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Ligand-bound RXR can mediate retinoid signal transduction during embryogenesis

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SUMMARY

Retinoids regulate various aspects of vertebrate development through the action of two types of receptors, the retinoic acid receptors (RARs) and the retinoid-X-receptors (RXRs). Although RXRs bind 9-*cis*-retinoic acid (9cRA) with high affinity, in vitro experiments suggest that RXRs are for the most part not liganded, but serve as auxiliary factors forming heterodimers with liganded partner receptors such as RAR. Here we have used RXR- and RAR-specific ligands 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-napthyl)ethenyl]benzoic acid (LG69) and (E)-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB), and show that, in the context of an embryo, liganded RXR can mediate retinoid signal transduction. This conclusion emerges from examining the induction of several retinoid-responsive genes in the limb bud (*Hoxb-6/-8, RARβ*) and in the developing central nervous system (*Hoxb-1, otx-2*). *RARβ* and *Hoxb-1* genes were most effectively activated by a combination of TTNPB and LG69, suggesting that the activation of these genes benefits from the presence of ligand-bound RAR and ligand-bound RXR. *Hoxb-6/-8* genes were most efficiently induced by LG69, suggesting that liganded RXR can activate these genes. The regulation of the expression of the *otx-2* gene was complex; expression was repressed by TTNPB, but such repression was relieved when LG69 was provided together with TTNPB, suggesting that ligand-bound RXR can overcome repression of transcription exerted by liganded RAR. Based on these findings, we propose that in our experimental system in which ligands are provided exogenously, transcriptional regulation of several genes involves liganded RXR.

Key words: retinoid receptors, RAR, RXR, retinoid agonists, *Hoxb-6* gene, *Hoxb-8* gene, *otx-2* gene, pattern formation, limb development, neurogenesis

INTRODUCTION

Two types of retinoid receptors transduce the retinoic acid signal, the retinoic acid receptors (RARs) and the retinoid-X-receptors (RXRs) (for reviews see Leblanc and Stunnenberg, 1995; Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995). These receptors are ligand-dependent transcription factors that belong to the nuclear hormone receptor superfamily. All-*trans*-retinoic acid (atRA) binds to RARs, while 9-*cis*-retinoic acid (9cRA) is a bifunctional ligand that binds to RXRs and RARs. RXRs can form heterodimers with RARs and with other nuclear receptors, including those for thyroid hormone and vitamin D. Mice carrying a RXR$\alpha^{-/-}$/RAR$\gamma^{-/-}$ double mutant provide evidence for RXR-RAR heterodimers mediating retinoid signaling in vivo (Kastner et al., 1994). It has been proposed that in these heterodimers, the RXR moiety is an unliganded auxiliary factor and that ligand-dependent transactivation is mediated by the heterodimeric partner. For example, in the case of RXR-RAR heterodimers, there is evidence that the RAR moiety binds its ligand, resulting in conformational changes that prevent 9cRA binding to RXR (Kurokawa et al., 1994, 1995; Forman et al., 1995a). However, RXR can also bind 9cRA in heterodimers consisting of RXR and certain orphan receptors (e.g. LXR, PPAR or NGFI-B/NURR; for reviews see Leblanc and Stunnenberg, 1995; Mangelsdorf and Evans, 1995).

Recently, coactivator and corepressor proteins have been identified that interact with nuclear hormone receptors (Chen and Evans, 1995; Hörlein et al., 1995; Oñate et al., 1995). It is now believed that, at least in the case of a RXR-RAR heterodimer bound to a DR5 responsive element (direct repeat with a spacer of five nucleotides), neither of the receptor subunits is initially liganded and that the corepressor masks the transcriptional activation function of the heterodimer (Baniahmad et al., 1995). Binding of ligand to the RAR moiety then evokes a conformational change that releases the corepressor and allows binding of the coactivator, which then results in transcriptional activation. In this case, RXR acts as a unliganded auxiliary protein. This model is derived from experiments using idealized response elements in transient transfection and electromobility shift assays.

In a living organism, retinoid-dependent regulation of endogenous genes may be more complex than in vitro experiments would suggest (reviewed in Kastner et al., 1995). For example, we have previously observed that 9cRA, an inducer of pattern duplications in the chick wing bud, has 25 times greater potency than atRA (Thaller et al., 1993). Likewise, 9cRA is more effective than atRA in causing posterior trans-

formations in the neural tube of *Xenopus* (Dawid et al., 1993; Creech Kraft et al., 1994). These results are not easily explained if 9cRA binds solely to the RAR subunit of a RXR-RAR heterodimer. Accordingly, we have proposed that the greater potency of 9cRA may be due to the fact that this isomer binds to RXR and RAR and thereby could activate both receptors, resulting in synergism (Thaller et al., 1993). In order to determine in vivo ligand requirements for retinoid-mediated signal transduction, we have studied several cases of retinoid-dependent responses. We used LG69 (Boehm et al., 1994; Kurokawa et al., 1994) and TTNPB (Mangelsdorf et al., 1990), two synthetic retinoids that specifically bind and activate RXRs and RARs, respectively. Unlike atRA and 9cRA, which interconvert in vivo through isomerization and thus cannot be used as receptor-specific agonists, TTNPB and LG69 neither interconvert nor isomerize (Thaller et al., 1993; Kojima et al., 1994). We found that both the induction of additional digits in the chick wing bud and posterior transformations in the neural tube of chick embryos were evoked most efficiently by a combination of LG69 and TTNPB, suggesting that the retinoid signal is transduced by liganded RXRs and RARs. Furthermore, we found that induction of expression of several retinoid-regulated genes (*Hoxb-1* in the neural tube and *RARβ* in the limb bud) occurred most effectively when LG69 and TTNPB were both present. In the case of the induction of *Hoxb-6* and *Hoxb-8* in the limb bud, LG69 was more effective than TTNPB, demonstrating that transactivation of these genes benefits from the presence of liganded RXR. A role of liganded RXR is also suggested in the regulation of expression of the *otx-2* gene. This gene was repressed by TTNPB, but providing LG69 together with TTNPB relieved the repression. Taken together, our studies of several types of responses in the embryo suggest that the presence of both a RXR and a RAR ligand can enhance retinoid responses through ligand-dependent synergism, and that for the genes examined, RXRs can function as ligand-regulated receptors and are not just unliganded partners in retinoid signal transduction pathways.

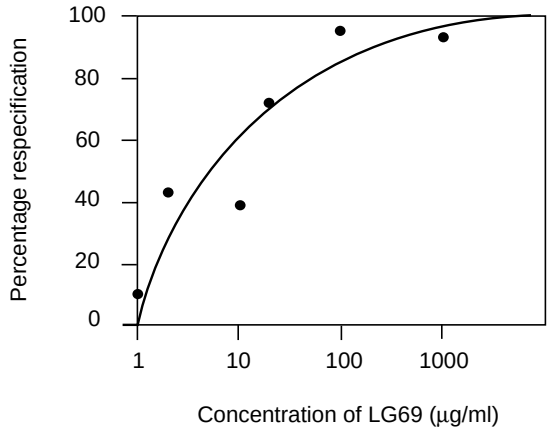

Fig. 1. Dose-dependence of induction of additional wing digits by LG69, a RXR-specific agonist. The percentage respecification values represent the extent of pattern duplication. 2234 patterns with an additional digit 2, score 25%; 32234 or 3234 patterns with an additional digit 3 at the anterior wing margin, score 50%; and 432234, 43234 or 434 patterns with an additional digit 4 at the anterior wing margin, score 100%. The ED_{50} (3234 or 32234 patterns) is 6 μg/ml.

MATERIALS AND METHODS

Retinoid stock solutions

4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-napthyl)ethenyl]-benzoic acid (LG69) and (E)-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) were dissolved in dimethylsulfoxide and stored at −70°C. LG69 was obtained from Dr R. Heyman, Ligand Pharmaceuticals, San Diego, CA, and TTNPB was a gift from Dr M. Klaus, Hoffman-LaRoche, Basel, Switzerland.

Retinoid treatment of limb buds

Fertile white Leghorn chicken eggs were purchased from Ideal Poultry (Cameron, Texas) and incubated at 38.5°C to develop to stage 20 (Hamburger and Hamilton, 1951). AG1-X2 ion-exchange beads were loaded with retinoid by soaking them in solutions of LG69 (1 μg/ml to 1 mg/ml) and/or TTNPB (0.04, 0.05 and 0.1 μg/ml) as previously described (Eichele and Thaller, 1987). After 3 or 6 hours of incubation, embryos were fixed in MEMFA (100 mM MOPS, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% w/v formaldehyde) overnight at 4°C and subjected to whole-mount in situ hybridization. Alternatively, embryos were allowed to develop to day 10 and then were fixed in trichloroacetic acid, stained for cartilage and scored for their wing patterns (Eichele and Thaller, 1987).

At soaking concentrations of 0.05 μg/ml TTNPB, the concentration of this agonist in the limb bud is about 1 nM (see Eichele et al., 1985 for calculation). Since LG69 has a structure similar to TTNPB and

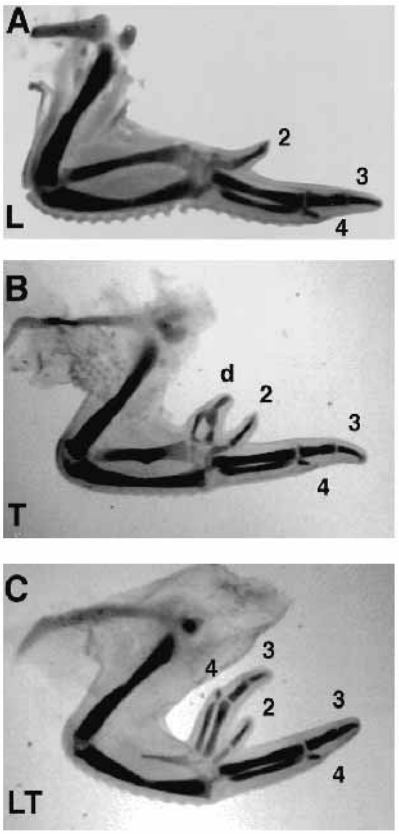

Fig. 2. Digit patterns resulting from treatment with LG69 and/or TTNPB. (A) Treatment with 1 μg/ml LG69 (L) resulted in a normal digit pattern. (B) 0.05 μg/ml TTNPB (T) evoked an additional element (d), which was proximally duplicated. (C) Fully duplicated pattern induced by a combination of 1 μg /ml LG69 and 0.05 μg/ml TTNPB (LT). 2, digit 2; 3, digit 3; 4, digit 4; d, digit.

thus similar release kinetics and half-life, its tissue concentration would be about 20 nM when a soaking concentration of 1 μg/ml is used. At such nanomolar concentrations, both agonists are highly receptor-selective (Mangelsdorf et al., 1990; Boehm et al., 1994; Kurokawa et al., 1994).

Retinoid treatment of primitive streak stage embryos

Culture of chick embryos and retinoid treatment were performed according to Sundin and Eichele (1992). White Leghorn chicken eggs were incubated at 38.5°C to reach stage 4. Embryos were isolated with a paper ring, cultured for 4 hours on albumin-agar plates containing high or low doses of LG69, TTNPB, or both, and overlayed with 20 μl YT (Yolk-Tyrode's solution, as defined in Sundin and Eichele, 1992) containing the same concentration of ligands as the agar. Embryos were treated with LG69 (0.2-2 μg/ml), TTNPB (0.01-0.1 μg/ml), or a mixture of LG69 and TTNPB. Retinoids were removed by washing the embryos for 30 minutes in three changes of YT at 38.5°C. Embryos were returned to agonist-free albumin-agar plates where they were incubated at 38.5°C for an additional 6-24 hours, then they were fixed for 90 minutes at 22°C in MEMFA and subjected to whole-mount in situ hybridization.

Concentrations of 0.2 μg/ml LG69 and 0.01 μg/ml TTNPB correspond to 573 nM and 28 nM, respectively. At these concentrations both agonists are receptor-selective (Mangelsdorf et al., 1990; Boehm et al., 1994; Kurokawa et al., 1994).

Whole-mount in situ hybridization

Syntheses of digoxigenin-tagged riboprobes of *RARβ* (nt 1 to nt 612, Smith and Eichele, 1991), *Hoxb-1* (nt 13 to nt 1237, Sundin et al., 1990), *Hoxb-6* (nt 13 to nt 278, Wedden et al., 1989), *Hoxb-8* (nt −51 to nt 726, unpublished data), *otx-2* (Bally-Cuif et al., 1995) were carried out with a Stratagene RNA transcription kit following the procedure described in Wilkinson (1993). Whole-mount in situ hybridization was performed with digoxigenin-labelled riboprobes as described by Li et al. (1994).

RESULTS

Synergism between RXR- and RAR-specific agonists in evoking wing pattern duplications

Application of atRA or 9cRA to the anterior margin of a chick wing bud induces an additional set of digits arranged in a mirror-symmetrical manner with respect to the normal digits (Tickle et al., 1982; Summerbell, 1983; Thaller et al., 1993). To determine which type of retinoid receptor mediates this process, a RXR-specific agonist (LG69) and a RAR-specific agonist (TTNPB) were applied to stage 20 chick wing buds. It has previously been reported that TTNPB is able to generate digit duplications in a dose-dependent manner (Eichele and Thaller, 1987). Low doses of TTNPB created 2234 digit patterns, whereas high doses evoked 43234 duplications. In the present study, we found that LG69 can also induce additional digits in a dose-dependent fashion (Fig. 1). The ED_{50} for LG69 was approx. 6 μg/ml (see Fig. 1) and that for TTNPB was approx. 0.2 μg/ml (Fig. 3 in Eichele and Thaller, 1987). To detect synergism between the two receptor-specific ligands, we used doses that were sufficiently low so as to elicit only a weak response (an additional digit 2) when ligands were provided separately. Thus at 1 μg/ml, LG69 evoked digit patterns that

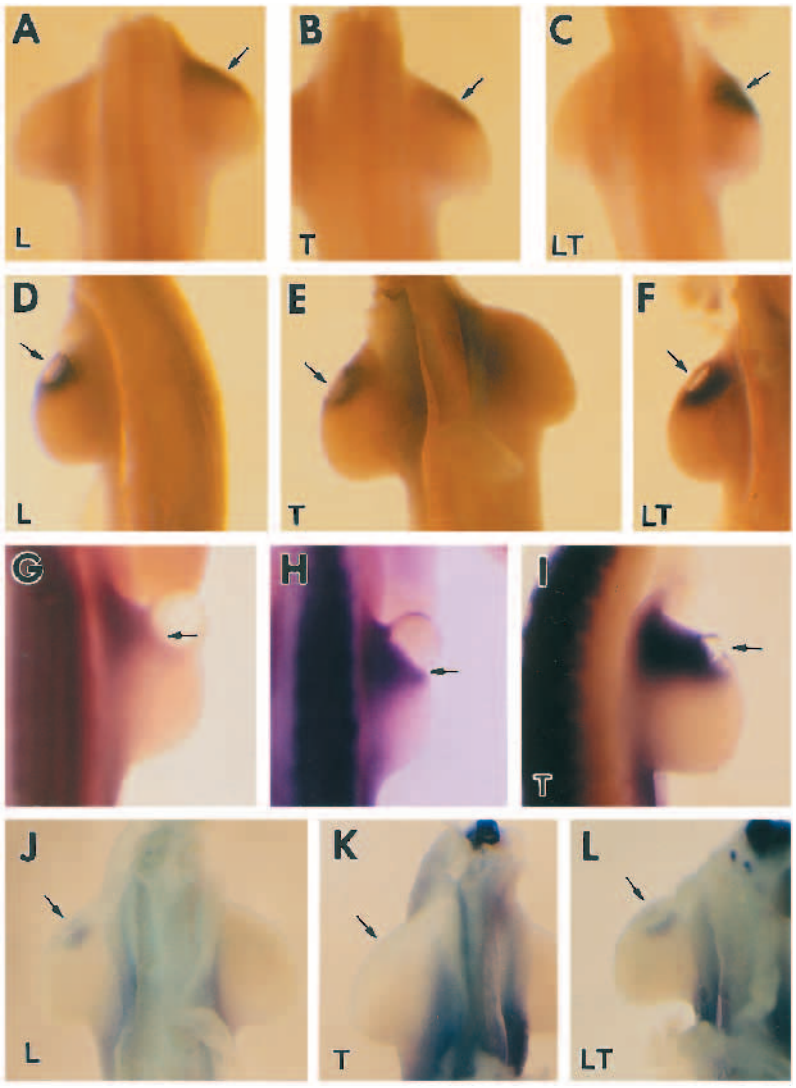

Fig. 3. Effects of atRA, LG69 (L), TTNPB (T) and a combination of LG69 and TTNPB (LT) on *RARβ* (A-F) and *Hoxb-6/-8* (G-L) expression in the chick wing bud. A-C and G-I are dorsal views, the other photographs show ventral aspects. (A,D), (B,E) and (C,F) are the same three embryos viewed from the dorsal and ventral sides. Arrows indicate the region of ectopic expression around the bead, which was removed prior to fixation except for specimens in G and H. (A,D) *RARβ* expression is induced anteriorly following 6 hours treatment with 1 μg/ml LG69. The untreated limb bud (e.g. left bud in A) endogenous *RARβ* is expressed only in the proximal region. (B,E) *RARβ* induction following 6 hours treatment with 0.05 μg/ml TTNPB. (C,F) *RARβ* induction following 6 hours treatment with 1 μg /ml LG69 and 0.05 μg/ml TTNPB. (G) *Hoxb-6* induction following 3 hours treatment with 100 μg/ml atRA. (H) *Hoxb-8* induction following 3 hours treatment with 100 μg/ml atRA. (I) *Hoxb-8* induction following 6 hours treatment with 5 μg/ml TTNPB. (J) *Hoxb-8* induction following 6 hours treatment with 1 μg/ml LG69. Note that the untreated bud does not express *Hoxb-8*. (K) *Hoxb-8* induction following 6 hours treatment with 0.05 μg/ml TTNPB. *Hoxb-8* induction is below the limit of detection. (L) *Hoxb-8* induction following 6 hours treatment with 1 μg /ml LG69 and 0.05 μg/ml TTNPB. The *Hoxb-8* expression domain is slightly larger than that seen in J.

Table 1. Synergistic effects of LG69 and TTNPB in evoking wing pattern duplications

Agonist and soaking concentration	Digit pattern				PRV*
	234	2234	32(2)34	432(2)34 4334	
LG69, 1 μg/ml	6	6	0	0	12.5%
TTNPB, 0.05 μg/ml	3	14	3	0	25%
LG69, 1 μg/ml TTNPB, 0.05 μg/ml	0	4	0	17	86%
LG69, 0.5 μg/ml TTNPB, 0.025 μg/ml	2	3	2	2	42%

*Percentage respecification value (see e.g. Eichele and Thaller, 1987).

were normal or had an additional digit 2 (Fig. 2A; Table 1). Likewise, with 0.05 μg/ml TTNPB, digit patterns had mostly an additional digit 2 and rarely a digit 3 (Fig. 2B; Table 1). However, when a mixture of agonists at final concentrations of 1 μg/ml LG69 and 0.05 μg/ml TTNPB were used, more than 80% of the wings developed full mirror-symmetrical duplication with additional digits 3 and 4 (Fig. 2C; Table 1). Application of a combination of LG69 and TTNPB, at final concentrations of 0.5 μg/ml LG69 and 0.025 μg/ml TTNPB, also yielded patterns with additional digits 3 and 4 but less frequently than with the higher dose (Table 1). Note that, when applied individually, 0.5 μg/ml LG69 or 0.025 μg/ml TTNPB always yielded normal patterns (see dose-response curves in Fig. 1 and in Fig. 3 in Eichele and Thaller, 1987). These data demonstrate that LG69 and TTNPB act synergistically in the digit pattern duplication assay.

Effects of RXR- and RAR-specific agonists on the ectopic expression of *RARβ* and *Hoxb-6/-8*

The induction of additional digits by retinoids is a multistep process requiring a sequential activation of genes in the mesenchyme and ectoderm (for reviews see Tickle and Eichele, 1994; Tabin, 1995). Early responses to atRA treatment of wing buds include the induction of *Hoxb-6*, *Hoxb-8* (Fig. 3G,H) and *RARβ* (Soprano et al., 1994, and references therein). This is followed by the ectopic activation of *sonic hedgehog*, *fgf-4*, *bmp-2* and *Hoxd-11* to *-13* (Tabin, 1995). The induction of these genes requires ≥18 hours of atRA exposure, suggesting that activation is indirect. The induction of *Hoxb-8* by atRA is particularly interesting since the ectopic expression of this gene in the mouse forelimb bud results in digit pattern duplications (Charité et al., 1994). Furthermore, *Hoxb-8* is expressed in the presumptive forelimbs of mouse (Charité et al., 1994) and chick (manuscript in preparation).

For our gene induction studies, we used doses of agonist that yielded full digit duplications when applied in combination but gave minimal responses when applied separately (Table 1). To determine which ligand induced *RARβ,* wing buds were treated for 6 hours with either 1 μg/ml LG69, 0.05 μg/ml TTNPB or with a combination of 1 μg/ml LG69 and 0.05 μg/ml TTNPB. In the normal embryo, *RARβ* is expressed only in the proximal

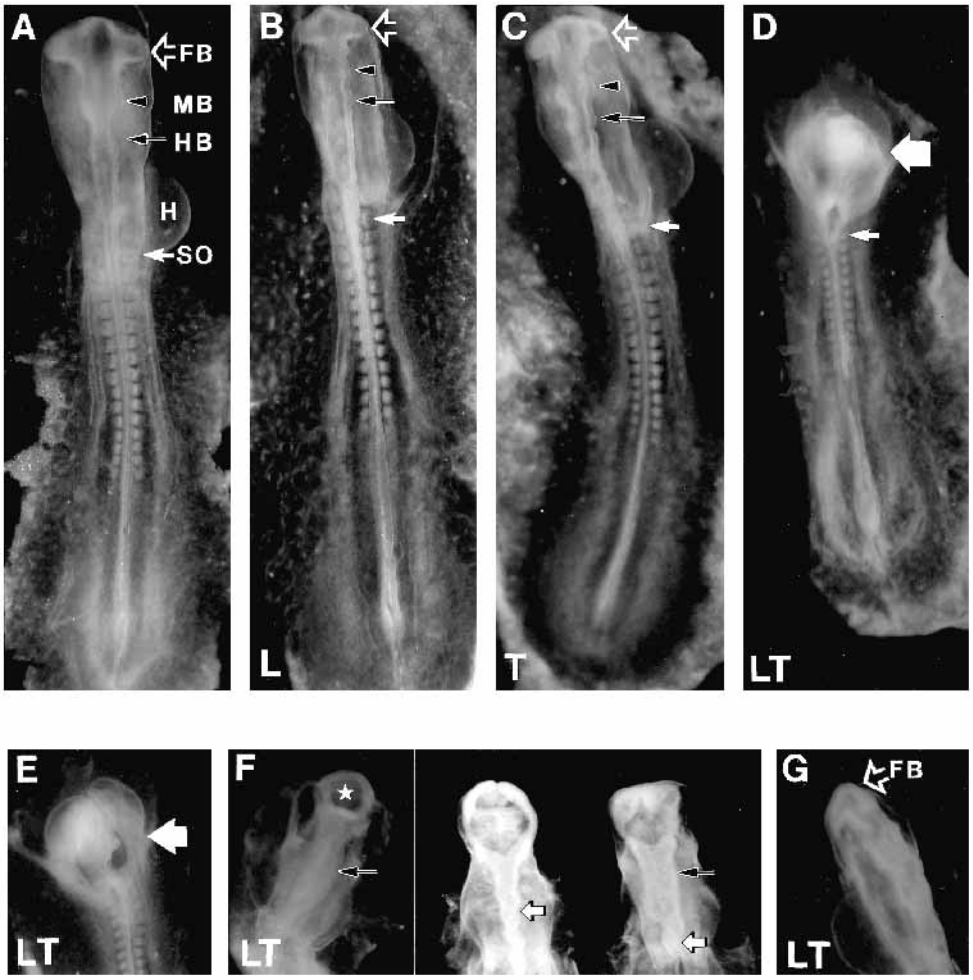

Fig. 4. Dysmorphogenesis of stage 12 embryos that were treated at stage 4 with LG69 (L, 0.2 μg/ml), TTNPB (T, 0.01 μg/ml), or a combination of LG69 and TTNPB (LT, 0.2 μg/ml and 0.01 μg/ml). (A) untreated embryo; (B) LG69-treated embryo; (C) TTNPB-treated embryo; (D-G) embryos treated with both agonists. Significant defects were observed in the anterior part of the central nervous system of embryos treated with the combination of LG69 and TTNPB. A dorsal view of the most severely affected case shows a ball-like deformed head (D, bold white arrow) but the posterior part of the embryo is normal. (E) Ventral view of the embryo shown in D. (F) The most frequently observed cases of embryos exposed to both agonists. Such embryos had big gaps in their dorsal forebrain or midbrain regions (white star), and exhibited a wider hindbrain (black arrow) or a kinked hindbrain (open black arrow). (G) Some embryos exposed to both agents had a small forebrain (open white arrow). Open white arrows/FB, forebrain; black arrowheads/MB, midbrain; black arrows/HB, hindbrain; small white arrow/SO, somite; H, heart.

mesenchyme of stage 20 wing buds (Fig. 3A, left wing bud, and e.g. Smith and Eichele, 1991). We found that *RARβ* is induced by LG69 alone (Fig. 3A, right wing bud, Fig. 3D, *n*=3) and to a lesser extent by TTNPB (Fig. 3B,E, *n*=3). However, the combination treatment strongly induced this gene (Fig. 3C,F, *n*=3), producing a *RARβ*-positive domain that extends deeper into the wing bud mesenchyme than that seen after treatment with a single agonist.

Hoxb-6/-8 are not expressed in stage 20 wing buds (bud on the right in Fig. 3J for *Hoxb-8*) but are induced by atRA within 3 hours (Fig. 3G,H). We found that *Hoxb-8* is activated by 1 μg/ml LG69 (Fig. 3J, *n*=3), but not by 0.05 μg/ml TTNPB (Fig. 3K, *n*=9), as judged by whole-mount in situ hybridization. Treatment with the combination of LG69 and TTNPB induced *Hoxb-8* in a domain slightly larger than that seen with LG69 alone (compare Fig. 3J and L, *n*=3), indicating moderate synergism. *Hoxb-6* showed the same ligand dependence as *Hoxb-8*; LG69 and the combination of LG69 and TTNPB induced this gene, while TTNPB alone had no effect (data not shown). When the concentration of TTNPB was increased 100-fold to 5 μg/ml, a dose which evoked full digit pattern duplications (Eichele and Thaller, 1987), *Hoxb-8* was strongly activated (Fig. 3I, *n*=3). As previously demonstrated, TTNPB can also activate RXR when used at high dose (Mangelsdorf et al., 1990). Thus, induction of *Hoxb-6* and *Hoxb-8* could be achieved by LG69 alone, but unlike the situation with *RARβ*, TTNPB alone did not induce these genes, unless a high dose was applied. This suggests that ligand requirements for the activation of *RARβ* and *Hoxb-6/-8* genes are different, but in all cases involve liganded RXR.

Effects of RXR and RAR agonists on the morphology of the anterior neural tube

The development of the CNS of vertebrate embryos is affected by retinoids, which cause a loss of anterior structures and posterior transformations (Durston et al., 1989; Sive et al., 1990; Holder and Hill, 1991; Papalopulu et al., 1991; Ruiz i Altaba and Jessell, 1991; Sundin and Eichele, 1992; Dawid et al., 1993; Creech Kraft et al., 1994; Simeone et al., 1995). Retinoids and their receptors are found in these early embryos (Durston et al., 1989; Ellinger-Ziegelbauer and Dreyer, 1991; Chen et al., 1992, 1994; Creech Kraft et al., 1994; Dollé et al., 1994; Smith, 1994), and the pattern of expression of RA-responsive genes (e.g. *Hoxb-1*, *otx-2)* is changed upon retinoid exposure (for a review see Conlon, 1995). To elucidate which receptors mediate these effects, primitive streak stage chick embryos (Hamburger-Hamilton stage 4) were treated with LG69 and/or TTNPB. To establish the appropriate dose range, embryos were treated for 4 hours with LG69 (0.2-2 μg/ml), TTNPB (0.01-0.1 μg/ml), or a mixture of LG69 and TTNPB. Thereafter, embryos were grown to stage 12 in agonist-free medium and morphological defects were assessed (Fig. 4). We found that a dose of 0.2 μg/ml LG69 or 0.01 μg/ml TTNPB resulted in minor morphologic defects (Fig. 4B,C). However, upon combination (final concentration of 0.2 μg/ml LG69 and 0.01 μg/ml TTNPB), the agonists evoked pronounced defects in the anterior part of the brain primordium (Fig. 4D-G). Some embryos had a big gap in the dorsal region of the forebrain (Fig. 4F), others had a misshapen forebrain, while a third group had a greatly diminished forebrain (Fig. 4G). In most specimens, the midbrain was significantly smaller or even absent; the hindbrain was longer and wider, and otic vesicles were absent (Fig. 4D-G). Similar to limb duplications, the posterior transformation induced by retinoids in the nervous system exhibits synergism.

Expression of *Hoxb-1* and *otx-2* in agonist-treated embryos

It had previously been shown that treatment of stage 4 chick embryos with atRA shifts the expression boundary of *Hoxb-1* in neuroectoderm and underlying mesoderm to a more cranial position and simultaneously represses the expression of *otx-2* in the anterior portion of the embryo (for a review see Conlon, 1995). While it is not known whether the *otx-2* gene is a direct retinoid target gene, *Hoxb-1* contains two retinoid responsive elements capable of binding RXR-RAR heterodimers (Marshall et al., 1994; Studer et al., 1994; Ogura and Evans, 1995a,b).

To determine the effect of receptor-selective agonists on *Hoxb-1* and *otx-2* expression, stage 4 chick embryos were exposed for 4 hours to 0.2 μg/ml LG69, 0.01 μg/ml TTNPB or 0.2 μg/ml LG69 and 0.01 μg/ml TTNPB, and cultured until they reached stage 8⁻ or 8. By this stage the anterior expression boundary of *Hoxb-1* was not affected when LG69 or TTNPB were provided alone (compare Fig. 5A with Fig. 5B,C). This is consistent with our morphological data (see above), in which no obvious defects were seen at these concentrations of agonists. However, the combination treatment caused a significant anterior shift of the rostral *Hoxb-1* expression boundary (Fig. 5D); there was ectopic expression of *Hoxb-1* anterior to the first somite (Fig. 5D) in a region in which *Hoxb-1* is normally not expressed (Fig. 5A).

At prestreak stages, the *otx-2* gene is expressed throughout the mouse and chick embryo, but between early and late streak stages, *otx-2* expression becomes progressively restricted to the anterior third of the embryo (Ang et al., 1994; Bally-Cuif et al., 1995). By the late headfold stage, *otx-2* is expressed from the presumptive mid-/hindbrain junction to the tip of the embryo (Fig. 6A). Treatment of early streak stage embryos with atRA resulted in a decrease of *otx-2* expression and simultaneously the posterior boundary of the *otx-2* expression domain shifted to a more anterior position than that in normal embryos (Ang et al., 1994; Bally-Cuif et al., 1995; Simeone et al., 1995). We found that the *otx-2* expression pattern was not affected in chick embryos exposed to 0.2 μg/ml LG69 (compare Fig. 6A and B), but *otx-2* was strongly suppressed in presumptive fore- and midbrain neuroectoderm by 0.01 μg/ml TTNPB (Fig. 6C). Unlike the situation with atRA, where a small *otx-2* expression domain remains, TTNPB abolishes all expression in neuroectoderm. Surprisingly, in combination treatments, the level of *otx-2* expression was the same as seen in untreated or in LG69-treated embryos (compare Fig. 6A,B and D), although the size of the *otx-2* expression domain was diminished as a result of the reduction in size of the fore- and midbrain primordia (Fig. 4D). In the untreated embryo *otx-2* mRNA was also found in head mesenchyme (Ang et al., 1994; Bally-Cuif et al., 1995); TTNPB did not affect *otx-2* expression in this tissue (Fig. 6C). In conclusion, our data demonstrate that the RAR agonist TTNPB repressed the *otx-2* gene, but that this inhibitory effect could be overcome by inclusion of the RXR-specific agonist. This situation greatly differs from that seen with *Hoxb-1*, where a straightforward synergism between liganded RXR and RAR was observed.

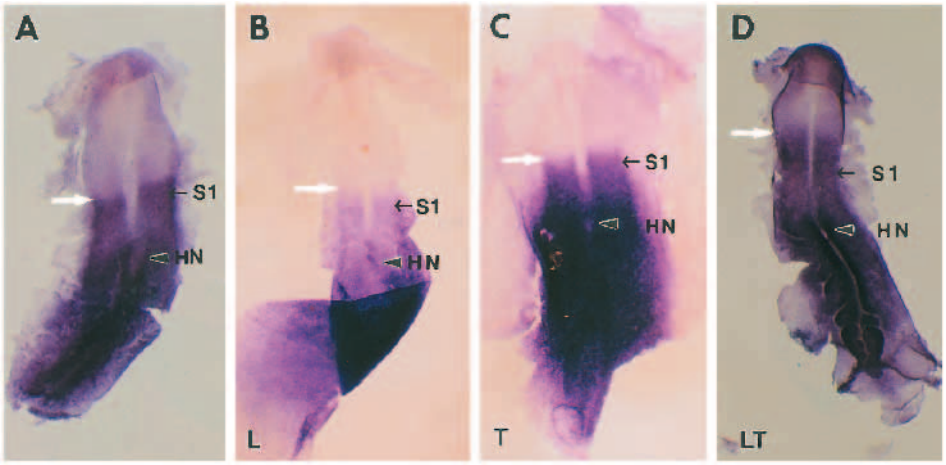

Fig. 5. The expression boundary (white arrow) of *Hoxb-1* shifts anteriorly upon treatment with the combination of LG69 and TTNPB. Embryos were treated at stage 4 and visualized for the expression of *Hoxb-1* by stage 8– using whole-mount in situ hybridization. (A) Untreated embryo; (B) LG69 (L, 0.2 μg/ml)-treated embryo; (C) TTNPB (T, 0.01 μg/ml)-treated embryo; (D) LG69 and TTNPB (LT, 0.2 μg/ml and 0.01 μg/ml)-treated embryo. The location of the anterior expression boundary of *Hoxb-1* in LG69- (B) or TTNPB- (C) treated embryos is the same as the untreated embryo (A). The combination treatment with LG69 and TTNPB (D) cause an anterior shifting of this expression boundary. Arrow/S1, position of first somite; HN, Hensen's node.

DISCUSSION

Retinoids regulate various aspects of vertebrate development (reviewed in Kastner et al., 1995). Here we have used two receptor-specific ligands, LG69 for RXR and TTNPB for RAR, to determine which type of retinoid receptor mediates a particular response in the context of an organism. Specifically, we have examined morphological effects caused by these receptor-specific agonists, alone and in combination, on the anteroposterior pattern of the limb and the structure of the anterior neural tube. In addition, we have studied a series of molecular markers that reveal cell fate changes in these tissues. We found that with regard to limb and CNS patterning, LG69 and TTNPB acted synergistically. Since pattern formation is a multistep process, such overall synergism could be caused by both ligands affecting one particular step. Alternatively, synergism could result from one step mediated by LG69 and another one mediated by TTNPB.

In order to determine how RXR- and RAR-specific retinoids affect the expression of an individual gene, we have investigated the inducibility of several retinoid-responsive genes by LG69 and TTNPB. *RARβ* and *Hoxb-1* genes were most effectively activated by a combination of both types of agonists, suggesting a synergistic action of liganded RXR and RAR. *Hoxb-6* and *Hoxb-8* genes were most efficiently induced by LG69, which implicates an involvement of liganded RXR. Transcription of the *otx-2* gene was repressed by TTNPB, but repression was relieved when LG69 was provided. Taken together, our studies make two points. First, liganded RXR and RAR can act synergistically. Second, since exogenously provided RXR ligand can induce (*RARβ Hoxb-6/8*) genes, we infer that in the context of an embryo, RXR can function as a ligand-regulated receptor. We emphasize that our experiments investigate responses that are evoked by exogenously provided ligands. It is conceivable that ligand-dependence of the corresponding processes occurring in the untreated embryos is at least in part, different from what we report in our studies. An added complication of experiments using exogenously provided agents is that these could synergize with their endogenous counterparts (see below for examples).

RARβ and *Hoxb-1* are synergistically activated by RXR and RAR ligands

In the limb bud, *RARβ* is activated by LG69, and to a lesser extent by TTNPB. Moreover, there is a significant synergism observed when both ligands are provided. The promoter region of the *RARβ* gene contains a direct repeat of a AGGTCA motif spaced by five nucleotides (DR 5) and a RXR-RAR heterodimer binds to this element (de Thé et al., 1990; Hoffmann et al., 1990; Sucov et al., 1990). How can the synergism

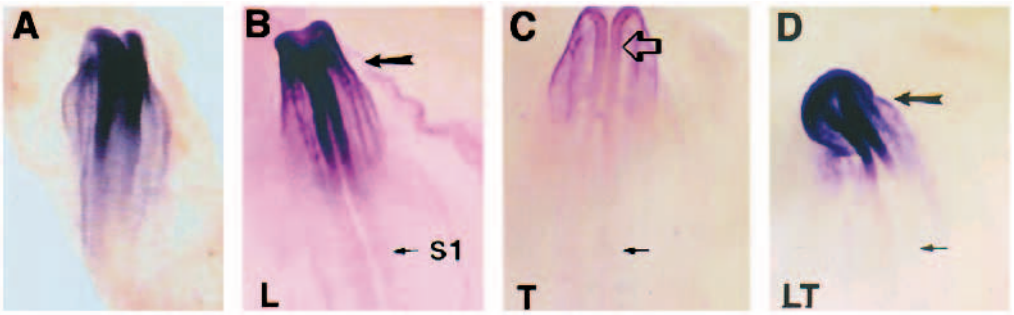

Fig. 6. *otx-2* is repressed upon the treatment with TTNPB while the treatment with LG69 or with the combination of LG69 and TTNPB had no effect. Embryos were treated at stage 4 and visualized for the expression of *otx-2* by stage 8 using whole-mount RNA in situ hybridization. (A) Untreated embryo, (B) LG69 (L, 0.2 μg/ml)-treated embryo; (C) TTNPB (T, 0.01 μg/ml)-treated embryo; (D) LG69 and TTNPB (LT, 0.2 μg/ml and 0.01 μg/ml)-treated embryo. In B and D, a large solid arrow points to the region expressing *otx-2* in the anterior part of the embryo. In a TTNPB-treated embryo (C), this region does not express *otx-2* (open arrow). The faint signal seen in this embryo is likely to represent the expression of *otx-2* in head mesenchyme (Ang et al., 1994; Bally-Cuif et al., 1995). Small arrow/S1, first somite.

observed in our experiments be explained? If both agonists are applied, then RAR and RXR are occupied by their cognate ligand and full transcriptional activation is achieved. If only LG69 is applied, it binds to RXR and in addition, endogenously present atRA (the atRA concentration in the limb bud is 20-50 nM, Thaller and Eichele, 1987) binds to RAR, thus giving rise to a RXR-RAR heterodimer with LG69 and atRA occupying the appropriate receptor. As a result of this, the *RARβ* gene is still efficiently transcribed. If TTNPB is provided, RAR is liganded, but since limb buds contain <1 nM 9cRA (our unpublished data) RXR is largely unoccupied (RXR has a K_d of 10 nM for 9cRA; Allenby et al., 1993) and therefore, the activation of *RARβ* gene is less pronounced. An alternative explanation for the synergistic activation of the *RARβ* gene is that this gene has two or even more RAREs. The known RARE could bind a RXR-RAR heterodimer activated by TTNPB and another yet unidentified element could bind a RXR-RXR homodimer or a RXR-orphan receptor heterodimer activated by LG69 (Mangelsdorf et al., 1991; Kliewer et al., 1992a,b; Zhang et al., 1992; Bourguet et al., 1995; Forman et al., 1995a,b; Leblanc and Stunnenberg, 1995; Perlmann and Jansson, 1995; Willy et al., 1995). The combined response of these two types of elements would also explain why a combination of TTNPB and LG69 is more effective. Roy et al. (1995) have examined the induction of the endogenous *RARβ* gene in EC cells by receptor-specific ligands and also found synergism.

The *Hoxb-1* gene contains two DR2 elements (direct repeats spaced by two nucleotides) which bind RXR-RAR heterodimers (Marshall et al., 1994; Studer et al., 1994; Ogura and Evans, 1995a,b). In the neural tube, this gene has a distinct anterior expression boundary coincident with the presumptive rostral end of rhombomere 4 (e.g. Sundin et al., 1990). In our assay, treatment with a combination of LG69 and TTNPB evoked an anterior shift of the *Hoxb-1* expression boundary, while each agonist by itself had no effect on the location of this boundary. The simplest explanation for synergism is that RAR and RXR bind their respective ligands and that such a dual receptor occupancy transactivates target genes in the embryo more strongly than a heterodimer in which only RAR is liganded.

In conclusion, the activation of *RARβ* and *Hoxb-1* genes through the DR5 and DR2 elements benefits from the presence of a liganded RXR and a liganded RAR. Current models of transcriptional regulation by retinoids assume that, in the case of DR5 elements, RXR-RAR heterodimers are complexed with a corepressor, and that binding of ligand to the RAR moiety induces conformational changes, which result in the release of the repressor and in binding of a coactivator (Chen and Evans, 1995; Kurokawa et al., 1995). Thus in this model RXR functions as a unliganded, silent partner and there is no room for ligand-dependent synergism. This mechanism has been established using transient transfection and in vitro DNA binding assays with idealized binding sites and micromolar ligand concentrations. The present study shows that the activation of endogenous genes by exogenously provided ligand exhibits synergism between liganded RXR and RAR. Similar conclusions were reached using differentiation and apoptosis of NB4 cells as an endpoint (Chen et al., 1996).

Expression of *Hoxb-6* and *Hoxb-8* is regulated by liganded RXR

At present, it is not known whether *Hoxb-6* and *Hoxb-8* genes contain a retinoic acid response element. However, since the induction of these genes in the limb bud occurs within 3 hours, a rate similar to that seen for *RARβ* (Soprano et al., 1994), which is known to have a RARE (see above), we propose a direct regulation. We found that even at low doses, LG69 effectively induced the expression of *Hoxb-6/-8*. Low dose treatment with TTNPB, however, did not induce these genes. As judged by in situ hybridization, low dose treatment with LG69 plus TTNPB does not increase the level of expression of either gene above that seen with LG69 alone, but the expression domain seems slightly enlarged. These findings raise the possibility that liganded RXR is required for the activation of *Hoxb-6/-8*.

Reciprocal regulation of *otx-2* expression by RXR and RAR ligands

At prestreak stages, the *otx-2* gene is expressed throughout the embryo, but by the late headfold stage, *otx-2* is expressed in the mid- and forebrain region of the mouse and chick embryo (Ang et al., 1994; Bally-Cuif et al., 1995). Treatment of embryos with atRA down-regulates the expression of *otx-2* (Ang et al., 1994; Bally-Cuif et al., 1995; Simeone et al., 1995). Our studies now reveal that this down-regulation is mediated by a liganded RAR, since treatment with TTNPB, but not with LG69, repressed the *otx-2* gene. While a retinoid response element has not yet been identified in the *otx-2* gene, its promoter region confers atRA-dependent repression (Simeone et al., 1995), raising the possibility that *otx-2* is directly regulated by retinoids. Several mechanisms can be envisaged by which TTNPB can repress *otx-2*. One possibility is that liganded RAR binds to a negative regulatory element in the *otx-2* promoter. Alternatively, liganded RAR could inhibit ligand-dependent activation of RXR by steric interference with the ligand binding domain of RXR. Kurokawa et al. (1995) provide an example for such a mechanism on a DR1 element; these authors show that liganded RAR in a RAR-RXR heterodimer prevents the release of co-repressor protein, thus inhibiting gene expression. A third possibility is that TTNPB induces the expression of a negative regulator, which would inhibit the expression of *otx-2*. To distinguish between these possibilities it will be necessary to fully characterize the *otx-2* regulatory region.

An unexpected feature of *otx-2* regulation by retinoids is that co-application of LG69 and TTNPB reversed the inhibitory effect exerted by TTNPB. There are two obvious explanations for this finding. First, liganded RAR could act as a repressor (see above), but in the presence of liganded RXR this repression would be released. This could occur through liganded RXR masking the repressor function of liganded RAR. Second, liganded RXR could be a positive regulator of *otx-2* expression. This raises the question of whether endogenous retinoids play a role in the regulation of *otx-2* expression. It has been suggested that endogenous atRA enriched in the hindbrain and trunk represses *otx-2* in these regions (Ang et al., 1994; Bally-Cuif et al., 1995; Conlon, 1995; Simeone et al., 1995). Such repression is not seen in the anterior regions of the embryo. One reason might be that atRA is not present in anterior tissue. In addition, tissue recombination experiments carried out in the mouse embryo revealed a factor in anterior mesendoderm that maintains the expression of *otx-2* in overlying neuroectoderm (Ang et al., 1994). The identity of

the factor is not known, but since LG69 (an analog of 9cRA) mimics its effects, it is possible that the unknown signal is either 9cRA or is induced by 9cRA. Intriguingly, 9cRA is enriched in the presumptive head region of the embryo (Creech Kraft et al., 1994) and binds with high affinity to RXR (Heymann et al., 1992).

In summary, we have used retinoid receptor-specific agonists to examine how such ligands influence the expression of several retinoid responsive genes. We intentionally used low agonist concentrations in these experiments and were able to reveal synergism between RXR and RAR ligands. We found that ectopically applied RXR ligand can transcriptionally regulate several of the genes examined, suggesting that liganded RXR plays a role in retinoid signal transduction. It should be pointed out that these conclusions are based on an approach using ectopically applied ligands that bind to endogenous receptors. While it is tempting to assume that applied ligands mimic their endogenous counterparts, our experiments do not prove this point. In other words, exogenous LG69 will induce *Hoxb-6/8* expression by binding to RXR. Whether its endogenous counterpart (presumably 9cRA) does this as well, remains an open question. Our studies also illustrate that, in addition to targeted mutagenesis of retinoid receptors (e.g. Kastner et al., 1995) and blocking of retinoid signaling by retinoid antagonists (Helms et al., 1996), receptor-specific agonists provide an additional and powerful tool to study the physiological roles of vitamin A and its derivatives in the context of a whole organism.

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