow tract septation (discussed in Kirby and Waldo, 1990).

(C) Respiratory tract defects in RXRα/RAR mutants

Severe bilateral hypoplasia of the lungs, or right lung hypoplasia with left lung agenesis, as well as absence of the oesophagotracheal septum, were consistently observed in Aα/Aβ2 and Aα/Aβ mutants (Mendelsohn et al., 1994a; Ghyselinck et al., unpublished observations), but were absent in Xα mutants (Kastner et al., 1994; compare fL and rL in Fig. 4a, b and d). The lungs of almost all Xα/Aα mutants were markedly hypoplastic (Fig. 4c) and, in addition, two thirds of these mutants lacked the oesophagotracheal septum (e.g. compare O and T with OT in Fig. 4e and f; Table 3). In contrast, in Xα/Aβ and Xα/Aγ mutants the size of the lungs was similar to that of their Xα littermates and the oesophagotracheal septum was always present (Table 3).

The morphogenesis of the tracheal cartilaginous rings is critically dependent on RARs, as extensive disorganisation of

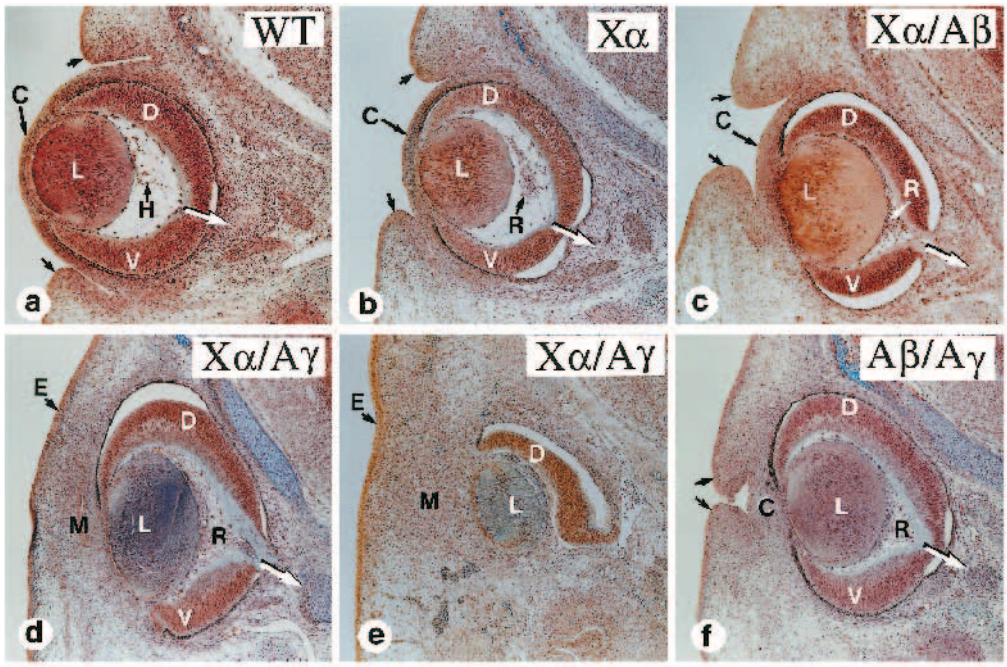


Fig. 2. Frontal histological sections of the ocular region of 14.5 dpc wild type (WT) and mutant fetuses (genotypes as indicated). C, cornea; D, dorsal retina; E, surface ectoderm; H, hyaloid vessels; L, lens; M, the abnormal mesenchyme interposed between the surface ectoderm and the lens; R, retrolenticular membrane; V, ventral retina. The long arrow indicates the level of the optic nerve exit point. The small arrows point to the eyelids. Magnification : ×32 (a-f). Note that the small changes in the size of eye components between the different micrographs are due to slight variations in: (1) the developmental state of the fetuses, (2) the planes of sectioning and (3) the dilation of the embedding medium.

these rings and/or ring fusions was observed in RAR compound mutants (Mendelsohn et al., 1994a), and to a lesser extent in RARγ single null mutants (Lohnes et al., 1993; see Fig. 6c). The trachea of three out of five Xα$^{+/-}$/Aγ mutants exhibited a tracheal ring disorganisation that was markedly more pronounced than in any RARγ single mutant (compare Fig. 6c and d). In addition, four out of eight Xα$^{+/-}$/Aα mutants displayed disorganised tracheal rings in the anterior portion of the trachea (AT in Fig. 6b; Table 4; note that tracheas of Xα$^{+/-}$ and Aα mutants appeared normal). No tracheal malformation was observed in Xα$^{+/-}$/Aβ2 mutants.

(D) Abnormalities of the urogenital system

(1) Kidney and ureter defects in RXRα/RAR mutants

Kidney hypoplasia has been previously observed in Aα/Aβ2 and Aα/Aγ mutants, as well as kidney agenesis (always accompanied by ureter agenesis) in Aα/Aγ mutants (Mendelsohn et al., 1994a). Amongst RXRα/RAR compound mutants, only Xα/Aα mutants showed an obvious and fully penetrant decrease in kidney size (compare K, Fig. 5a,c with b,d; Table 3). Kidney and ureter agenesis was rare and associated only with the Xα/Aα genotype (Table 3).

Agenesis of the caudal ureter and/or ectopic ureteral

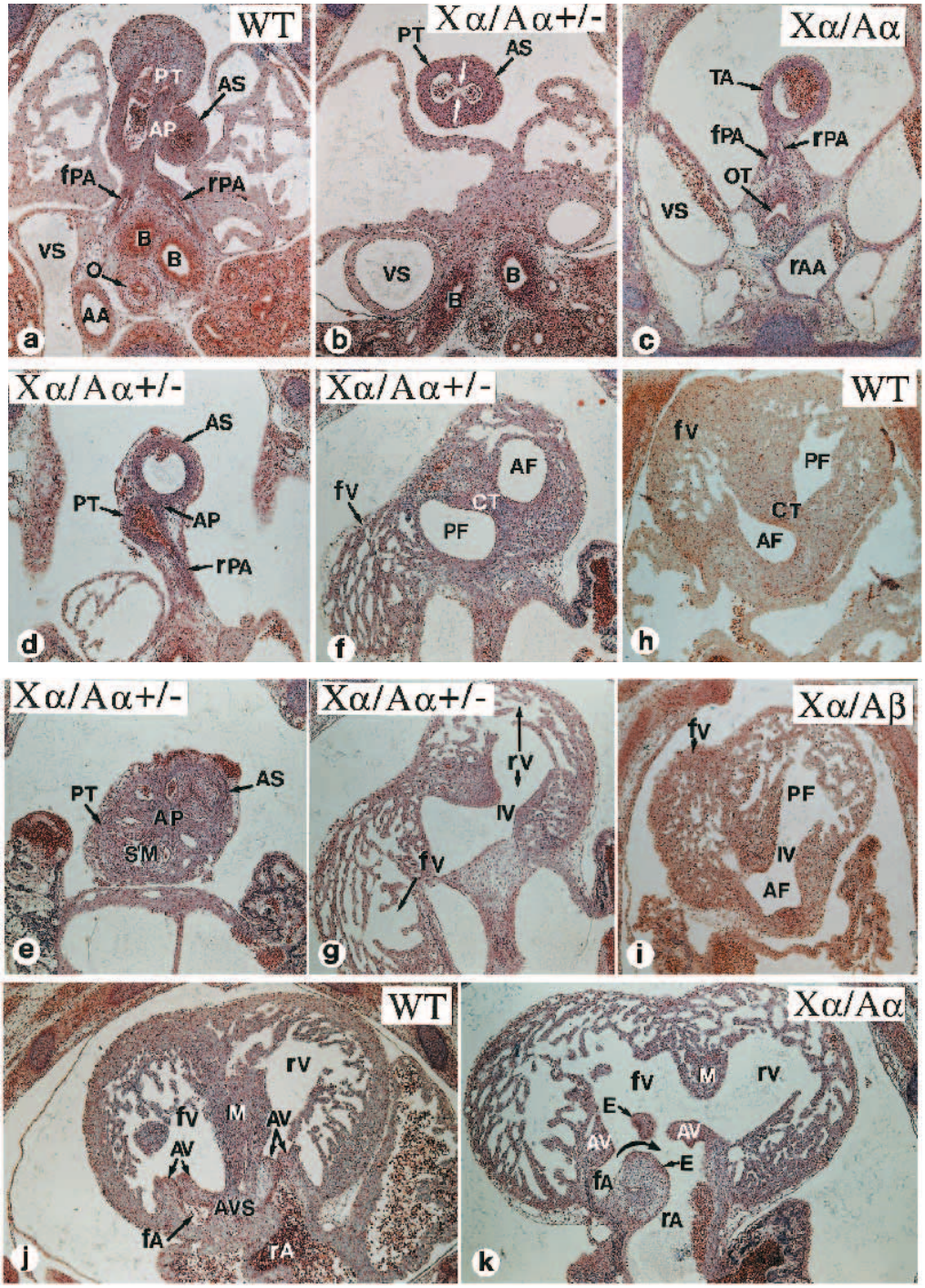


Fig. 3. Comparison of the heart and heart outflow vessels on transverse histological sections of 14.5 dpc wild type (WT) and mutant fetuses (genotypes as indicated). (a-c) Sections at (a,c) or slightly caudal to (b) the level of origin of the pulmonary arteries (fPA and rPA). (d-g) Adjacent sections through the same mutant heart (d: most rostral; g: most caudal), illustrating the transposition of the great vessels: (d) level of origin of the right pulmonary artery (rPA); (e) level of the semilunar valves (SM), corresponding to the junction between the arterial and ventricular portions of the cardiac outflow tract; (f) level of the conotruncus; (g) section through the trabeculated portions of the ventricles (rV and fV). (h,i) Sections at similar levels of the conotruncus. (j-k) Sections at the level of the atrioventricular canals. rA and fA, right and left atrium respectively; AA, arch of the aorta; rAA, right-sided arch of the aorta; AF, aortic infundibulum; AS, ascending aorta; AP, aortico pulmonary septum; AV, atrioventricular valves; AVS, atrioventricular septum; B, stem bronchi; CT, conotruncal septum; E, unfused atrioventricular cushions; IV, interventricular communication; M, muscular ventricular septum; O, oesophagus; OT, oesophagotrachea; rPA and fPA, right and left pulmonary arteries respectively; PF, pulmonary infundibulum; PT, pulmonary trunk; SM, semilunar valve in the pulmonary trunk; rV and fV, trabeculated portions of the right and left ventricles respectively; TA, persistent truncus arteriosus; VS, left superior vena cava. The white arrows in b point to the aorticopulmonary window and the curved arrow in k crosses the persistent atrioventricular canal. The ventralmost structures are located at the upper border of each micrograph. Magnification ×30 (a-k).

Table 3. Abnormalities of the cardio-vascular, repiratory and genito-urinary systems and glandular defects in RXRα$^{-/-}$/RAR compound mutants

Abnormalities	RXRα$^{-/-}$/RAR (X/A) mutant genotypes (number of 14.5 dpc fetuses analysed)									Genotype of RAR double (A/A) mutants exhibiting similar abnormalities
	Xα/Aα (6)	Xα/Aα$^{+/-}$ (6)	Xα/Aβ (8)	Xα/Aβ$^{+/-}$ (5)	Xα/Aγ (6)	Xα/Aγ$^{+/-}$ (6)	Xα/Aβ2 (5)	Xα/Aβ2$^{+/-}$ (7)	Xα (22)	
Heart and arterial defects										
•Commun atrioventricular canal	2/6	2/6	1/8	0	0	0	0	0	0	Aα/Aβ2,Aα/Aγ
• Conotruncal ventricular septal defect										Aα/Aβ2*, Aα/Aγ*
- partial (proximal)	NA	1/6	2/8	2/5	0	2/6	0	0	1/22	
- complete	6/6	1/6	6/8	3/5	5/6	0	1/5	0	7/22	
• Persistent truncus) arteriosus (NCC)										Aα/Aβ2*, Aα/Aγ*
- partial (aorticopulmonary window)	NA	1/6	0	0	1/6	0	0	0	0	
- complete	6/6	0	2/8	0	2/6	0	0	0	0	
• Transposition of great vessels (NCC)	NA	1/6	0	0	0	0	0	0	0	0
• Abnormal arteries† (NCC)	5/6	1/6	2/8	0	1/6	1/6	0	0	0	Aα/Aβ2*, Aα/Aγ*
Respiratory tract defects										
• Lung hypoplasia	5/6	0	0	0	0	0	0	0	0	Aα/Aβ2*
• Lack of oesophagotracheal septum	4/6	0	0	0	0	0	0	0	0	Aα/Aβ2*
Thymic agenesis	0	0	U:2/8	0	0	0	U:3/5	0	0	0
Submandibular gland hypoplasia	0	0	0	0	6/6	0	0	0	0	Aα1/Aγ
Genito-urinary tract defects										
• Müllerian duct agenesis										
- partial (caudal portion missing)	NA	U:1/6 B:4/6	B:7/8	B:3/5	B:2/6	U:1/6	U:1/5 B:3/5	0	0	Aα/Aγ* (partial) Aα/Aβ2* (complete)
- complete	B:6/6	0	0	0	0	0	0	0	0	
• Ectopic ureteral openings	U:2/6 B:4/6	B:1/6	U:1/8 B:7/8	B:3/5	U:1/6 B:2/6	B:1/6	B:5/5	0	0	Aα/Aβ2
• Ureter agenesis										Aα/Aβ2, Aα/Aγ*
- partial (caudal portion missing)	0	0	U:1/8	0	0	0	0	0	0	
- complete	U:1/6	0	0	0	0	0	0	0	0	
• Kidney hypoplasia	U:1/6 B:5/6	0	0	0	0	0	0	0	0	Aα/Aβ2*
• Kidney agenesis	U:1/6	0	0	0	0	0	0	0	0	Aα/Aγ

*These defects are fully penetrant. U, unilateral; B, bilateral. NCC: these defects are likely to be caused by abnormal migration, proliferation, death or differentiation of cardiac neural crest cells.

†Arteries derived from aortic arches, including: right-sided arch of the aorta, i.e. the miror image of the normal arterial pattern (Xα/Aα: 2/6; Xα/Aβ: 1/8; Xα /Aγ$^{+/-}$: 1/6); double (left and right) arch of the aorta (Xα/Aα: 2/6); retroesophageal right subclavian artery (Xα/Aα$^{+/-}$: 1/6; Xα/Aβ: 1/8); aberrant origin of one pulmonary artery from the innominate artery or from the arch of the aorta (Xα/Aα: 1/6; Xα/Aγ: 1/6). For further details see the text, Mendelsohn et al. (1994a) and Lohnes et al. (1994). Note also that only two of the six Xα/Aα$^{+/-}$ mutants displayed a conotruncal septal defect instead of four out of six, as indicated in Table 3 of Kastner et al. (1994), due to a typing error.

openings in the urethra have been previously detected in most Aα/Aβ2 and all Aα/Aγ mutant mice analysed at 18.5 dpc (Mendelsohn et al., 1994a). With the exception of Xα/Aβ2$^{+/-}$ mutants, ureters (U) opening in the caudal urogenital sinus (US, the future pelvic urethra) at the same level as the Wolffian ducts (WD) were found in all types of 14.5 dpc RXRα$^{-/-}$/RAR compound mutants (e.g. compare U, US and WD in Fig. 5e-h; Table 3). Thus, this failure of the caudal Wolffian duct to incorporate into the dorsal wall of the urinogenital sinus (Larsen, 1993 and references therein), which is the defect underlying ectopic ureteral openings, can be generated by the absence of any of the RARs in mice lacking RXRα.

(2) Müllerian duct agenesis

We have previously reported that Aα/Aβ2 double mutants consistently exhibit a complete absence of the Müllerian ducts (MD), leading in females to the absence of oviducts, uterus and cranial portion of the vagina (Mendelsohn et al., 1994a). Likewise, a complete bilateral agenesis of these ducts was observed in all Xα/Aα mutants (compare MD, Fig. 5c and d; Table 3). In addition, a sometimes unilateral, partial agenesis of the caudal portion of the Müllerian duct (see MD, Fig. 5e and f) was seen in most RXRα$^{-/-}$/RAR compound mutant genotypes (Xα/Aα$^{+/-}$, Xα/Aβ2, Xα/Aβ, Xα/Aβ$^{+/-}$, Xα/Aγ$^{+/}$, Xα/Aγ; Table 3). Therefore in mice lacking RXRα, agenesis of the rostral Müllerian duct only occurs upon further inactivation of RARα, whereas in the same context agenesis of the caudal Müllerian duct can be generated by inactivation of any the RARs.

(E) Glandular abnormalities

Thymic agenesis was observed in some Aα/Aβ mutants (Ghyselinck et al., unpublished observations) and agenesis of the submandibular gland in some Aα/Aγ mutants (Lohnes et al., 1994). In the present study, absence of one thymic lobe was only detected in some Xα/Aβ and Xα/Aβ2 mutants and hypoplasia of the submandibular gland anlage was seen in all Xα/Aγ mutants (Table 3 and data not shown).

(F) Skeletal abnormalities

(1) Axial skeleton

The analysis of RAR single and compound mutants has revealed an important role of RARs in the patterning of the axial skeleton in the cervical region: malformations (including homeotic transformations) of cervical vertebrae occur frequently in RARγ and occasionally in RARα null mutants,

whereas the cervical region is markedly disorganised in Aα/Aγ mutants (Lohnes et al., 1994). We have examined here the effect on cervical region patterning of the inactivation of one RXRα allele within several RAR mutant backgrounds. Remarkably, $Xα^{+/-}/Aγ^{+/-}$, as well as $Xα^{+/-}/Aα^{+/-}$ newborn skeletons, exhibited a high frequency of defects in their cervical region, since 7/11 $Xα^{+/-}/Aγ^{+/-}$ and 10/15 $Xα^{+/-}/Aα^{+/-}$ skeletons had at least one defect affecting cervical vertebrae (Table 4). $Xα^{+/-}/Aα$ mutants also exhibited a much higher incidence of defects than Aα single mutants, in which cervical vertebrae defects were found only rarely (Table 4; Lohnes et al., 1994). In several cases, an anterior process (AAA*) was present on C2, reminiscent of the anterior arch of the atlas (AAA), which is likely to correspond to a partial homeotic transformation of C2 into C1 (Fig. 6e,f; Table 4; see Lohnes et al., 1993). The presence of a cartilaginous extention fusing C2 to the anterior arch of the atlas was also frequently observed, as well as a fusion between C2 and C3 (Table 4). Interestingly, this latter defect seemed to arise preferentially in the compound mutants between Xα and Aα, and may have a high penetrance in $Xα^{+/-}/Aα$ mutants. Other defects observed in RXRα/RARα or RARγ compound mutants were C2 dysymphisis, fusion of the basioccipital bone with the anterior arch of the atlas or to the axis dens and C7 to C6 transformation (revealed by the occurence of the tuberculum anterior on C7). Control mice also occasionally exhibited cervical vertebrae malformations, but at a much lower frequency than the corresponding compound mutants (see Table 4). Together, these observations strongly suggest a synergistic effect between the RXRα and the RARα or γ mutations for generating defects during cervical vertebrae morphogenesis. It is noteworthy that the defects observed affected preferentially C2 (see Table 4); thus the morphogenesis of this vertebra appears to be highly sensitive to reduced retinoid receptor gene dosage.

Fusion of the basioccipital bone to the anterior arch of the atlas was occasionally observed in Aγ mutants (Lohnes et al., 1993; Table 4). In some instances, this fusion did not occur, but instead an outgrowth was present on the ventral side of the basioccipital bone, budding towards the anterior arch of the atlas (Table 4). Interestingly, a similar budding, as well as one case of fusion, was observed in several $Xα^{+/-}/Aγ^{+/-}$ skeletons (open arrow in Fig. 6h, and Table 4), suggesting that a synergy between the effects of the two heterozygote mutations can lead to perturbation of basioccipital bone morphogenesis.

(2) Limb skeletal defects

Aα/Aγ double mutants display a number of limb skeletal abnormalities, which include size reduction of the scapula, perforated scapula, radius agenesis and abnormal digit number (Lohnes et al., 1994). Interestingly, one of two Xα/Aγ 14.5 dpc mutant skeletons exhibited a large hole in its left scapula, a feature never seen in Xα or Aγ mutants (Fig. 6i,j; and data not shown). As such a malformation was never observed in any of the hundreds of skeletons of various genotypes examined (except Aα/Aγ), it probably reflects a synergy between the effects of the RXRα and the RARγ mutations. Note that humerus, radius and ulna, as well as digit number, were not affected in any of the RXRα/RAR compound mutants (Fig. 6j, and data not shown).

(3) Cranio-facial skeletal abnormalities

A cartilaginous or ossified rod linking the alisphenoid bone to the incus has been observed in Aα/Aγ and Aα/Aβ2 mutants (Lohnes et al., 1994). This abnormal supernumerary skeletal element is homologous to the pterygoquadrate bone present in the reptilian ancestors of mammals (discussed in Lohnes et al., 1994). A unilateral pterygoquadrate element was also seen in about 10% of the Aα mutants (our unpublished data). Interestingly, several $Xα^{+/-}/Aα$ mutants exhibited this atavistic structure, which was often bilateral (Table 4, and data not shown). These observations indicate a synergy between the effects of RXRα and RARα mutations for the occurrence of a pterygoquadrate element. In contrast, $Xα^{+/-}/Aγ$ and $Xα^{+/-}/Aβ2$ mutants did not exhibit this atavistic malformation.

Table 4. Skeletal abnormalities in newborn RXRα/RAR compound mutants

	Mutant genotypes and number of skeletons analysed											
Abnormalities	WT (14)	$Xα^{+/-}$ (13)	$Aγ^{+/-}$ (14)	$Aγ^{-/-}$ (12)	$Xα^{+/-}$ $Aγ^{+/-}$ (11)	$Xα^{+/-}$ $Aγ^{-/-}$ (5)	$Aα^{+/-}$ (8)	$Aα^{-/-}$ (3)	$Xα^{+/-}$ $Aα^{+/-}$ (15)	$Xα^{+/-}$ $Aα^{-/-}$ (8)	$Xα^{+/-}$ $Aβ_2^{-/-}$ (5)	$Aβ_2^{-/-}$ (5)
C2 malformations												
Anterior process on C2	0	0	0	3	4	2	0	0	3	1	0	0
Cartilaginous fusion of C2 to AAA	0	2	1	0	6	1	0	0	4	3	0	0
C2-C3 fusion	1	0	1	3	1	1	0	0	3	6	0	0
C2 dysymphisis	0	0	0	0	0	2	0	0	2	0	0	0
Axis dens-basioccipital fusion	0	0	0	0	0	0	0	0	1	0	0	0
Basioccipital bone												
Ventral budding (V)	0	V:1	0	V:2	V:3	V:1	0	0	0	0	0	0
Fusion to AAA (F)				F:4	F:1	F:3						
Tuberculum anterior on C7	0	0	0	0	0	0	0	0	1	0	0	0
Pterygoquadrate	0	0	0	0	0	0	0	U:1	0	B:3 U:1	0	0
Enhanced tracheal malformations	0	0	0	0	0	3*	0	0	1†	4†	0	0

*With respect to the corresponding RXRα or RARγ single mutant genotype.
†Affecting only the anterior portion of the trachea.
U, unilateral; B, bilateral; AAA anterior arch of the atlas.

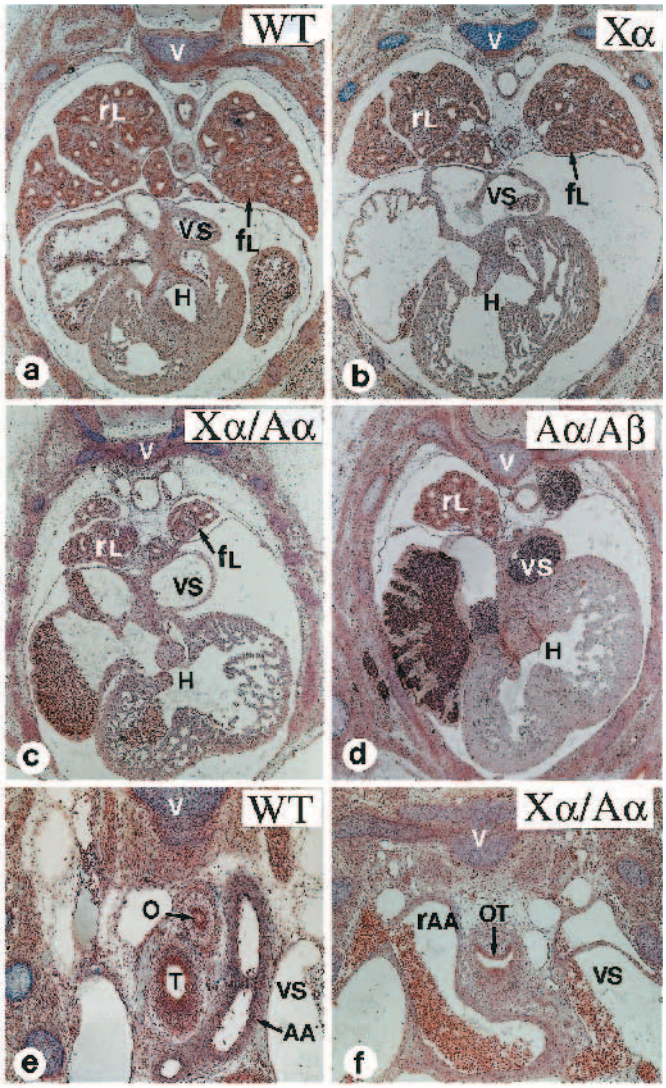


Fig. 4. Comparison of the lungs and trachea on transverse histological sections at comparable levels of wild type (WT) and mutant 14.5 dpc fetuses (genotypes as indicated). Note the complete absence of the left lung in the Aα/Aβ mutant. AA, arch of the aorta; rAA, right arch of the aorta; H, heart; O, oesophagus; OT, oesophagotrachea; T, trachea; V, vertebra; VS, left superior vena cava. The ventralmost structures are located at the bottom of each micrograph. Magnification: ×16 (a-d) and ×30 (e,f).

One out of the 6 Xα/Aγ mutant analysed on serial histological sections displayed a duplication of the nasal septum, a defect also observed in all Aα1/Aα2$^{+/-}$/Aγ mutants and corresponding to a deficiency in cranial neural crest cells (discussed in Lohnes et al., 1994). Aside from the mutants mentioned above, the abnormality was absent in the fetuses of various genotypes that we have analysed, and therefore it might reflect a synergism between the effects of the RXRα and the RARγ mutations.

(G) Interdigital webbing

Interdigital webbing was observed with an incomplete penetrance in Aα and Aγ mutants (Lufkin et al., 1993; Fig. 7). This phenotype affects mostly the areas between digits 2-3 and 3-4 in the hindlimbs, and was generally more pronounced between digits 2 and 3 (see panels II and III in Fig. 7A). Whenever an animal was affected, the two hindlimbs were generally affected to a similar extent; forelimbs were mildly affected only in animals that exhibited extensive or severe hindlimb webbing. A mild interdigital webbing was also observed at a low frequency in Xα$^{+/-}$ mutants (Fig. 7). The frequency of webbing increased markedly upon inactivation of one RARα or RARγ allele within the Xα$^{+/-}$ background, as about 50% of Xα$^{+/-}$/Aα$^{+/-}$ or Xα$^{+/-}$/Aγ$^{+/-}$ animals were affected. In addition, a significant proportion of these double heterozygotes displayed an extensive webbing (see Fig. 7A, panel III), which was never observed in Xα$^{+/-}$ animals (Fig. 7B). This synergy between the effects of RXRα and RAR mutations was also evident from the occurrence of a complete webbing of digits 2-3-4 in most Xα$^{+/-}$/Aγ and Xα$^{+/-}$/Aα$^{+/-}$/Aγ$^{+/-}$ mice (see Fig. 7A, panel IV; note that Aα$^{+/-}$/Aγ$^{+/-}$ only occasionally exhibited a mild webbing). No interdigital webbing was seen in Xα$^{+/-}$/Aβ or Xα$^{+/-}$/Aβ2 mice. Therefore, even though RARβ is selectively expressed in interdigital tissues during development (Dollé et al., 1989), this receptor does not appear to play an important role in the involution of the interdigital tissue. However, the role of RARβ was apparent in some mutant backgrounds, as (1) Aγ$^{+/-}$/Aβ2 mice often exhibited moderate interdigital webbing, (2) Xα$^{+/-}$/Aγ$^{+/-}$/Aβ2 mice always displayed a severe webbing (data not shown).

(H) Absence of synergistic effects of mutations in RARs and RXRβ or RXRγ

Xβ/Aα, Xβ/Aβ2 and Xβ/Aγ double null mutants were viable, and did not not exhibit an increased lethality when compared to the corresponding RAR single null mutants (Lohnes et al., 1993; Lufkin et al., 1993; Chambon, 1994). Xβ/Aα and Xβ/Aβ2 double mutants appeared morphologically normal, and females were fertile (all RXRβ$^{-/-}$/RAR compound mutant males were sterile, a consequence of the RXRβ, RARα or RARγ gene disruption). Xβ/Aγ double mutants were not more runted than single RARγ null mutants. Analysis of serial histological sections of 18.5 dpc double mutants of each of these genotypes did not reveal any of the abnormalities present in the various RAR compound mutants. In addition, skeletons of two 18.5 dpc Xβ/Aγ mutants did not appear more affected than those of Aγ mutants.

All three types of RXRγ/RAR compound mutant exhibited the same viability as RAR single mutants. Morphological defects were not detected upon dissection of adult double mutants or by analysis of serial histological sections of 18.5 dpc fetuses (performed in the case of Xγ/Aβ and Xγ/Aγ mutants). In addition, Xγ/Aβ mutants were fertile, as well as Xγ/Aα females.

Thus, it appears that there is no obvious synergy between the effects of RAR mutations and mutations of either RXRβ or RXRγ for generating the developmental defects resulting from RAR mutations on their own.

DISCUSSION

RXRα/RAR heterodimers as functional units in vivo

We have shown here that the inactivation of one or both RXRα alleles, combined with that of one or both alleles of a given

RAR isotype, can lead to specific defects that do not occur in the corresponding RXRα or RAR single mutant mice. Importantly, each of these defects has been previously observed in the context of one or several RAR isotype double mutations (Lohnes et al., 1994; Mendelsohn et al., 1994a; Luo et al., 1996; Ghyselinck et al., unpublished observations). In other words, in the genetic background of a given RAR, RXRα can become essential to enable the remaining RAR(s) to functionally replace the knocked-out RAR. Thus, as many of these defects belong to the fetal VAD syndrome, these synergistic effects of compound RXRα/RAR mutations strongly support the conclusion that RXR/RAR heterodimers act as functional units transducing the retinoid signals in vivo.

Alternatively, RXRs and RARs may act independently on the expression of specific subset(s) of target genes encoding proteins exhibiting distinct functions, but synergizing for the realization of a given developmental process. This would correspond to distinct molecular events occurring in a single cell (cell-autonomous) or different cells (non-cell autonomous). Although such more complex possibilities are not excluded, they do not easily account for two sets of observations: (1) compound RAR isotype mutants and RXR/RAR mutants very often exhibit the same defects, which can be readily interpreted only in the light of the heterodimer hypothesis (which implies that RAR and RXR act on the same molecular events), provided that there is some functional redundancy amongst RARs (see below); (2) in several cases, the synergistic effect of an RXRα and an RAR isotype mutation is already apparent in mice heterozygous for one (or even both) of the two receptors, which can also be readily interpreted in the light of the heterodimer hypothesis, while, in the context of more complex scenarios, it would imply that two distinct synergizing events are markedly sensitive to gene dosage.

The present genetic evidence, which supports the conclusion that RXRα/RAR heterodimers act as functional units transducing the retinoid signals, is in full agreement with previous studies showing conclusively that RXR/RAR heterodimers bind more tightly and specifically than RARs on their own to retinoid response elements in vitro, and also with results from transfection studies in cells cultured in vitro, which have demonstrated that RXR/RAR act as functional units (Leid et al., 1992; Nagpal et al., 1993; reviewed in Chambon, 1996). Importantly, more physiologically relevant studies of RA-induced differentiation and of target gene expression using receptor-specific synthetic retinoids and F9 embryonal carcinoma cells bearing single and compound RAR and RXR mutations, have also led to the conclusion that RXR/RAR heterodimers can mediate the retinoid signal in a cell-autonomous system (Roy et al., 1995; Taneja et al., 1996; Clifford et al., 1996; Chiba et al., unpublished observations). A similar conclusion was also reached in the case of the cultured NB4 human acute promyelocytic leukemia (APL) cells (Chen et al., 1996) or retinoid-induced apoptosis of T cell hybridomas (Bissonnette et al., 1995). Moreover, it has been shown that heterodimers between the drosophila RXR

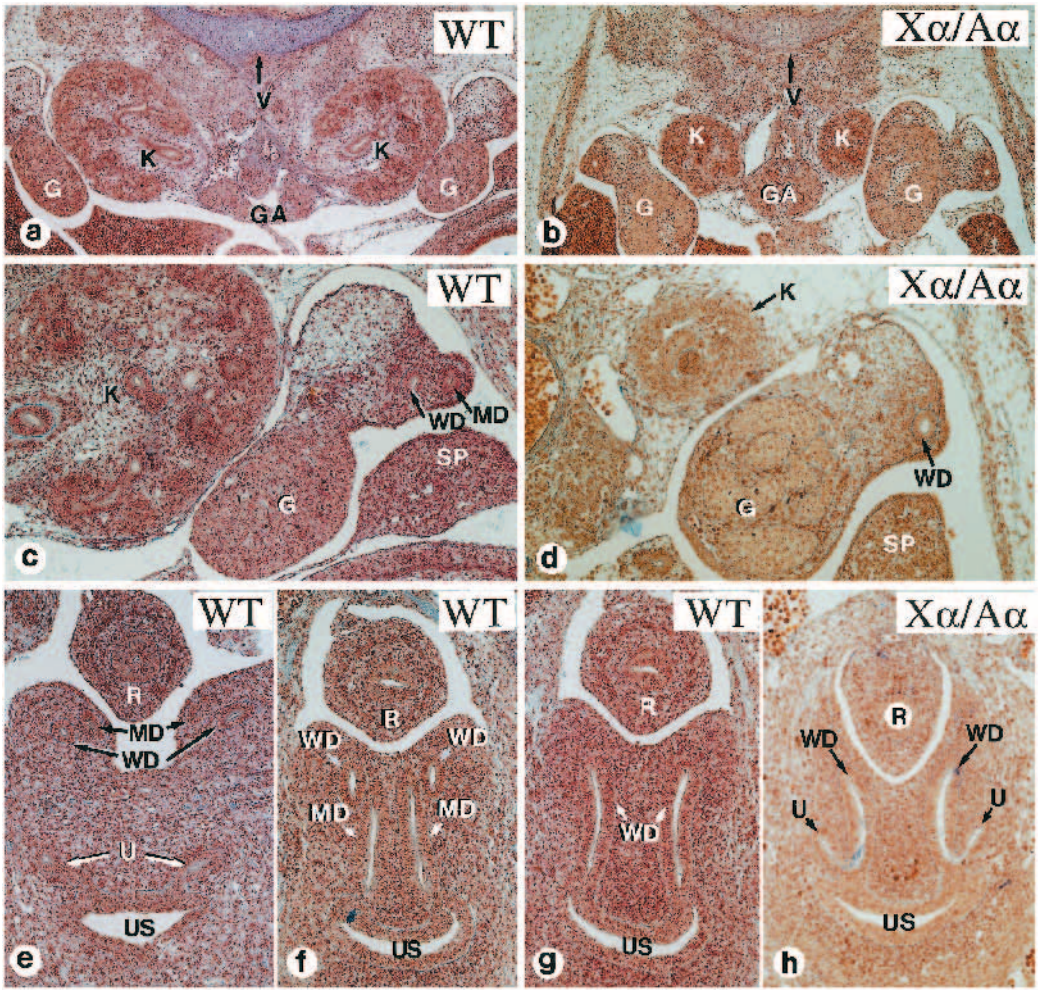


Fig. 5. Comparison of the kidney and of the urogenital tract between 14.5 dpc wild type (WT) (a,c,e-g) and Xα/Aα (b,d,h) fetuses. (a-d) Transverse sections at similar levels of the kidneys (K) and gonads (G). (e-g) Transverse sections at slightly different levels (e: most rostral; g: most caudal) of the normal fetal urogenital sinus (US), illustrating the relative positions of the openings of the ureters (U), Müllerian ducts (MD) and Wolffian ducts (WD) in the dorsal wall of this structure. (h) A typical example of common openings of the Wolffian duct and ureters. G, gonad; GA, sympathetic ganglion; K, kidney; MD, Müllerian duct; R, rectum; SP, spleen; U, ureter; US, urogenital sinus; V, vertebra; WD, Wolffian duct. Magnification: ×16 (a,b) and ×64 (c-h).

homologue ultraspiracle (usp) and the ecdysone receptor (ECR) are likely to be the functional units involved in the control of expression of ecdysteroid target genes, as both ECR and usp have been shown to be colocalized at ecdysone target loci on salivary gland chromosomes (Yao et al., 1993). Thus, taken together with our present data, all of these studies firmly establish the prevalence of RXR heterodimerisation in vivo.

RXRα is the functionally predominant RXR in vivo

The present data show a clear synergy between the effects of RAR and RXRα mutations, whereas in contrast, no such synergy was observed when RXRβ or RXRγ mutations were combined with RAR mutations, thus indicating that RXRα could be the essential RXR involved in RAR function during ontogenesis. Several additional observations point to a prominent role for RXRα: heterozygocity for RXRα can lead on its own to several abnormal phenotypes (growth deficiency, cardiac abnormalities, digital webbing; this study, Kastner et al., 1994; Gruber et al., 1996; and our unpublished results); furthermore, the RXRα null mutation results in an array of developmental defects affecting most notably the heart and the eyes. RXRα is therefore critically required (both alleles in certain cases) for several developmental events. In contrast, RXRβ and RXRγ might be largely dispensible for development, since null mutants for these receptors, as well as Xβ/Xγ double null mutants, appear essentially normal (Krezel et al., 1996). Moreover, Xβ/Xγ/Xα$^{+/-}$ mice are viable and, with the exception of a marked reduction in size, not more affected than RXRβ$^{-/-}$ mice (which exhibit a spermatogenesis defect in males; Krezel et al., 1996), indicating that a single RXRα allele is sufficient to perform the essential RXR developmental functions. In this respect, it is noteworthy that all defects of the fetal VAD syndrome are recapitulated by the various RXRα/RAR isotype compound mutations. However, a tight functional specificity of RXRα for heterodimerisation with RARs is unlikely, since many RAR-dependent developmental processes proceed normally in Xα/Xγ double null mutants, i.e. in mutants that express RXRβ as the only RXR (Krezel et al., 1996). In any event, it is important to remember that the present study does not rule out that RXRβ and RXRγ may be involved in RAR functions not tested in the present study, but which could be crucial in a natural environment (for further discussion, see Kastner et al., 1995).

Differential susceptibility of RA-dependent processes to retinoid receptor deficiency

The various RA-dependent developmental processes have been shown to exhibit marked differences in susceptibility to genetic RAR isotype deficiency. The occurrence of a first class of events is impaired in mild receptor deficiency, when a single receptor isotype or even, in some cases, a single receptor isoform is lacking. Such processes include the patterning of cervical vertebrae, involution of interdigital tissues (both affected in Aγ and Aα single null mutants; Lohnes et al., 1993, 1994; Lufkin et al., 1993; this report), involution of the retrolenticular mesenchyme (affected in Aβ2 and Aβ mutants; Grondona et al., 1996; Ghyselinck et al., unpublished observations), or patterning of the tracheal rings and laryngeal cartilages (abnormal in Aγ and Aγ1 mutants; Lohnes et al., 1993; our unpublished data). It is noteworthy that malformations belonging to this class of events are also found in RXRα single null mutants, which all exhibit a retrolenticular membrane, digital webbing and often cervical vertebrae and tracheal defects (Kastner et al., 1994; our unpublished data). Remarkably, the presence of a retrolenticular membrane and digital

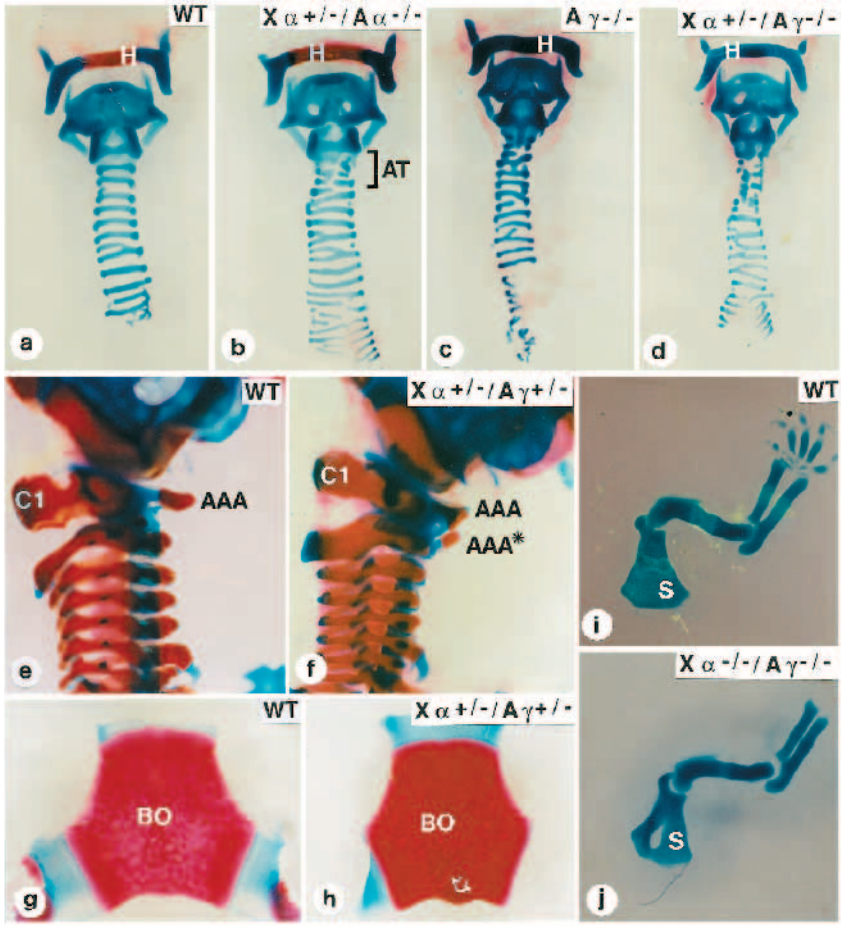
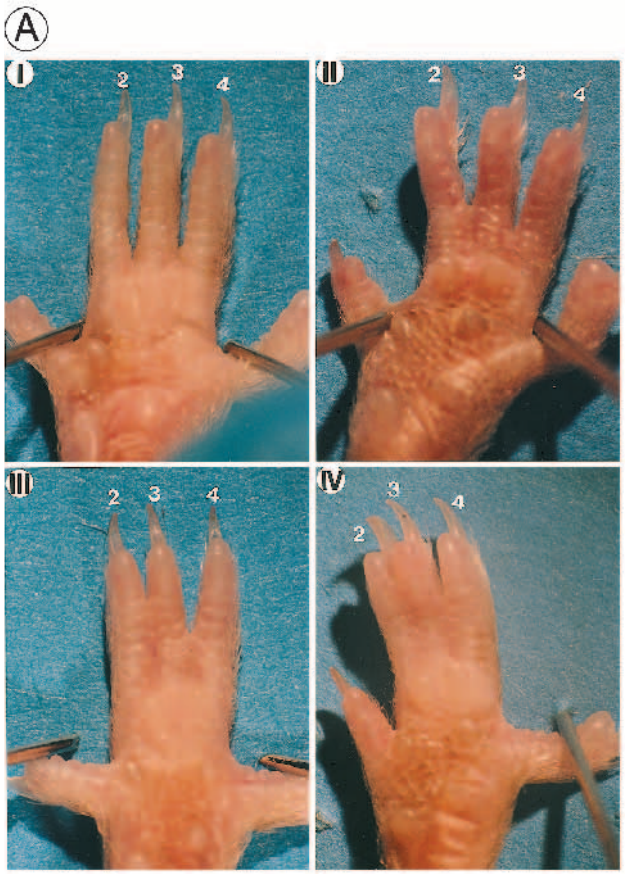


Fig. 6. Tracheal and skeletal defects in RXR/RAR compound mutants. (a-h) Derived from skeletons of newborn animals, (i and j) are from skeletons from 14.5 dpc fetuses. (a-d) Isolated tracheas. The absence of one lesser horn of the hyoid bone (H) in the Aγ$^{-/-}$ mutant is due to loss upon dissection. Note also the retardation in the ossification of this bone in the Xα$^{+/-}$/Aγ$^{-/-}$ mutant. The bracket in (b) points to the affected anterior trachea (AT) region. (e and f) Correspond to cervical regions. The Xα$^{+/-}$/Aγ$^{+/-}$ mutant exhibits an anterior arch of the atlas-like process (AAA*) on C2. Note also the thickening of C2 in this mutant. (g and h) Represent dissected basioccipital bones (BO). The Xα$^{+/-}$/Aγ$^{+/-}$ basioccipital bone exhibits a budding on its ventral side (open arrow). (i and j) Correspond to left forelimbs. Digits of the Xα/Aγ mutant were lost in the course of manipulation. AAA, anterior arch of the atlas; C1, first cervical vertebra or atlas; S, scapula.

webbing were also occasionally observed in RXRα$^{+/-}$ animals, suggesting that some events are highly sensitive to a decrease in RXRα levels. Our present data show that these events are also highly sensitive to the synergistic effects of RAR and RXRα mutations, as they appear to be frequently affected in double heterozygotes (cervical vertebrae transformations in Xα$^{+/-}$/Aγ$^{+/-}$ and Xα$^{+/-}$/Aα$^{+/-}$ mutants; retrolenticular membrane in Xα$^{+/-}$/Aβ$^{+/-}$ mutants; digital webbing in Xα$^{+/-}$/Aγ$^{+/-}$ and Xα$^{+/-}$/Aα$^{+/-}$ mutants). Thus, relatively high levels of both RARs and RXRs are necessary for the completion of the corresponding processes. Interestingly, the retrolenticular membrane phenotype is also the most frequently observed defect of the fetal VAD syndrome (Wilson et al., 1953), indicating that the retinoid-induced disappearance of the primary vitreous is highly sensitive to reduced levels of both receptors and ligand.

A second class (by far the most frequent) of RA-dependent developmental events corresponds to processes usually not affected in single RAR isotype null mutants, and whose impairment requires the concomitant loss of two RAR isotypes (see Lohnes et al., 1994; Mendelsohn et al., 1994a). In addition, the corresponding defects are sometimes not fully penetrant even in double mutants (e.g. lens agenesis; see Lohnes et al., 1994), which suggests that in some instances the third remaining receptor isotype can at least partially carry through the function of the two missing RARs. With the exception of ventricular myocardial hypoplasia and of some ocular defects, these abnormalities are usually not observed in RXRα single null mutants. However, many of them arise in RXRα/RAR compound mutants, and require in general the inactivation of both alleles of the two receptors. Therefore, RXRα single mutants, as well as RAR and RXRα/RAR compound mutants, indicate that events belonging to this second class are much harder to perturb by genetically decreasing the amount of functional RAR and/or RXR. In this respect, it is noteworthy that not all defects selectively seen in RAR double mutants could be reproduced in the present RXR/RAR compound mutants, thus probably indicating that the RXR/RAR activity was still high enough for mediating the retinoid signal, rather than the dispensability of RXR in the corresponding processes.

A third class of RA-dependent events may exist, which would require even more drastic receptor deficiencies than those that can be achieved in RAR/RXR or RAR double mutants. That the correct expression of RARβ is apparently not affected in Aα/Aγ embryos (Lohnes et al., 1994), indicates that even in these severely affected mutants, some RA transduction function can still operate normally (RARβ expression is induced by RA in the embryo; Ward, 1994). In this respect,



B

	digital webbing			
	absent (I)	mild (II)	extensive (III)	severe (IV)
WT	100%	0%	0%	0%
Xα$^{+/-}$	~95%	~5%	0%	0%
Aα$^{+/-}$	100%	0%	0%	0%
Aγ$^{+/-}$	100%	0%	0%	0%
Aα$^{+/-}$/Aγ$^{+/-}$	~95%	~5%	0%	0%
Aα$^{-/-}$	+[a]	+[a]	+[a]	0%
Aγ$^{-/-}$	+[a]	+[a]	+[a]	0%
Aβ$^{-/-}$	100%	0%	0%	0%
Xα$^{+/-}$/Aγ$^{+/-}$	~50%	~40%	~10%	0%
Xα$^{+/-}$/Aα$^{+/-}$	~50%	~50%	~5%	0%
Xα$^{+/-}$/Aβ$^{-/-}$	~95%	~5%	0%	0%
Xα$^{+/-}$/Aα$^{+/-}$/Aγ$^{+/-}$	0%	0%	~30%	~70%
Xα$^{+/-}$/Aγ$^{-/-}$	0%	0%	~30%	~70%

(a) relative frequencies could not be estimated due to a high variability in the phenotype and the low number of animals observed (<20)

Fig. 7. Hindlimb digital webbing phenotype in various RXRα/RAR compound mutants. (A) The four webbing phenotypes that are categorized in the table in (B). Genotypes of the mutants displayed in panels I-IV are: panel I: Xα$^{+/-}$; panels II and III: Xα$^{+/-}$/Aγ$^{+/-}$; panel IV: Xα$^{+/-}$/Aα$^{+/-}$/Aγ$^{+/-}$. Digits 2, 3 and 4 are indicated. (B) Table describing the estimated frequencies of the four webbing phenotypes in mice of various genotypes. Frequency estimates are not given for Aα$^{-/-}$ and Aγ$^{-/-}$ mutants, because the number of observed animals of these groups was low (less than 20) and the phenotypes very variable.

note that a number of studies have suggested that RA plays an important role in some early developmental processes, including early aspects of cardiogenesis, patterning of the hindbrain or vascular development (Heine et al., 1985; Bavik et al., 1996; Marsh-Armstrong et al., 1995; Costaridis et al., 1996; Twal et al., 1995; Maden et al., 1996), none of which were affected in the available RXR/RAR or RAR double mutants. It is also puzzling that tooth morphogenesis, which is critically dependent on the presence of vitamin A in whole embryo and organotypic cultures (Wolbach and Howe, 1933; Mellanby, 1940; Mark et al., 1992), is unaffected in the double mutants that have been analyzed. Interestingly, double null Xα/Xβ mutants display extensive early developmental defects not seen in Xα mutant (our unpublished data), which could possibly correspond to the abrogation of this putative third class of RA-dependent events.

At the molecular level, this differential susceptibility of particular events to retinoid receptor deficiency could reflect variations in receptor levels among different tissues, variation in the binding affinities of the receptor heterodimers for distinct RAREs present in different target genes, and/or differences in functional redundancies between the various receptors for triggering distinct events (see below), and/or differences in the availability of factors synergizing with the receptors in the regulation of transcription. In this context, it is noteworthy that a similar differential sensitivity to RAR and RXR deficiency has been observed for RA-target genes in the F9 embryonal carcinoma cell system, as different RA-responsive genes were differently affected in their expression in RARα, RARγ and RXRα single or double mutant cell lines (Boylan et al., 1993, 1995; Clifford et al., 1996; Taneja et al., 1996; Chiba et al., unpublished observations). Interestingly, the RA inducibility of RARβ was also weakly affected in these mutant cell lines.

Functional specificity and redundancy among RARs and RXRs

The multiple RAR and RXR isotypes (and isoforms) are conserved among species throughout vertebrate evolution and exhibit differential patterns of expression during development and in the adult (Ruberte et al., 1990, 1991; Dollé et al., 1990, 1994). Furthermore, molecular biology studies have shown that they exhibit some specific functional characteristics in transfected cultured cells (Nagpal et al., 1992, 1993). Taken together, these observations have suggested that the basis for the highly pleiotropic effect of retinoids may ultimately reside in the control of different subsets of retinoid-response promoters by specific combinations of RARs and RXRs (see Chambon, 1994). However, only a limited number of defects were present in mice lacking a single RAR or RXR isotype (for references see Kastner et al., 1995). In contrast, a large number of defects (including the complete spectrum of congenital abnormalities of the fetal VAD syndrome) were generated by one or the other of the various combinations of two RAR isotype null mutations. This suggested that the different receptors could be largely functionally redundant, and therefore that the transcriptional control of most RA target genes would only require that a certain threshold level of RAR and RXR heterodimer is achieved through any combination of RAR and RXR isotypes (or isoforms).

In fact, our present study reveals that there is much less functional redundancy between RARs in an RXRα mutant background. In many instances, the phenotypes of the present RXRα/RAR mutants point to specific heterodimeric pairs as being preferentially involved in a given process, since in many cases complete penetrance and expressivity of a given defect was observed only in mutants for a given RXRα/RAR pair. For instance, the study of RAR double mutants has suggested that RARα and RARβ2, as well as RARα and RARγ, are functionally redundant for the formation of the aortico-pulmonary septum (Mendelsohn et al., 1994a), whereas the complete absence of this septum in all Xα/Aα double mutants shows that, in an RXRα mutant background, there is little functional redundancy between the RAR isotypes, since RARα can never be functionally replaced by either RARγ or RARβ2. Similarly, only Xα/Aγ mutants exhibit, with full penetrance and expressivity, defects in the eye anterior segment. Furthermore, the predominant role of particular RXR/RAR pairs in the realisation of some events is also suggested by the observation that a number of defects occur only in specific types of RXR/RAR compound mutants (even though not necessarily with complete penetrance; e.g. thymic agenesis in Xα/Aβ mutants or lack of oesophago-tracheal septation in Xα/Aα mutants). The crucial role of particular RXRα/RAR heterodimers indicated by these various observations could merely reflect the fact that the corresponding RXR and RAR partners may be quantitatively predominant in the target tissue, their absence leading to a drastic decrease in heterodimer levels, which would fall below the critical threshold. On the other hand, these observations may be accounted for by assuming that a specific RXR/RAR heterodimer performs most efficiently a given function, but that the other RAR and/or isotypes (and isoforms) could still be functionally close enough to substitute for the inactivated receptor and perform a number of its functions, albeit possibly with a lower efficiency.

The latter idea that a specific RXR/RAR heterodimer could preferentially be involved in mediating particular retinoid-dependent events has been strongly supported by recent observations obtained with the F9 embryonal carcinoma cell model of differentiation. Using RARα$^{-/-}$, RARγ$^{-/-}$, RXRα$^{-/-}$, RXRα$^{-/-}$/RARα$^{-/-}$ and RXRα$^{-/-}$/RARγ$^{-/-}$ F9 cells, as well as synthetic retinoids specific for RAR isotypes and RXRs, it has indeed been shown that, in this cell-autonomous system, specific RXRα/RAR pairs are differentially involved in different physiological events (differentiation, growth inhibition, apoptosis), as well as retinoid-induced expression of subsets of target genes (Boylan et al., 1993, 1995; Taneja et al., 1995, 1996; Roy et al., 1995; Clifford et al., 1996; Chiba et al., unpublished observations). Similarly, the preferential involvement of a particular RAR/RXR pair has been recently demonstrated for the differentiation of APL NB4 cells (Chen et al., 1996). In addition, using the above mutant F9 cells, it has been conclusively shown that at least one case of functional redundancy observed after knockout of one RAR isotype does not occur in WT cells (the induction of the RARβ gene; see Taneja et al., 1996). Thus, we propose that much of the functional redundancy seen in knockout experiments (particularly in the case of RAR isotypes) does not reflect an actual lack of selectivity in the wild-type situation, but rather potentialities linked to the complexity of the heterodimeric retinoid transducing system, which are revealed under the ‘artefactual’ conditions of particular gene knockouts.

Even though many of our data point to specific roles for

given RXR/RAR heterodimers, it is noteworthy that several developmental events were perturbed in more than one type of RXRα/RAR compound mutant. This multiplicity, which indicates that more than one RXRα/RAR pair may normally be involved in the completion of these events, is not easy to interpret, as the underlying developmental processes are likely to be complex, involving interaction between several cell types, as well as the activation of multiple RA target genes. Thus, the apparent requirement of several distinct RXR/RAR pairs may result from their involvement in distinct cellular or molecular events, or alternatively, could reflect cases of true redundancy, in which the different RARs would exert similar functions.

Phenotypic examination of mice bearing somatic mutations resulting from spatio-temporally controlled knockouts of given RXR/RAR pairs, as well as analysis of the expression of RA target genes, are necessary to establish whether the abnormalities generated by these mutations result from cell-autonomous or non-cell-autonomous developmental processes, and to unequivocally demonstrate the functional specificity of RXR/RAR heterodimers in vivo.

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