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Transgenic indicator mice for studying activated retinoic acid receptors during development

(mouse embryos/differentiation/transcription)

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ABSTRACT **Retinoic acid (RA) receptors (RARs) are ligand-inducible transcription factors that bind to specific DNA sequences associated with the regulatory regions of RA-regulatable genes. Since RA has been implicated as an important factor both in normal development and in teratological studies, one would like to have a model system that detects the presence of activated receptors during development. We have constructed a recombinant reporter gene that has three copies of the RA response element (RARE) from the RARβ-2 promoter 5′ to the herpes simplex virus thymidine kinase promoter; this regulatory region is coupled to the bacterial β-galactosidase reporter gene. This construct was RA inducible in transient transfection assays in F9 embryonal carcinoma cells. Transgenic embryos with this reporter gene construct exhibited restricted and reproducible patterns of β-galactosidase activity during embryogenesis, beginning between gestational ages day 7.5 and 8.5. At day 8.5, β-galactosidase activity was detected in the closed neurotube and somites. Day 8.5 embryos, from pregnant females fed RA 14 hr earlier, exhibited a greater intensity and distribution of β-galactosidase activity. Similarly, at later stages of gestation, maternal RA exposure resulted in enhanced embryonic β-galactosidase expression. This type of transgenic indicator mouse should be useful in detailing the role of activated RARs during embryonic development.**

Retinoic acid (RA) is involved in normal developmental patterning in the chick wing bud, is a potent teratogen in rodents and humans, and plays a role in normal cellular differentiation and gene regulation (see refs. 1–3 for reviews). Several receptors for RA have been identified. These retinoic acid receptors (RARs) are members of the superfamily of ligand-inducible transcription factors, which includes the steroid hormone, thyroid hormone, vitamin D_3, and retinoid receptors. DNA response elements have been identified to which individual receptors bind. The association of a receptor with these specific DNA sequences in the regulatory region of a gene can result in transcriptional activation and/or repression of the gene. Response elements with specificities for the RARs have been identified (4–9), and one of the more specific DNA sequences is a direct repeat found in the human and mouse RARβ-2 promoter (5–7). In transient cotransfection studies, this sequence coupled to a minimal promoter driving a reporter gene confers RA inducibility to the gene (5–7). It is an attractive hypothesis that the RARs and/or the retinoid-X receptors (10, 11) mediate some of these biological processes.

We have been directing our efforts towards defining the roles that RARs and retinoid-X receptors play during development and differentiation. One approach has been to introduce expression vectors for truncated RARs into F9 embryonal carcinoma (EC) cells. These expression vectors produce a dominant-negative effect upon endogenous RARs (12) resulting in an inhibition of RA-induced differentiation. Additionally, we have produced a constitutively active RAR vector that, when transferred to cells in culture or examined in transgenic mice, can produce an effect independent of RA (13). In this report we describe an approach to produce transgenic mice that allowed us to indirectly localize activated RARs. Current understanding on how RARs function is that an activated receptor would require not only the presence of the receptor but also its direct interaction with RA. By coupling a DNA element through which RARs act to a minimal promoter-reporter construct, one might be able to identify the presence of activated receptors during development.

Using the above approach, we report here the presence of reproducible reporter gene expression at different times of gestation and in specific embryonic regions in transgenic mice. The reporter construct used encodes bacterial β-galactosidase. When RA was administered to pregnant females carrying transgenic embryos, there was an increase in β-galactosidase activity in the regions observed in uninduced embryos along with expression in other regions of the embryos. The transgene was also shown to be RA inducible in embryonic fibroblasts isolated from transgenic embryos. This type of basic approach should be useful for identifying the tissues and developmental targets of activated RARs.

MATERIALS AND METHODS

Mice. FVB/N mice were obtained from Taconic Laboratory Animals.

Cell Culture and Transfections. F9 EC cells were cultured and transfected as previously described (14).

Recombinant DNA Constructions. The basic vector for making the transgene was pGEM-9Zf(−) (Promega). A *Bam*HI fragment containing the simian virus 40 (SV40) intron and polyadenylylation signal from pSV2 (15) was adapted to *Pst* I ends with a *Bam*HI to *Pst* I adaptor, and this fragment was cloned in the *Nsi* I site of the plasmid. A *Bam*HI site was added between the *Spe* I and *Hin*dIII sites of the polylinker. The resulting plasmid was named pW1. Three copies of the RARβ-2 promoter repeat were inserted into the *Sal* I site of the plasmid (5′-TCGAGGGTAGGGTTCACCGAAAGTTCAC-3′ and 5′-TCGAGTGAACTTTCGGTGAACCCTACCC-3′, based upon the sequence in ref. 5). A *Bgl* II to *Bam*HI fragment containing a thymidine kinase promoter luciferase fragment was inserted into the *Bam*HI site of the plasmid. The luciferase insert was removed through a *Pst* I to *Bam*HI restriction. A *Pst* I to *Bam*HI adapter was used to ligate the *Pst* I site to the 3′ *Bam*HI site, and a *Bgl* II fragment containing the bacterial *lacZ* gene was cloned in the *Bam*HI site. This final construct has in 5′ to 3′ order: three copies of the RARβ-2 promoter repeat (the first two copies in reverse

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Abbreviations: RA, retinoic acid; RAR, RA receptor; RARE, RA response element; SV40, simian virus 40.

tandem orientation; see Fig. 1*B*), the thymidine kinase promoter, and the *lacZ* gene, followed by the SV40 intron plus the poly(A) addition signal. The final construct was digested with *Not* I and *Sfi* I and the transgene was separated from the plasmid sequences by agarose gel electrophoresis.

β-Galactosidase Activity. β-Galactosidase activity was measured from cell extracts by using the procedure of Miller (16). The embryos were stained for β-galactosidase activity by using a modification of the Sanes *et al.* (17) protocol as described by Balkan *et al.* (13). Reactions were allowed to proceed for the times indicated at room temperature in a humidified chamber.

Pronuclear Injections. Ova were obtained from superovulated FVB/N females mated to FVB/N males. Mice were maintained on a 14-hr light/10-hr dark cycle and received food and water ad lib. Transgenic mice were generated by introducing linearized DNA into one of the pronuclei of recently fertilized mouse ova at 1.5 ng/μl. Zygotes were transferred into the oviducts of pseudopregnant B6SJL mice.

Embryonic RA Exposure. Hemi- or homozygous transgenic males were mated to normal FVB/N females. Pregnant females were gavage-fed either RA in sesame oil at 80 mg/kg or sesame oil alone. Embryos were isolated 14 hr later.

Embryonic Fibroblasts. The transgenic line was bred to homozygosity. Homozygous indicator males were mated with normal FVB/N females and embryos were isolated at gestational ages day 12.5 or 13.5. Primary cultures of embryonic fibroblasts were prepared from transgenic embryos (18). Primary isolates or second-pass fibroblasts were grown in media with or without 0.1 μM RA for 24 hr. Cell extracts were prepared and assayed for β-galactosidase activity as described above.

Histology. Transgenic embryos were stained for β-galactosidase activity as described above. Day 13.5 embryos were postfixed with neutral buffered Formalin, dehydrated, and embedded in paraffin. Sections were counterstained with alcoholic eosin.

RESULTS

The current model for RAR function assumes interaction of RA with the ligand-binding domain of the receptor, followed by binding of a receptor dimer to a specific DNA sequence associated with the gene to be regulated (see Fig. 1*A*). This interaction can result in transcriptional enhancement or repression, depending upon the sequences involved. The RARE from the RARβ-2 promoter is an extremely sensitive indicator element for the presence of activated RARs in cell culture transfection studies (5–7). Although activated thyroid hormone receptors have been shown to bind to the RARE, it has been reported that they do not activate expression of reporter genes coupled to this response element and basal promoters (5, 6). Fig. 1*B* illustrates the DNA construct used in the transgenic studies described below. The construct was composed of three copies of the RARE element 5′ to the herpes simplex virus thymidine kinase promoter and coupled to the structural gene encoding bacterial β-galactosidase (*lacZ*). The SV40 intron and polyadenylylation signal were 3′ to the *lacZ* gene. The construct was tested by transfecting F9 EC cells, followed by treatment of the cells with and without 0.5 μM RA. *In situ* staining of the cells for *lacZ* activity (data not shown) illustrated RA inducibility. Cell extracts prepared from the transfected cells showed a 17-fold RA induction of the reporter gene (see Fig. 1*C*).

Its RA inducibility having been demonstrated, the DNA construct was microinjected into the pronuclei of fertilized FVB/N eggs. Four transgenic founder animals were generated, three of which transmitted the transgene to their offspring. One of these lines was selected and bred to homozygosity for the indicator transgene (an independent transgenic line produced a pattern of expression shown by the selected founder line; data not shown). FVB/N females were mated with males hemizygous or homozygous for the transgene. The females were sacrificed at various times after the identification of the fertilization plug. Noon of the morning after mating was designated day 0.5 of gestation. We could not detect β-galactosidase expression in day 7.5 embryos. Transgenic embryos between days 8.5 and 12.5 exhibited specific and reproducible patterns of β-galactosidase expression (see Fig. 2). Distinct staining in the closed neural tube was seen in the day 8.5 embryo (Fig. 2 *A* and *B*). When females carrying day 8.0 transgenic embryos were gavage-fed RA at 80 mg/kg and the embryos were isolated 14 hr later (day 8.5), we observed a greater intensity and distribution of the β-galactosidase compared with non-RA-exposed embryos. There was an extension of the anterior–posterior borders of the β-galactosidase expression, plus an extension of the stain to both the neural folds and the simple heart tube (see Fig. 2*B* for a comparison of untreated and RA-treated embryos). The arrows in Fig. 2*B* point out the anterior and posterior borders of the β-galactosidase activity in the untreated embryo. Sections through embryos at these stages (data not shown) demonstrated β-galactosidase expression associated with both the neural tube and the somites. Fig. 2 *C* and *D* illustrate RA-treated and untreated day 9.5 embryos. RA treatment continued to produce both an enhancement of the uninduced patterns and an extension of the pattern in an anterior–posterior fashion. The optic cup exhibited β-galac-

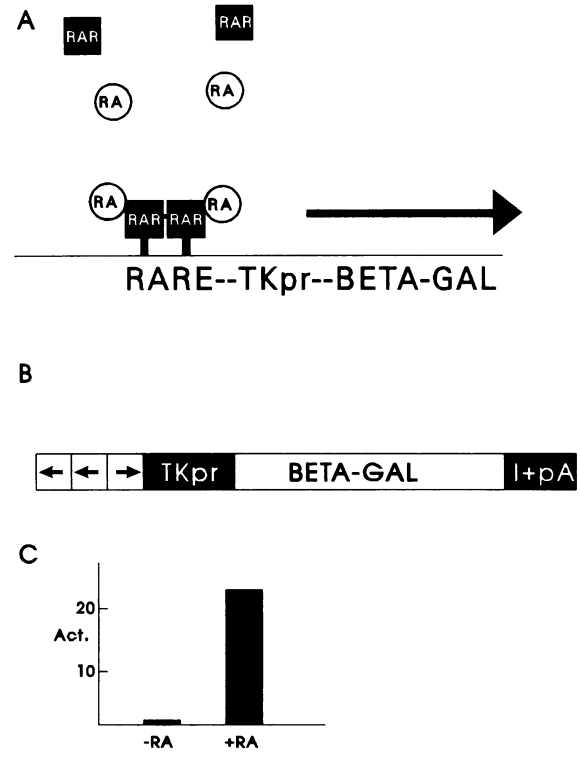

FIG. 1. (*A*) Current model for gene activation by a ligand-inducible transcription factor. In transgenic animals, expression of reporter gene (β-galactosidase, BETA-GAL) serves as an indicator for activated receptors. RARE, RA response element; TKpr, herpes simplex virus thymidine kinase promoter. (*B*) Specific DNA construct, used to detect activated RARs in cell culture and in transgenic mice. I + pA, SV40 intron plus polyadenylylation signal. Arrows represent orientation of the RARβ-2 direct repeat element. (*C*) β-Galactosidase activity from F9 embryonal carcinoma cells transfected with 20 μg of the DNA described in *B*. Act., specific activity [units (16) per mg of protein per min]. The data represent the average of three separate transfections. Transfections were for 24 hr in the absence (−) or presence (+) of 0.5 μM RA.

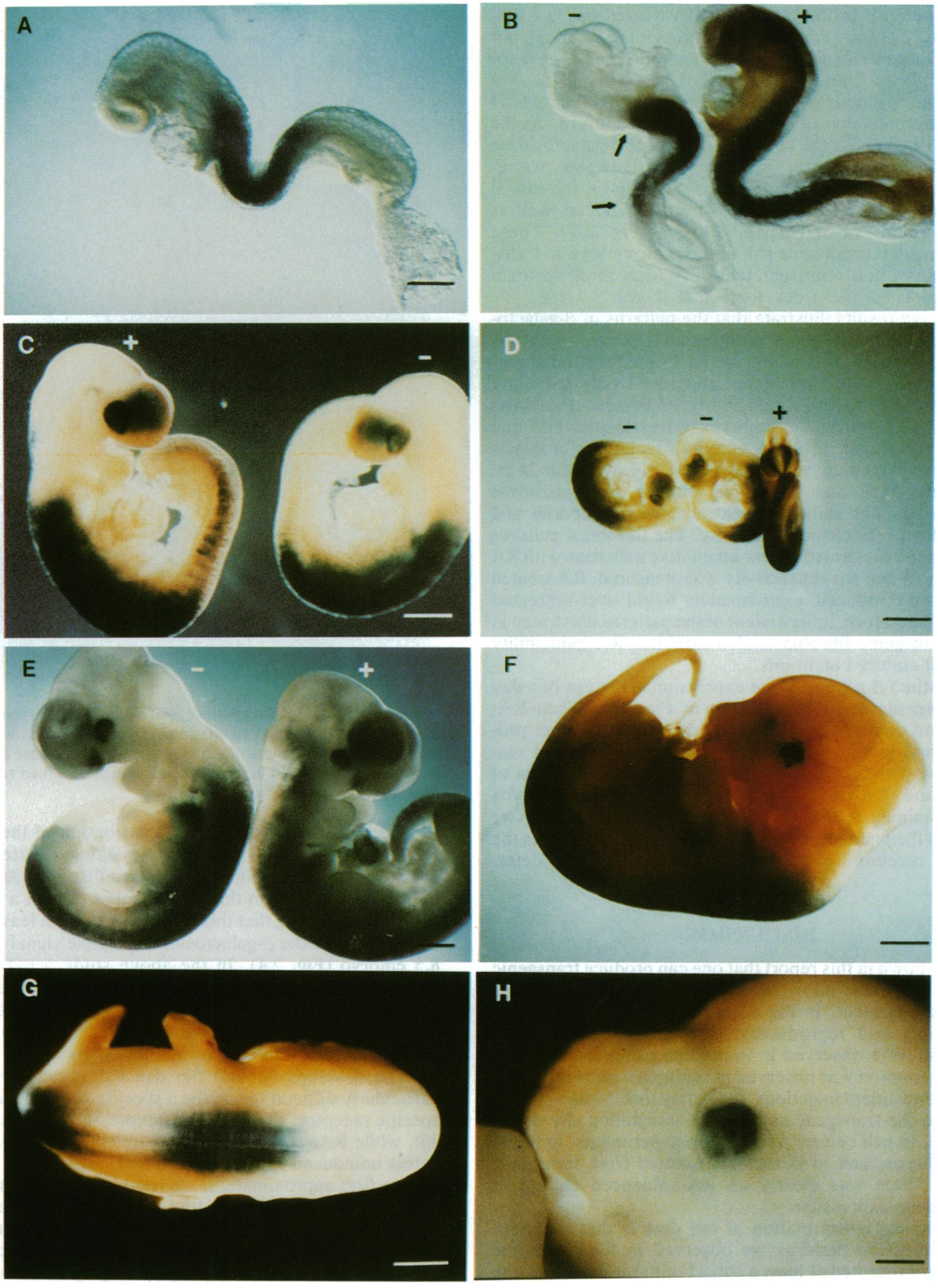

FIG. 2. β-Galactosidase activity in transgenic embryos carrying the DNA construct in Fig. 1*B*. The activity stains dark blue. (*A*) Day 8.5 embryo. (Bar = 200 μm; 4-hr staining for β-galactosidase activity.) (*B*) Day 8.5 embryos. (Bar = 200 μm; stained for 4 hr.) The − and + in this and subsequent panels indicate embryos whose mothers were untreated or treated with RA, respectively. Arrows represent anterior and posterior boundaries of β-galactosidase activity in the untreated embryo. Note the extended staining in the treated embryo. (*C*) Day 9.5 embryos from untreated (−) and RA-treated (+) mothers. (Bar = 500 μm.) (*D*) Day 9.5 embryos from untreated (−) and RA-treated (+) mothers. (Bar = 1 mm.) The embryos in *C* and *D* were stained for 5.5 hr for β-galactosidase activity. Note the enhancement and extension of the β-galactosidase activity in the RA-treated embryos. (*E*) Day 10.5 embryos from untreated (−) and RA-treated (+) mothers. (Bar = 500 μm; stained for 18 hr for β-galactosidase activity.) (*F–H*) Embryos from untreated mothers. (*F*) Day 12.5 embryo. (Bar = 500 μm; stained for 18 hr for β-galactosidase activity.) (*G*) Day 12.5 embryo. (Bar = 500 μm; stained for 3.5 hr for β-galactosidase activity.) (*H*) Day 12.5 embryo. (Bar = 250 μm; stained for 18 hr for β-galactosidase activity.) In *B*, *C*, *D*, and *E*, mothers carrying transgenic embryos were gavage-fed RA at 80 mg/kg in sesame oil for 12–16 hr. All patterns are representative of results obtained from a minimum of three independent experiments for each stage.

tosidase activity in untreated and RA-treated day 9.5 embryos (Fig. 2 *C* and *D* and sections through the embryos, not shown). In general, RA treatment extended the β-galactosidase activity posteriorly to the end of the tail in both day 9.5 (Fig. 2 *C* and *D*) and day 10.5 (Fig. 2*E*) embryos. Fig. 2 *F–H* shows different views of untreated day 12.5 embryos. As the embryos developed, the specific patterns of β-galactosidase expression became increasingly complex. However, we repeatedly detected two major foci of activity on the dorsal aspect of day 12.5 embryos (shown in Fig. 2*G*) as well as distinct regions of activity in the eye (Fig. 2*H*). These patterns were reproducible from transgenic embryo to transgenic embryo and representative of at least three separate sets of studies at these developmental stages.

The above results illustrate that the patterns of β-galactosidase expression observed in untreated transgenic embryos changed when RA was administered to pregnant mice carrying transgenic embryos. Since this result only indirectly measures β-galactosidase inducibility, embryonic fibroblasts from day 12.5 transgenic embryos were isolated (18) and assayed for β-galactosidase activity. Isolated fibroblasts were grown in medium with and without 0.1 μM RA for 24 hr. Cells were fixed and stained *in situ* for β-galactosidase activity (data not shown) or extracts were prepared and assayed for β-galactosidase activity. The fibroblast cultures stained *in situ* illustrated a clear qualitative induction with RA treatment. When enzyme activity was measured, RA-treated cultures were induced approximately 6-fold over untreated cultures. Therefore, independent of the patterns illustrated in Fig. 2, transgenic fibroblasts also exhibited RA inducibility under cell culture conditions.

The distinct β-galactosidase expression in the eye of a day 12.5 transgenic embryo is shown in Fig. 2*H*. When RA-treated and control day 13.5 transgenic embryos were processed for β-galactosidase expression, embedded, and sectioned, we detected distinct staining in the neural retina of control and RA-treated embryos. RA-treated embryos showed staining in the neural retina, lens, and, to a lesser degree, in the pigmented retinal layer (Fig. 3). What roles the activated receptors play in this tissue remain to be determined.

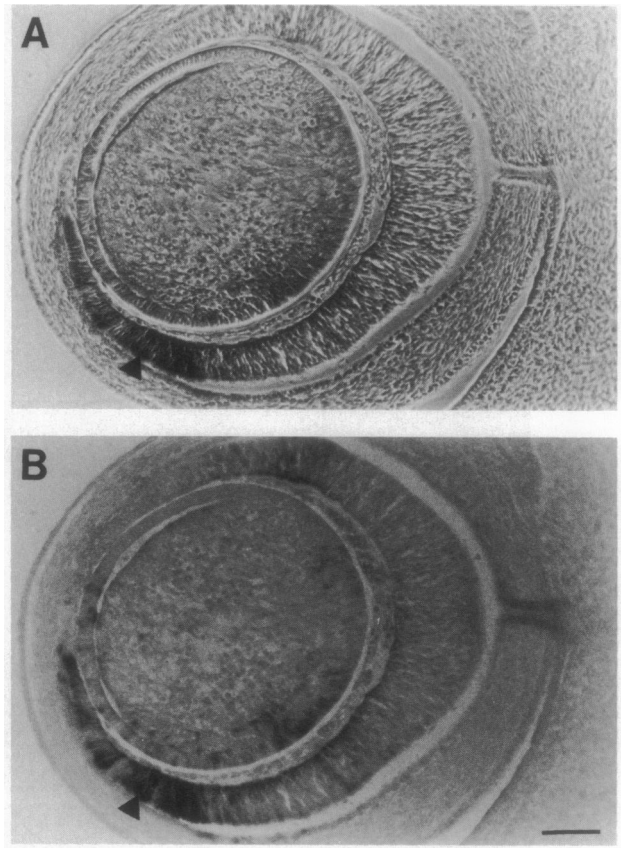

FIG. 3. Sections through the eye of a day 13.5 transgenic embryo. Phase-contrast (*A*) and brightfield (*B*) micrographs show β-galactosidase staining in the eye of a transgenic embryo whose mother was treated with RA for 14 hr. Arrowheads indicate stained region in the neural retinal layer. (Bar = 50 μm.)

DISCUSSION

We have shown in this report that one can produce transgenic indicator mice that allow for the study of activated RARs during development. In the absence of exogenously added RA, distinct and reproducible patterns of reporter gene expression were observed in transgenic embryos. This pattern of expression was observed in transgenic mice produced by two independent injections, indicating that it was a pattern specific to the transgene and not the integration site of the transgene. In cell culture transfection experiments, in transgenic embryos, and in fibroblasts isolated from transgenic embryos, there was clearly an RA-influenced change in reporter gene expression.

The simplest interpretation of our data is that the uninduced expression patterns we observed in the transgenic embryos represented at least a subset of tissues in which the existing RARs were activated. We assume that the activation is through natural RA interaction with existing receptors, an assumption that is supported, in part, by our observations that RA treatment of pregnant females carrying the transgenic embryos resulted in an enhancement of the uninduced pattern of expression (in addition to an extension of the expression pattern to other areas of the embryos). Comparing our patterns with published reports of *in situ* hybridization of RNAs for RARs in developing embryos (19, 20) suggests that areas of β-galactosidase activity correlated with localization of RAR mRNAs. Given the nature of this system, it is difficult to ascertain the corresponding contribution of the different RARs to a specific β-galactosidase staining pattern. However, Ruberte *et al.* (20) have shown a distinct RARβ *in situ* hybridization signal in the closed neural tube of a 14-somite embryo, suggesting that this receptor may be at least partially responsible for the β-galactosidase-positive signal in the day 8.5 embryo (Fig. 2*A*). In the above study a hybridization signal for RARγ mRNA was detected in the open caudal neuropore, suggesting that the posterior extension of the β-galactosidase activity in RA-induced embryos may be mediated either directly or indirectly by RARγ. As the embryo develops beyond the day 8.5 stage, it would be particularly difficult to ascribe a specific staining pattern to a specific receptor, since RARα is expressed ubiquitously (19, 20), while RARβ is expressed in several regions where we detect uninduced expression of β-galactosidase.

As a first approximation, we have used a minimal thymidine kinase promoter to restrict the expression of a reporter gene to inducible events. A more complex promoter, such as the whole RARβ-2 promoter, might contain a complex combination of transcription factor binding sites that could obscure the indicator transgene approach we have described in this study. Any choice of natural promoter might bias the type of results obtained, since the co-interaction of activated RARs with other transcription factors that might bind to a basal promoter is not very well understood at this time. Since promoters available for transgenic research are usually characterized only in cell lines, it is difficult to predict the variety of regulatory events occurring within each cell type and the differentiation processes that take place during development. In this context, a report was recently published describing a transgenic approach similar to ours. Rossant *et al.* (21)

described an RA-inducible transgene that included three repeats of the RARβ-2 element; however, their construct was coupled to the mouse 68-kDa heat shock protein (HSP68) promoter (22). While their patterns resemble ours early (day 8.5), there are apparent differences at later stages. These differences may reflect the more complex promoter these investigators used, since their promoter has been found to be both heat inducible and arsenite inducible within the context of transgenic animals (23). These added elements in the mouse HSP68 promoter may themselves be indirectly inducible by RA-mediated events *in vivo*. The differences illustrate the care that must be taken in the future in defining minimal promoters for use in transgenic studies. As these processes become better understood, we believe this type of transgenic approach will be generalized for several specific transcription factor binding sites as a means to dissect the roles of gene regulators during development.

The expression patterns observed in these transgenic embryos under uninduced and RA-induced conditions provide a direction for focusing attention to the regions of the embryos where activated receptors occur. For example, the neural retina would be a likely target for directing constructs to interfere with RA-inducible events. Both dominant-negative (3, 12) and constitutively active (3, 13) expression vectors for RARs are available for such experiments.

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