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Original Article

Embryonic stem cells stably transfected with mRARß$_2$-lacZ exhibit specific expression in chimeric embryos

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ABSTRACT **Using the embryonic stem (ES) cell/chimera approach, we have studied the activity of the mouse retinoic acid receptor ß$_2$ (mRARß$_2$) promoter during ES cell differentiation and during embryonic development. Stable ES clones were isolated after introduction of a 1.8 kb mRARß$_2$-lacZ expression cassette. LacZ expression in these stable clones was specifically induced by retinoic acid (RA) in a similar fashion as the endogenous RARß$_2$ gene. Following introduction of three different ES clones into blastocysts, an integration-independent mRARß$_2$-lacZ expression pattern was obtained in chimeric embryos similar to that described by *in situ* hybridization and transgenic studies. Moreover, mRARß$_2$-lacZ expression was also detected at some additional sites not described before, e.g. body wall, ureter, mesonephric duct and optic stalk. Maternal RA administration at 8.5 days of pregnancy extended lacZ expression to more anterior and posterior regions. Transgenic mice were generated from germ-line transmission of the transfected ES cells; expression pattern and changes in expression upon RA induction in these transgenic embryos were identical to those in chimeric embryos. We conclude that by using the ES/chimera approach, the proximal 1.8 kb of the mRARß$_2$ promoter produces a reliable and reproducible expression pattern of the reporter gene, and that the ES cell/chimera approach is invaluable for the study of gene expression and regulation.**

KEY WORDS: *chimeric embryos, embryonic stem cells, mRARβ$_2$-lacZ expression, retinoic acid*

Introduction

RA has a wide spectrum of effects on embryonic development. Treatment of embryos with excess RA induces malformations of different tissues including hindbrain, neural tube, craniofacial tissue, vertebrae and limbs in different vertebrates (Kochhar, 1973; Sulik *et al.*, 1988; Tibbles and Wiley, 1988; Durston *et al.*, 1989; Kessel and Gruss, 1991; Morriss-Kay *et al.*, 1991). Local application of high concentrations of RA to the chicken limb bud mimics the zone of polarizing activity (Tickle *et al.*, 1982). *In vitro*, RA can induce differentiation of a variety of cells (Breitman *et al.*, 1980; Lotan, 1980), including embryonal carcinoma (EC) cells (Strickland and Mahdavi, 1978; Edwards and McBurney, 1983) and ES cells (Smith and Hooper, 1987).

It is now known that RA functions through binding to specific receptors (RARs) which are members of the steroid/thyroid hormone receptor superfamily (Evans, 1988). RARs act as nuclear transcriptional activators or repressors on target gene promoters (Gudas, 1992). Three different RARs, α, ß, γ (Giguere *et al.*, 1987; Petkovich *et al.*, 1987; Benbrook *et al.*, 1988; Brand *et al.*, 1988; Krust *et al.*, 1989; Zelent *et al.*, 1989) and more recently a second class of related receptors, designed RXRs, α (Mangelsdorf *et al.*, 1990), ß (Yu *et al.*, 1991), and γ (Leid *et al.*, 1992) have been discovered. The fact that transcripts of the different RARs exhibit unique expression patterns in the embryo suggests that each receptor may have a distinct function during development (Dolle *et al.*, 1989, 1990; Ruberte *et al.*, 1990, 1991). RARß expression is mainly detected in the central nervous system, the facial areas, the digestive tract, the urogenital system, in the limbs and sense organs (Dolle *et al.*, 1990, Ruberte *et al.*, 1991).

Different isoforms were identified for members of the RAR subfamily which are generated by differential splicing and/or different promoter usage (Kastner *et al.*, 1990, Leroy *et al.*, 1991, Zelent *et al.*, 1991). For the RARß gene, three isoforms have been described with different exons encoding the A domain of the receptor: RARß$_1$ and RARß$_3$ are transcribed from a distinct promoter more than 20 kb upstream of the RARß$_2$ promoter, and RARß$_2$ is the most active form in RA-treated EC or ES cells and in embryos (Zelent

Abbreviations used in this paper: BRL-CM, buffalo rat liver cell-conditioned medium; EC, embryonal carcinoma; ES, embryonic stem; kb, kilobase pair; lacZ, bacterial ß-galactosidase gene; MEM, minimal essential medium; RA, retinoic acid; RAR, retinoic acid receptor; RARE, RA responsive element; RXR, retinoid X receptor.

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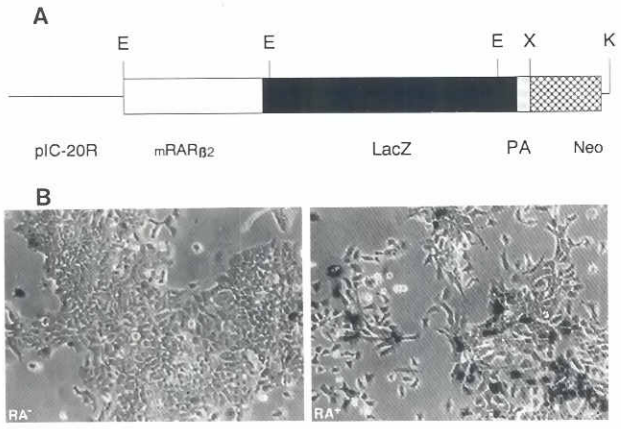

Fig. 1. Structure of the mRARß$_2$-lacZ expression cassette (A) and its transient expression in P19 EC cells (B). (A) *Schematic representation of the construct containing 1.8 kb of mRARß$_2$ promoter (open box), 3.4 kb of lacZ coding sequence (filled box) and 1.1 kb of pMC1Neo (hatched box). The restriction sites used for linearization of the plasmid (KpnI) and digestion of genomic DNA for Southern hybridization (EcoRI and XhoI) are marked with K, E and X, respectively.* **(B)** *mRARß$_2$-lacZ expression in transiently transfected P19 EC cells in the absence (RA$^-$) or presence of RA (RA$^+$) at a concentration of 1 μM for 24 h.*

et al., 1991), indicating an important role of the RARß$_2$ gene during early development.

In previous studies we have characterized both human and mouse RARß$_2$ promoters in P19 EC cells and found that, in addition to the RA-responsive element (RARE) at position -59 (de The *et al.*, 1990; Hoffmann *et al.*, 1990; Sucov *et al.*, 1990), more upstream sequences contribute to the RA-inducibility as well (Kruyt *et al.*, 1991; Shen *et al.*, 1991). Although the EC cell is a very useful system for studying RARß$_2$ regulation *in vitro*, its regulation during embryonic development is not understood. Rossant *et al.* (1991) have recently produced transgenic embryos using a lacZ reporter gene under the control of three copies of the RARß$_2$-RARE coupled to a heat-inducible (hsp68) promoter. In a recent study, transgenic mice carrying a 3.8 kb mRARß$_2$ promoter-lacZ fusion gene were reported by Mendelsohn *et al.* (1991).

To study RARß$_2$ promoter regulation *in vivo*, we have chosen the ES cell/chimera approach similar to that described by Lovell-Badge *et al.* (1987). ES cells derived from the inner cell mass of the blastocyst resemble both inner cell mass and EC cells in many respects (Rossant and Papaioannou, 1984). After re-introduction into embryos, they can participate in all tissues originated from three germ layers including functional gametes. Single copy integration of a reporter gene, rather than multiple integration usually occurring upon microinjection of DNA into pronuclei of fertilized eggs, can be achieved relatively easily in ES cells by electroporation, without interference with their developmental potency. Both integration and expression patterns of the reporter gene can be determined *in vitro* before returning cells to embryos. In addition, ES cells can be maintained as undifferentiated stem cells in conditioned medium from Buffalo rat liver cells (BRL-CM), or can be differentiated into different cell types by transfer to minimal essential medium (MEM) or by addition of RA (Mummery *et al.*, 1990). Therefore, this approach is the only method making it possible to connect *in vitro* and *in vivo* studies, and this is the first study of this type for a member of the steroid/thyroid receptor superfamily.

In this report we describe the isolation of stable ES clones containing the mRARß$_2$-lacZ transgene in one or multiple copies. Transgene expression was studied during ES cell differentiation *in vitro* and during development in chimeric embryos as well as in ES cell-derived transgenic embryos. The results show that 1.8 kb mRARß$_2$ promoter can direct lacZ expression both in stable ES cells and in embryos in a manner similar to that of the endogenous RARß$_2$ gene. Moreover, maternal administration of RA extends the borders of lacZ expression to more anterior and posterior regions.

Results

The mRARß$_2$-lacZ vector and its expression in vitro

As we described before (Shen *et al.*, 1991), 1.8 kb of the most proximal sequence of the mRARß$_2$ promoter contains sufficient information to direct transient lacZ expression in EC cells in a RA-inducible manner. To investigate the RARß$_2$ promoter activity during embryonic development through the ES cell/chimeric embryo approach, we constructed an expression cassette by coupling the mRARß$_2$-lacZ construct to the neomycin-resistance gene (Fig. 1A). This construct was tested by a transient expression assay in P19 EC cells. As can be seen in Fig. 1B, lacZ was hardly expressed in transfected cells in the absence of RA (Fig. 1B, RA$^-$), while after addition of RA at a concentration of 1 μM for 24 h, expression was significantly induced (Fig. 1B, RA$^+$).

To obtain ES cells with stable integration of the expression cassette, the construct was linearized at the 3' end of the neomycin-resistance gene and introduced into ES cells by electroporation. Resistant clones were isolated after three weeks of G418 selection. Subsequently, these clones were treated with RA and stained with X-gal to detect lacZ expression in comparison with cells maintained in BRL-CM. RA-inducible lacZ expression was observed after 48 h of RA treatment in most isolated clones.

Among these clones, three (1F5, 2E5 and 2F3) were characterized by Southern hybridization (Fig. 2). When genomic DNA from these clones was digested with *EcoR*I and hybridized with the lacZ coding sequence, a 3.0 kb DNA fragment was detected in all clones as expected (Fig. 2B). When *EcoR*I-digested genomic DNA was probed with the mRARß$_2$ promoter sequence, a 1.8 kb hybridization signal was observed besides the 4.2 kb endogenous fragment (see Fig. 2A and Shen *et al.*, 1991). Quantification of the ^{32}P signal revealed that both 2E5 and 1F5 clones, which were isolated from two independent electroporations, contain a single copy integration. However, the 2F3 clone has multiple integrations of the reporter gene (not shown).

ES cells can be differentiated by addition of RA to the BRL-CM or by maintaining cells in MEM (Mummery *et al.*, 1990). However, RARß transcripts are only expressed in RA-treated cells, but not in cells differentiated in MEM (our unpublished observation). Consistent with endogenous RARß induction, the mRARß$_2$-lacZ gene was strongly expressed in cells in the presence of RA, but there was practically no expression in cells either maintained in BRL-CM or differentiated in MEM (Fig. 3). LacZ expression in these ES clones is relatively sensitive to RA treatment as 10^{-9} M RA can induce lacZ expression significantly (data not shown). Among different clones, there was no significant difference in the transgene expression when the ß-gal activity was quantified by assaying cell extracts with O-nitrophenyl-ß-galactopyranoside (not shown). Rapid induction of

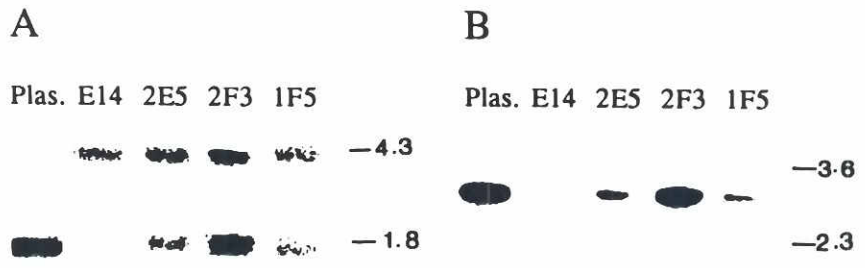

Fig. 2. Southern hybridization of stably transfected ES cell lines. *Ten μg of genomic DNA from three independent clones (2E5, 2F3 and 1F5) as well as negative control DNA from non-transfected ES cells (E14) were loaded per lane. As a positive control, 15 ng plasmid DNA (plas.) was added to 10 μg of E14 genomic DNA. Genomic DNA was digested with EcoRI restriction enzyme and hybridized with the 1.8 kb of [^{32}P]-labeled mRARß$_2$ promoter* **(A)** *or 3 kb lacZ coding sequence* **(B)**. *DNA size is indicated in kilobase. The integration copy number of the transgene for 2E5 and 1F5 clones was determined on the Phosphorimager by the ratio of the transgene signal (1.8 kb) over the endogenous gene signal (4.2 kb, 2 copies) in each cell line.*

lacZ expression in combination with its down-regulation after 5 days of RA treatment indicates that RARß$_2$ is an early response gene in the RA-mediated differentiation pathway.

In conclusion, these results demonstrate that the 1.8 kb of the mouse RARß$_2$ promoter sequence can regulate lacZ expression in a fashion similar to that of the endogenous RARß gene in these stably transfected ES clones.

Mouse RARß$_2$-lacZ expression in chimeric mouse embryos

To investigate the RARß$_2$ promoter-directed lacZ expression during embryonic development, the three independent clones described above were injected into a total of 316 blastocysts obtained from superovulated females. After transfer of 272 (86.1%) manipulated embryos into uteri of foster mothers, 168 (61.8%) embryos were recovered at mid-gestation stages and 127 (75.6%) chimeric embryos were identified based on X-gal staining (Table 1).

A potential problem in studying gene expression in chimeric embryos is that in a given embryo, information may be lost due to a restricted distribution of ES cells. We tried to overcome this problem by introducing a total of 20 to 30 ES cells into each blastocyst in order to increase the ratio of ES/inner cell mass cells. In this way, transgene-like chimeric embryos with highly identical lacZ expression patterns were obtained (Fig. 4F). Moreover, the results showed that 1.8 kb of the mRARß$_2$ promoter expressed the lacZ reporter gene in an integration-independent manner, since chimeric embryos with different clones of ES cells revealed identical and age-dependent staining patterns (see below, Fig. 7A,C and E). In addition, the lacZ transgene was detected in all organs of a newborn chimeric mouse by Southern hybridization (Fig. 5). These data together with other independent evidence demonstrate that ES/chimera is a reliable and reproducible approach for the study of gene expression during development (see discussion).

By 9.5 days, mouse embryos possess 21-29 somites with apparent forelimb buds at the level of 8-12th somite, while the hindlimb buds have not appeared yet. The anterior neuropore is closed but the posterior neuropore still remains open. In chimeric embryos at this stage, mRARß$_2$-lacZ was strongly expressed in the spinal cord with a sharp anterior border, caudal to the third pharyngeal arch at the closure point of the neural tube (Fig. 4A). However, the posterior border of lacZ expression was located at the level of 12th somite, neither identical to the RARß transcripts detected by *in situ* hybridization (Ruberte *et al.*, 1991) nor similar to expression in transgenic animals (Mendelsohn *et al.*, 1991; Rossant *et al.*, 1991) in which it was localized to the posterior neuropore. In the spinal cord caudal to the 12th somite, expression was reduced abruptly. In agreement with *in situ* hybridization data (Dolle *et al.*, 1990), ß-gal activity was observed in the fronto-nasal region (fn, Fig. 4A). In the region of the forelimb bud, strong staining appeared in the anterior part at the base of the limb bud (Fig. 4A). In sections, staining was detectable in developing gut and heart (Fig. 6G). The lateral body wall ventrally to the somites expressed the lacZ transgene in a thin line along the anterior/posterior (A/P) axis caudal to the forelimb buds; expression at this position continued up to 12.5 days and disappeared in day 13.5 embryos (arrows in Figs. 4 and 7).

A day 10.5 embryo consists of 35-39 somites with the appearance of hindlimb buds as an important feature. At this stage, lacZ expression in the anterior part of the neural tube was similar to that in day 9.5 embryos and remained very strong (Fig. 4B), and staining also appeared at the position of hindlimb buds. However, in the spinal cord between fore- and hind-limb buds ß-gal activity continued to be weak. In contrast, staining at the lateral body wall (arrow, Fig. 4B) became more intense, with stronger expression near the forelimb buds. In intact embryos, high ß-gal activity was observed in spinal ganglia in the regions corresponding to lacZ expression in the neural tube, while sections revealed that staining was mainly limited to the outer layer of spinal ganglia (not shown). Expression at the base of the forelimb bud was reduced compared to day 9.5 embryos, whereas very weak staining appeared at the base of the hindlimb bud. In sections mRARß$_2$-lacZ expression was also observed in developing gut, lung bud and heart (not shown).

In day 11.5 chimeric embryos, expression at the anterior part of the spinal cord was similar to that in day 10.5 embryos, while at the posterior part of the neural tube corresponding to developing hindlimb buds (between the 21st and 25th somite), staining was becoming stronger (Fig. 4C). LacZ expression along the body wall was increased caudally in the direction of the hindlimb bud. In sagittal sections, high ß-gal activity was detected in mesonephros and metanephros, and weak expression was present in the developing lung and eye (not shown).

By 12.5 days, the hand-plates are becoming indented, the tongue is delimited from the jaw, and the gonads are formed and

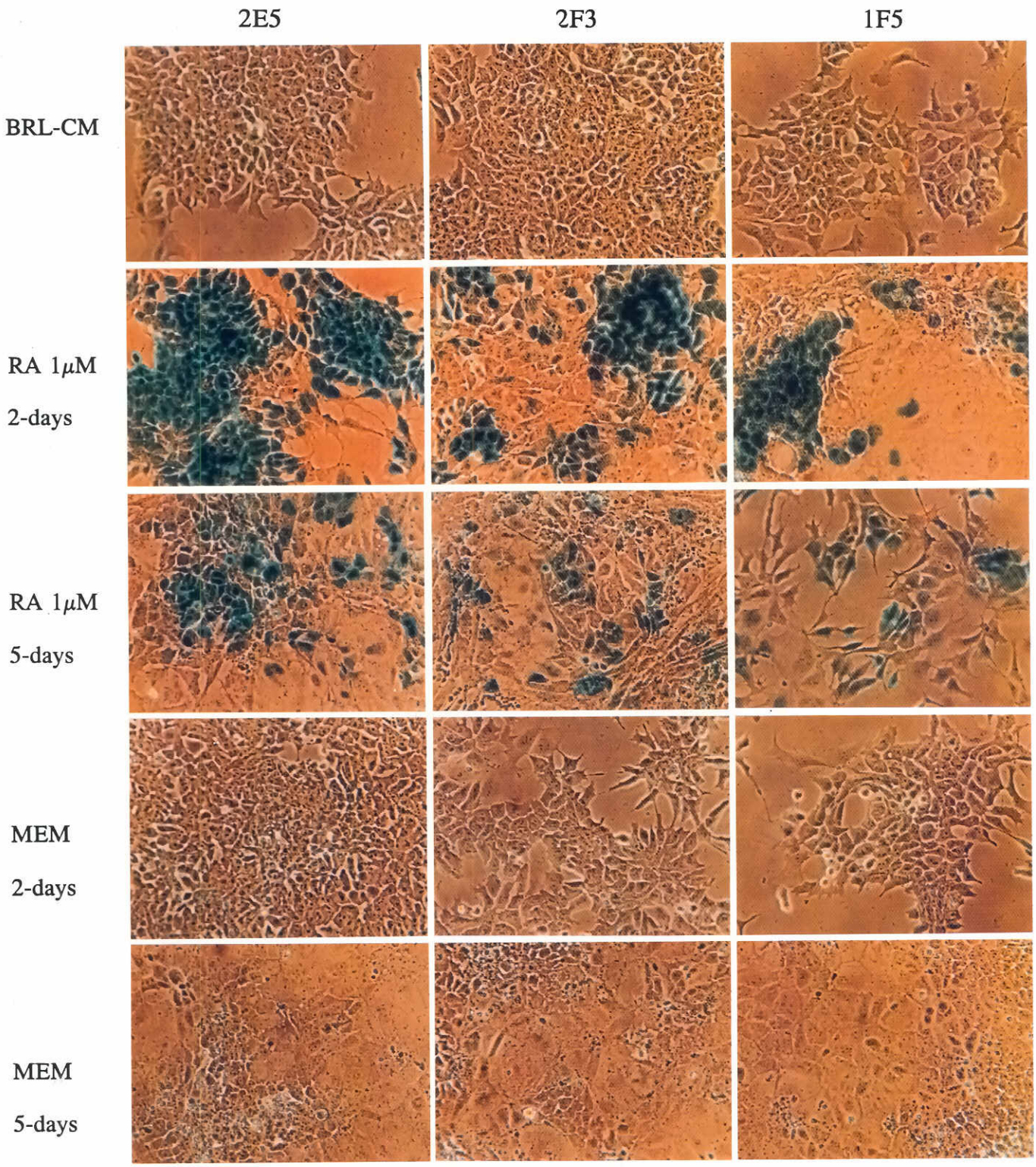

Fig. 3. mRARß$_2$-lacZ expression in stable ES clones is specifically induced by RA. *Three stable clones (2E5, 2F3, and 1F5) were maintained in BRL-CM, differentiated in the presence of RA (1 μM) or differentiated spontaneously in MEM for 2 or 5 days as indicated. Cells were fixed and stained with X-gal.*

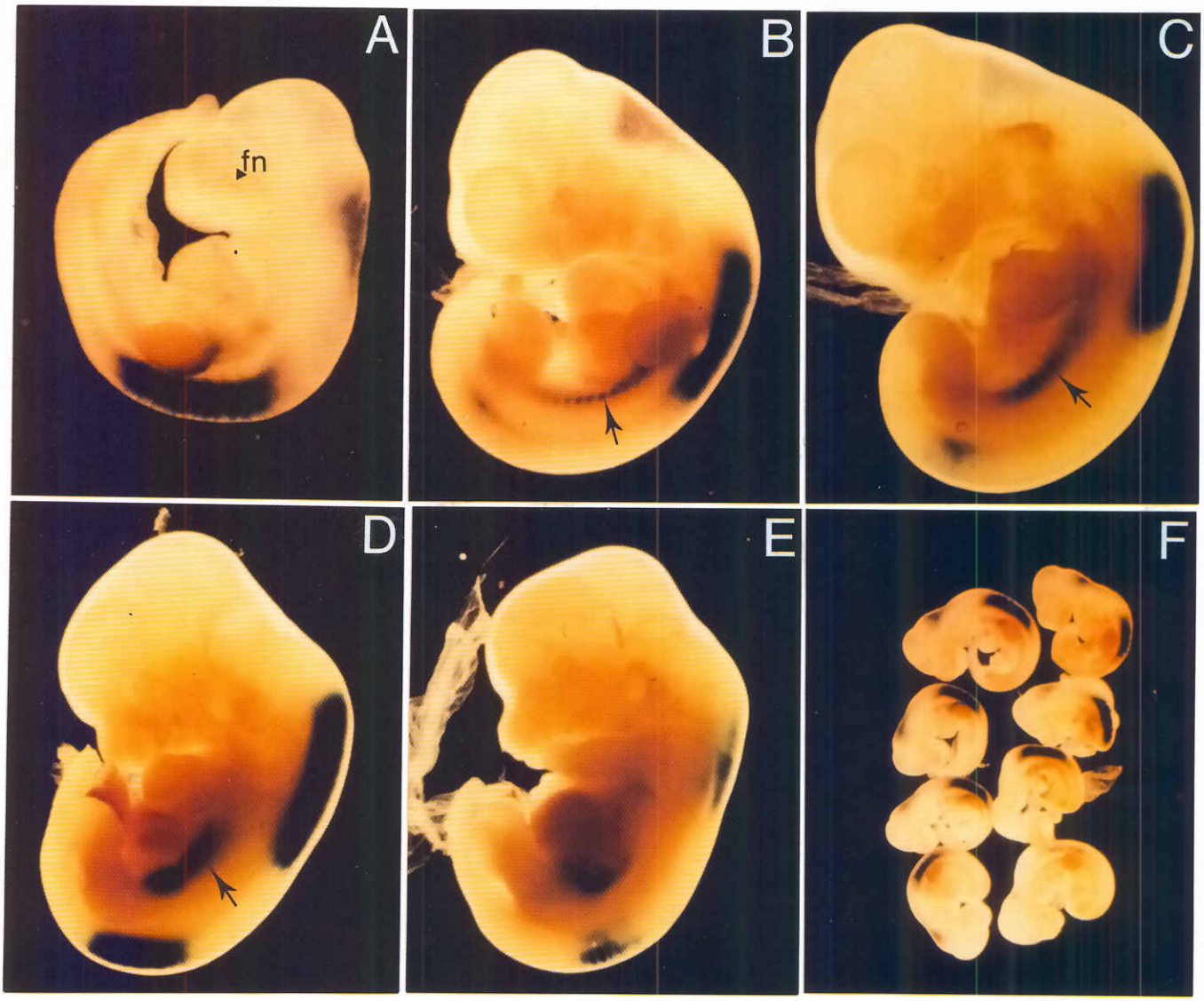

Fig. 4. mRARß$_2$-lacZ expression in chimeric mouse embryos. *ES cells resulting from stable transfection of the mRARß$_2$-lacZ construct were introduced into embryos by blastocyst injection. Developing chimeric embryos were dissected from foster mothers at mid-gestation and stained with X-gal. Embryos from day 9.5 to 13.5 are depicted from A to E, respectively. Under F a litter of day 9.5 chimeric embryos is shown. The frontonasal region is marked with fn (A), and arrow indicates staining of the body wall.*

separated from the mesonephros. In the forelimb region from the level of inner ear in the hindbrain to that of the 11th somite, the spinal cord was strongly stained (Figs. 4D and 6A). Expression in the neural tube at the level of the hindlimb bud (from the 19th to 26th somite) became as strong as that in the forelimb region, while between these two domains ß-gal activity remained very low. Staining on the body wall along the A/P axis reached its highest level at this stage during embryonic development (Figs. 4D and 6C). In developing sense organs, the lacZ gene was expressed in the retinal layer (not shown) and in the optic stalk (on, Fig. 6B), in the epithelium and adjacent mesenchymal cells of the nasal chamber (Fig. 6A) and in the inner ear (e, Fig. 6D). Strong staining was found at the back of the tongue and adjacent mandible (Fig. 6A), while jaw and oral epithelium stained weakly. In the brain, lacZ expression was absent except for weak staining in the myelencephalon and in Rathke's pocket (not shown). In sections low ß-gal activity was observed in the lung (lu, Fig. 6F) and in the trachea and the pleural sac (ps, Fig. 6F). Strong expression was found in mesonephros (m, Fig. 6I), kidney (k, Fig. 6H), ureter (u, Fig. 6H) and mesonephric duct (md, Fig. 6H) as well as at the base of the genital eminence (ge, Fig. 6H). The ß-gal activity was also present in the urethra and umbilical cord, but almost undetectable in the allantoic bladder (ab, Fig. 6G), except where connected to ureter and mesonephric duct. In the digestive system, staining was detectable in esophagus (not shown), stomach (st, Fig. 6C and G), intestine (in, Fig. 6G) and rectum. The mRARß$_2$-lacZ expression was also observed in the

Fig. 5. Distribution of the lacZ transgene in different organs of a newborn chimeric mouse containing transfected ES cells (1F5) detected by Southern hybridization of genomic DNA with lacZ probe. *Lanes 1 and 3 contain 10 μg control DNA from non-transfected E14 cells and normal mouse liver, respectively. Five μg genomic DNA from 1F5 cells was loaded on lane 2 as a positive control. Ten μg genomic DNA from head, forelimb, hindlimb, tail, skin, heart, lung, liver, kidney, reproductive system, digestive system and the remaining tissues were added in lane 4 to 15, respectively.*

heart (he, Fig. 6E) throughout 9.5 to 12.5 days, while weak staining was also observed in the pericardial layer of day 12.5 embryos. However, staining in the interdigital region of limbs was hardly detectable (not shown). Consistent with *in situ* hybridization data (Dolle *et al.*, 1990), expression in liver, gonads, somites, ribs or tail was completely lacking.

In general, day 13.5 (Fig. 4E) and 14.5 (not shown) chimeric embryos showed progressively limited lacZ staining. The main expression domains were found in the spinal cord at the position of the fore- and hind-limbs, in mesonephros and kidney, while the intensity of staining in the spinal cord was much weaker compared to embryos of 12.5 days.

RA-induced lacZ expression in chimeric embryos

The data presented above demonstrated that ES cells with stable integration of the mRARß$_2$-lacZ construct express the lacZ gene in a RA-inducible manner. On the other hand, RA is well-known to cause malformations in a variety of embryonic tissues when applied during pregnancy (Kochar, 1973; Sulik *et al.*, 1988; Tibbles and Wiley, 1988; Kessel and Gruss, 1991; Morriss-Kay *et al.*, 1991) and RA-induced abnormal development has been correlated with changed *Hox* gene expression (Kessel and Gruss, 1991; Morriss-Kay *et al.*, 1991). To reveal mRARß$_2$-lacZ expression caused by excess RA, pregnant animals carrying chimeric embryos were treated with RA at 8.5 day of gestation. After 20 h, embryos were removed and stained with X-gal to visualize RA-induced RARß$_2$-lacZ expression. Control chimeric embryos were obtained from mothers which received oil injection only.

The most striking effect of RA treatment was the shift in lacZ expression to more anterior and posterior regions. In control embryos with three independent clones, a distinct and reproducible anterior border of mRARß$_2$-lacZ expression was observed at the closure point of the neural tube, posterior to the otic vesicle (arrow head, Fig. 7). In chimeric embryos with ES cells containing a single copy of the reporter gene (clone 1F5 and 2E5), this border was shifted to the metencephalon (Fig. 7F). Sections of RA-treated embryos demonstrated that staining was present in all organs except the forebrain and developing tail bud (Fig. 7F). In chimeric embryos with 2F3 cells — containing multiple copies of the reporter gene — the whole embryo was strongly stained after RA treatment (Fig. 7B), similar to the results obtained in transgenic mice with the RARE-hsplacZ or RARE-tklacZ transgene (Rossant *et al.*, 1991; Balkan *et al.*, 1992). In addition, application of 1 mg of all-trans RA to the mother at the 8.5 day pregnancy caused a delay in development: half of the embryos recovered on the next day were practically arrested at the stage of RA application (not shown).

LacZ expression in transgenic embryos obtained from germ-line transmission of chimerae

Forty-five chimeric males were born after introduction of three ES clones (1F5, 2E5 and 2F3) derived from E14 (isolated from agouti mouse strain 129/Ola, Handyside *et al.*, 1989) into MF1 albino embryos. Agouti mice were obtained from 1F5-containing chimeric males after breeding with MF1 albino females. Southern hybridization showed the transmission of lacZ transgene in approximately half of agouti mice (not shown) and expression of the lacZ transgene was examined in transgenic mice (Fig. 8).

The staining patterns of 10.5 (Fig. 8A) and 13.5 (Fig. 8B) day transgenic embryos were very similar to those of chimeric embryos (Fig. 4B and E). As in the chimeric embryos, lacZ expression in the interdigital region was not found in these transgenic embryos (Fig. 8B). To reveal the mRARß$_2$-lacZ expression also in early embryonic development, transgenic embryos at 4.5, 6.5 and 8.5 days were examined. In day 4.5 embryos, lacZ was weakly expressed in trophectoderm cells (not shown). *In vitro* culture of blastocysts in M16 medium in the presence of 1 μM RA overnight resulted in weak induction of staining in primitive endoderm cells (not shown). When 6.5 day egg cylinders were cultured in MEM medium containing 7.5% fetal calf serum, staining was hardly visible, however, in the presence of 1 μM RA, X-gal activity was slightly induced in the extraembryonic part (not shown). To be sure that RA can be active in these embryos under the culture conditions employed, 8.5 day embryos were incubated overnight in MEM containing 7.5% fetal calf serum with or without 1 μM RA. X-gal staining revealed stronger and more extended lacZ expression (Fig. 8D) in comparison to non-RA-treated embryos (Fig. 8C), consistent with the situation in chimeric embryos (Fig. 7).

Discussion

ES cells have been widely used for studies on cell differentiation (Smith and Hooper, 1987; Mummery *et al.*, 1990), gene inactivation by homologous recombination (see review Capecchi, 1989) as well as for promoter trapping (Gossler *et al.*, 1989; Friedrich and Soriano, 1991). In this paper we extend the ES cell/chimera approach (Lovell-Badge *et al.*, 1987) to the study of a ligand-inducible and developmentally regulated gene. *In vitro*, expression of the reporter gene can be characterized in the transfected ES cells. *In vivo*, ES cells introduced in blastocysts were virtually contributing to all embryonic organs and tissues as was supported by four independent lines of evidence. First, similar expression patterns of the transgene were obtained among chimeric embryos with three different ES clones (Fig. 7A,C and E). Second, maternal administration of RA induced mRARß$_2$-lacZ expression in the whole embryo (Fig. 7D). Third, hybridization of genomic DNA from a newborn chimeric mouse showed that the mRARß$_2$-lacZ transgene was present in all organs tested, including those devoid of lacZ staining such as head, limbs, tail, liver and reproductive system (lanes 4, 5 and 6, 7, 11 and 13, respectively, in Fig. 5), although there is a difference in the intensity of the transgene (ES cell) distribution

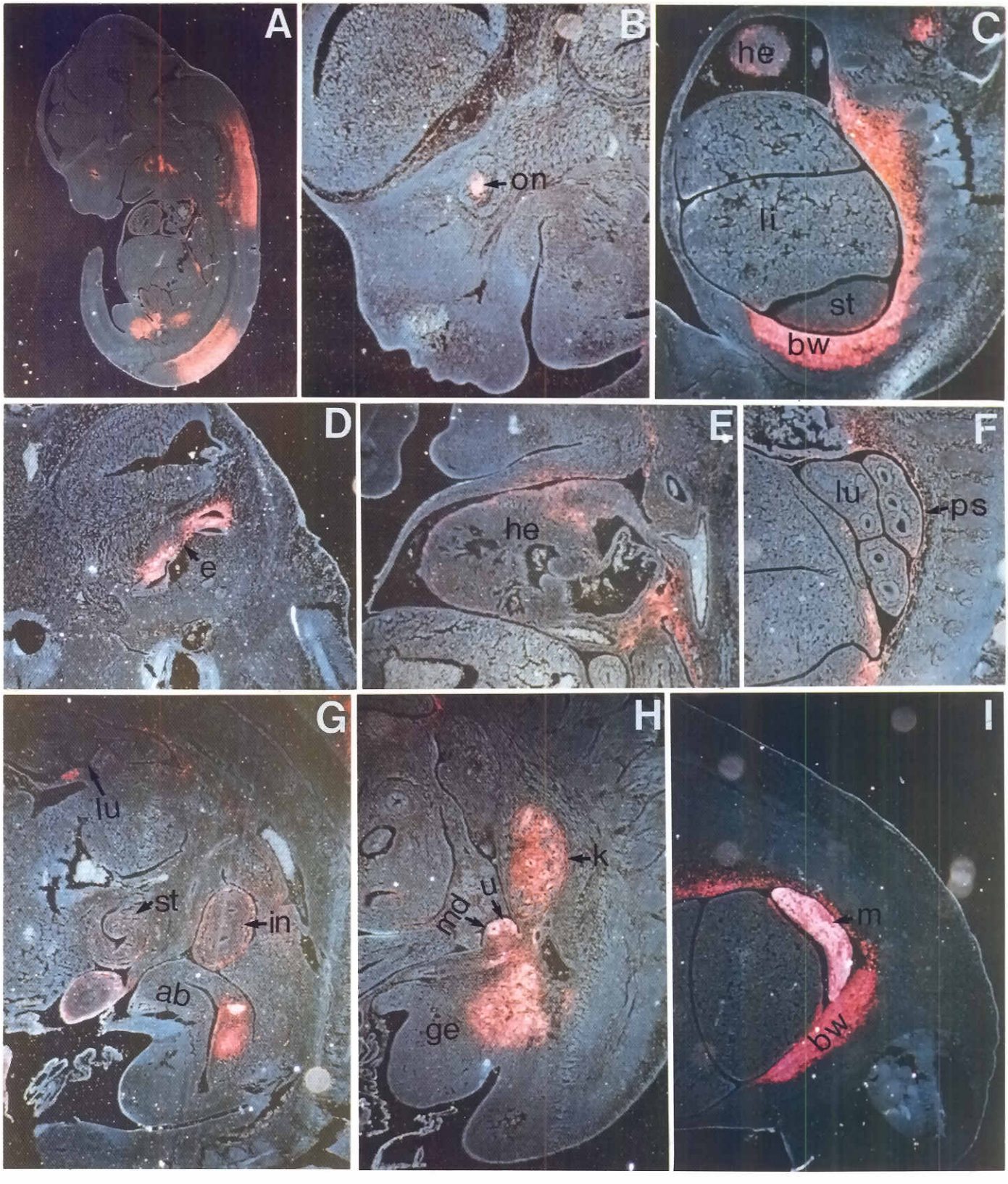

Fig. 6. mRARß$_2$-lacZ expression in sagittal sections of a day 12.5 chimeric embryo. *ab, allantoic bladder; bw, body wall; e, inner ear; ge, genital eminence; he, heart; in, intestine; ki, kidney; li, liver; lu, lung; m, mesonephros; md, mesonephric duct; on, optic stalk; ps, pleural sac; st, stomach; and u, ureter.*

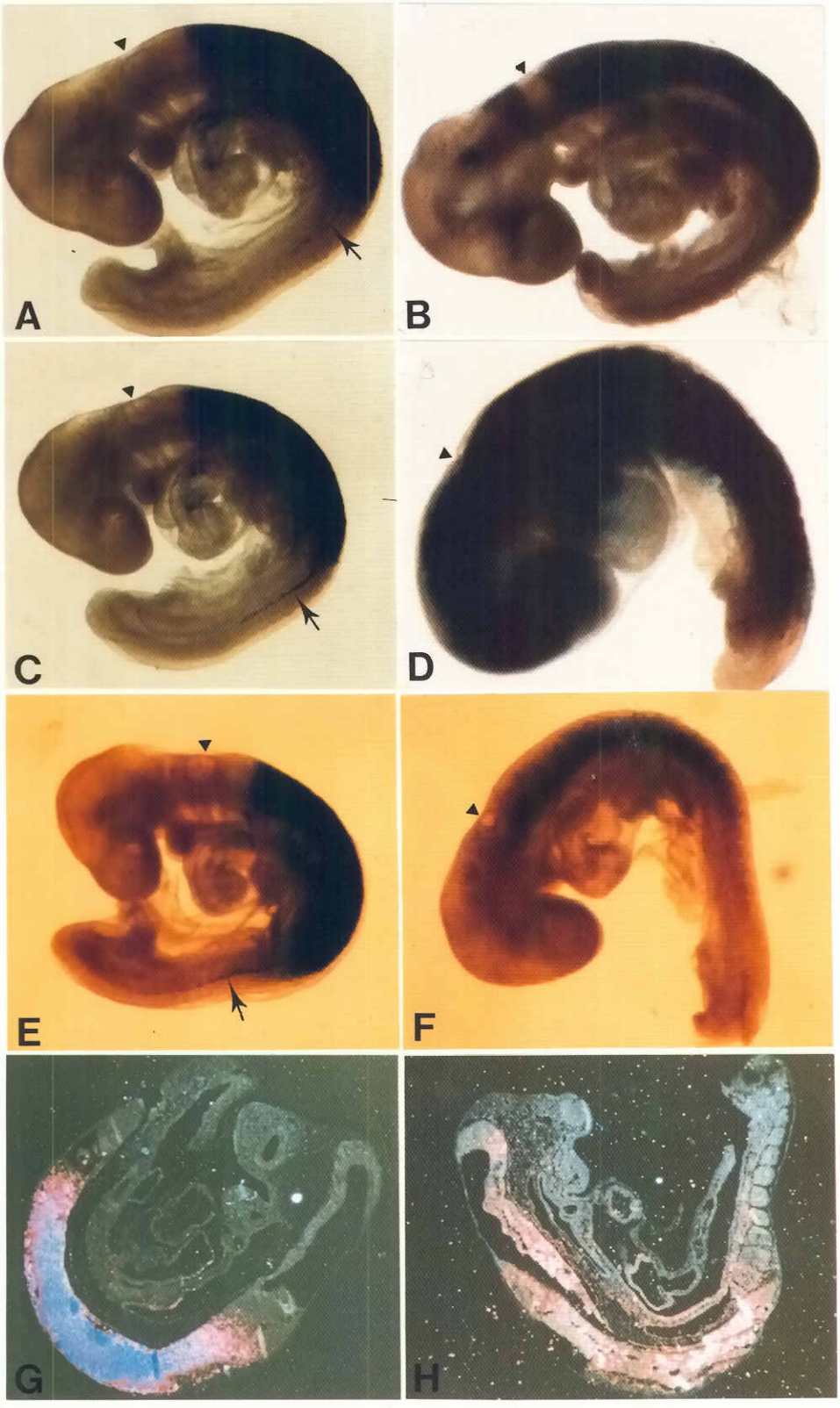

Fig. 7. RA-induced mRARß$_2$-lacZ expression in chimeric embryos. *RA-treated embryos containing 2E5, 2F3 and 1F5 cells are shown in B, D and F, respectively with their corresponding control embryos in A, C and E. Under G and I sections are shown from embryos in E and F, respectively. Arrow head indicates otic vesicle, and arrow indicates body wall staining.*

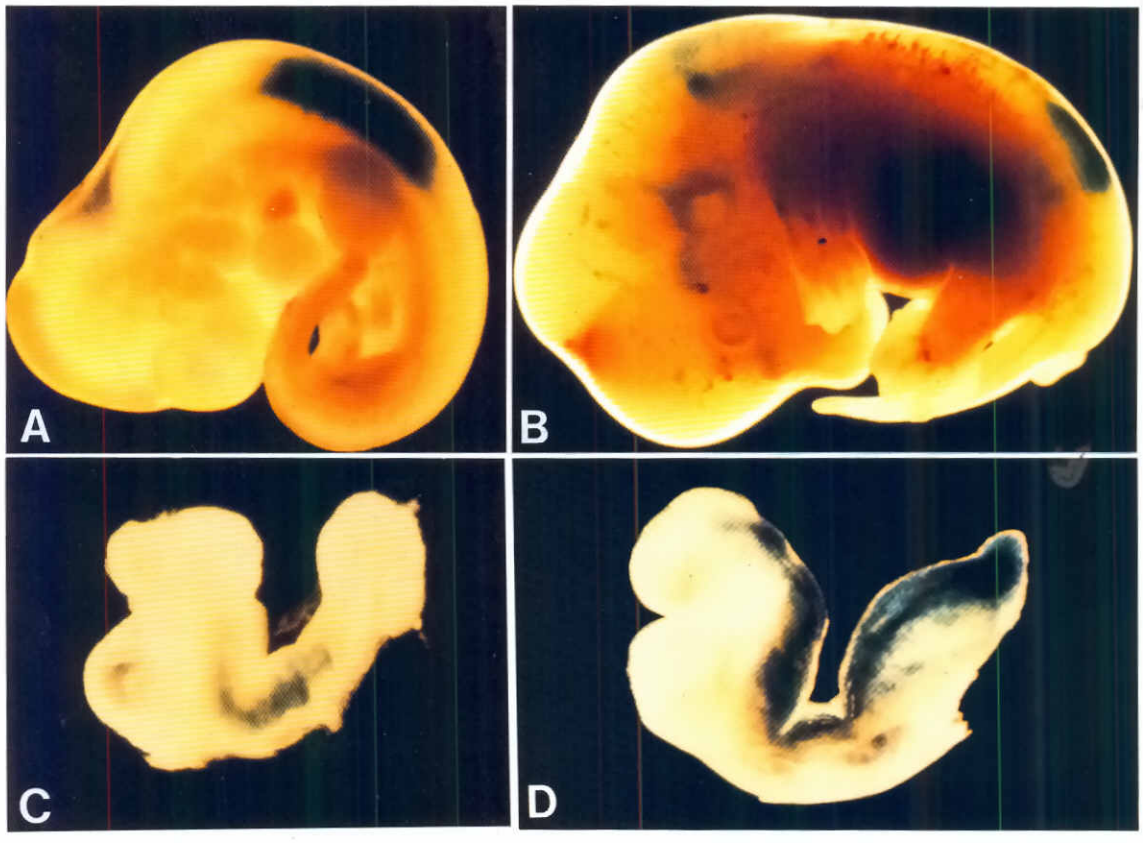

Fig. 8. LacZ expression in ES cell (1F5)-derived transgenic embryos. (A) *A day 10.5 embryo.* **(B)** *A day 13.5 embryo.* **(C)** *A day 8.5 embryo cultured for 20 h in MEM containing 7.5% (v/v) fetal calf serum.* **(D)** *A day 8.5 embryo from the same litter cultured under the same condition as in C, but with the addition of 1 µM RA.*

among different organs. Fourth, transgenic mice derived from transfected ES cells showed lacZ staining pattern comparable to chimeric embryos (see Figs. 4 and 8). Therefore, undetectable expression of the reporter gene in these organs of chimeric embryos is unlikely to be due to the absence of ES cells, but more likely reflects gene regulation *in vivo*. In this way we studied the expression pattern of a specific promoter, the mRARß$_2$. The results showed that in general, the 1.8 kb mRARß$_2$ promoter sequence was sufficient to direct lacZ expression in a pattern similar to that of endogenous RARß gene. In stably transfected ES cells, significant induction of lacZ expression by RA was already detected at 10^{-9} M, in agreement with the endogenous RARß induction in EC (Kruyt *et al.*, 1990) or ES cells (unpublished results).

In the majority of tissues from chimeric embryos, the pattern of mRARß$_2$-lacZ expression was consistent with results obtained by *in situ* hybridization (Dolle *et al.*, 1990; Ruberte *et al.*, 1991) or by transgene studies (Mendelsohn *et al.*, 1991; Rossant *et al.*, 1991) (for a comparison see Table 2). In the head region, mRARß$_2$-lacZ expression was observed in the retinal layer, in the epithelium of the nasal chamber and in the inner ear, tongue, mandible and jaw. In internal organs, strong expression was detected in mesonephros, kidney and at the base of genital eminence, while relatively weak staining was seen in lung, gut, stomach, intestine, rectum and pancreas. On the other hand, no expression was found in brain, liver, or gonads. In contrast to RARE-hsplacZ or RARE-tklacZ transgene studies (Rossant *et al.*, 1991; Balkan *et al.*, 1992), no ß-gal activity was detected in somites. In addition, we observed strong expression in the optic stalk, ureter, mesonephric duct and some specific regions of the body wall similar to the results obtained with transgenic mice by Reynolds *et al.* (1991) and weak expression in myelencephalon and Rathke's pocket which may represent expression in the embryonic brain detected by Northern hybridization (Zelent *et al.*, 1991).

The mRARß$_2$-lacZ transgene expression on the body wall was developmentally and spatially regulated. Expression first appeared in day 9.5 embryos as a sharp line caudally to the forelimb buds and ventrally to somites. The intensity of expression was stronger towards the forelimb bud in day 9.5 and 10.5 embryos. Following the formation and further growth of the hindlimb buds, staining in the direction of the hindlimb buds was increased concordantly. At this position, lacZ expression reached a maximum in day 12.5 embryos but disappeared abruptly within the next day. This expres-

TABLE 1

NUMBER OF CHIMERIC EMBRYOS OBTAINED AFTER BLASTOCYST INJECTION WITH ES CLONES CONTAINING MRARß$_2$-LACZ REPORTER GENE

		No. Embryos (percentage)			
Clone	Age (days)	Injected	Transferred	Recovered	X-gal Stained
2E5	9.5	50	42	27	26
2E5	10.5	42	35	26	19
2E5	11.5	27	22	9	7
2E5	12.5	10	10	3	3
2E5	13.5	15	15	5	3
2F3	9.5	26	26	20	18
2F3	10.5	27	19	14	11
2F3	11.5	9	9	7	7
2F3	12.5	9	9	5	5
1F5	9.5	42	31	22	4
1F5	10.5	16	11	6	4
1F5	11.5	7	7	4	3
1F5	12.5	20	20	13	13
1F5	14.5	16	16	7	4
Total		316	272(86.1%)	168(61.8%)	127(75.6%)

sion seems not to be ectopic, since the pattern was observed with all ES cell lines tested (Fig. 7A,C and E). It remains possible that sequences located distally from the 1.8 kb promoter are responsible for the repression of RARß$_2$ expression on the body wall.

As can be seen from Table 2, there are locations in which the mRARß$_2$-lacZ expression is different from the previous reports (Dolle *et al.*, 1990; Mendelsohn *et al.*, 1991; Rossant *et al.*, 1991; Ruberte *et al.*, 1991). In the spinal cord, RARß transcripts (Dolle *et al.*, 1990; Ruberte *et al.*, 1991) as well as expression of RARE-hsplacZ transgene were detected continuously along the A/P axis. However, in chimeric embryos, the mRARß$_2$-lacZ was predominantly expressed in the spinal cord with the same anterior border, but with a different posterior border at the level of the 12th somite in day 9.5 embryos. Later, in day 12.5 embryos a second expression domain appeared at the region where hindlimbs were developing, while staining between fore- and hind-limbs or posterior to the 25th somite was very weak or undetectable. If the RARß$_2$ promoter contains an enhancer activating its expression in the middle part of the neural tube, this enhancer is not present in the adjacent 2 kb upstream sequence since the gap in lacZ expression was also reported in day 11.5 and 12.5 transgenic embryos with 250 base pair, 625 base pair, 3.1 kb (Reynolds *et al.*, 1991), 3.5 kb (our unpublished observation), or 3.8 kb (Mendelsohn *et al.*, 1991) of the promoter. It is more likely that the discontinuous mRARß$_2$-lacZ expression in the spinal cord indicates that different RARß isoforms specify distinct domains along the neural tube during development since all transgenic mice with different lengths of mRARß$_2$ promoter (from 7.5 kb to 187 base pair) revealed the same expression pattern in the spinal cord (data not shown).

We observed lacZ expression in the heart of chimeric embryos, which was also reported in transgenic embryos with the RARE-hsplacZ (Rossant *et al.*, 1991) and in adult heart by Northern hybridization (Zelent *et al.*, 1991). Expression in the pericardial membrane is consistent with the findings using a 3.8 kb mRARß$_2$ promoter sequence (Mendelsohn *et al.*, 1991), while staining in the pleural sac may result from differential stability of RARß$_2$ and lacZ transcripts and/or diffusion of ß-gal activity.

Although mRARß$_2$-lacZ expression in the spinal cord, spinal ganglia, and body wall was closely related to the position of the limb buds, expression in the interdigital regions as observed in other studies (Dolle *et al.*, 1990, Mendelsohn *et al.*, 1991, Rossant *et al.*, 1991) was undetectable in this study. It is unlikely due to the absence of ES cells in the interdigital regions because the mRARß$_2$-

TABLE 2

COMPARISON OF mRARß$_2$-LACZ EXPRESSION IN CHIMERIC MOUSE EMBRYOS WITH DATA OBTAINED BY *IN SITU* HYBRIDIZATION[1] AND TRANSGENE EXPRESSION STUDIES[2,3]

Organs	in chimeric embryos (1.8 kb)	*In situ* [1]	Transgene expression	
			3.8 kb [2]	RARE [3]
Spinal cord	two domains	+	+	+
Eye				
retina	+	+	+	+
lens	-	-	-	+
optic stalk	+	n.d	n.d	n.d
Nose				
epithelium	+	+	+	+
mesenchyme	+	+	-	n.d
Inner ear				
epithelium	+	+	+	+
mesenchyme	+	+	-	n.d
Tongue	+	+	n.d	n.d
Mandible	+	+	n.d	+
Jaw	+	n.d	+	+
Lung	+	+	-	n.d
Pleural sac	+	-	n.d	n.d
Heart	+	-	-	n.d
Pericardial membrane	+	-	+	n.d
Gut	+	+	+	n.d
Stomach	+	+	+	+
Kidney	+	+	+	+
Mesonephros	+	+	+	n.d
Ureter	+	n.d	n.d	n.d
Mesonephric duct	+	n.d	n.d	n.d
Urethra	+	+	n.d	+
Genital eminence	base	+	n.d	n.d
Limb				
base mesenchyme	+	+	+	+
interdigital	-	+	+	+
Brain	-	-	-	telencephalon
Liver	-	-	-	-
Gonad	-	-	-	-
Somite	-	-	-	+
Body wall	+	n.d	n.d	n.d
RA Induction	whole embryo	n.d	limited	whole embryo

[1] Dolle *et al.*, 1990.
[2] Mendelsohn *et al.*, 1991.
[3] Rossant *et al.*, 1991.
+/- indicates the presence/absence of lacZ staining or RARß transcripts.
n.d., not described.

lacZ transgene was detected in both fore- and hindlimb (lane 5 and 6 in Fig. 5, respectively) in a new-born chimera. Furthermore, we have generated transgenic mice from germ-line transmission of the 1F5 cell line and staining in the interdigital regions does not appear in the transgenic offspring either (data not shown). Interestingly, staining in this region was variable among a litter of embryos dissected from a female crossed with a transgenic male carrying a lacZ transgene under the control of 3.5 kb mRARß$_2$ promoter sequence (unpublished observation).

The mRARß$_2$-lacZ expression pattern observed may reflect endogenous levels of retinoids since in cultured cells lacZ expression is very sensitive to RA treatment, and *in vivo*, administration of excess RA to mothers induces lacZ expression in whole embryo. Moreover, addition of RA to *in vitro* cultured transgenic embryos resulted in a comparable pattern of lacZ induction (see Figs. 7 and 8). However, it seems retinoids are not the only factor determining mRARß$_2$-lacZ expression. Recent reports have demonstrated that expression of RXRs as well as other co-regulators are possibly involved in RARß$_2$ regulation and that formation of RAR/RXR heterodimers is critical for binding to RAREs (Glass *et al.*, 1989, 1990; Yu *et al.*, 1991; Kliewer *et al.*, 1992; Leid *et al.*, 1992; Zhang *et al.*, 1992). Additional regulatory sites on the upstream part of the promoter for tissue-specific factors may also exist. Identification of the complexes responsible for differential RARß$_2$ regulation and systematic characterization of RARß$_2$ promoter activity during embryonic development will be useful for a full understanding of the function of RARs. Availability of RARß-lacZ transfected ES cells and ES cell-derived transgenic mice as described above will assist in further understanding of the RA network: endogenous retinoids, retinoid receptors and unknown accessory factors, the relationship between RARß$_2$ expression and cell differentiation, and the effect of dominant negative mutants during development.

Materials and Methods

The mRARß$_2$-lacZ expression cassette and Southern hybridization

The *Xho*I-*Hind*III fragment of the neomycin-resistance gene from the pMC1NeoPA vector (Strategene) was inserted into the same restriction sites of the pIC-20R vector (Marsh *et al.*, 1984), pIC-Neo. A 5.6 kb of mRARß$_2$-lacZ (*Xho*I-*Xba*I) fragment from pMRß-lacZ-PA (Shen *et al.*, 1991) was cloned into the *EcoR*V site of pIC-Neo. The construct was linearized by *Kpn*I before electroporation.

Genomic DNA from ES cell pellets and mouse tissues were isolated according to the protocol described by Hogan *et al.* (1986). Southern hybridization of the genomic DNA was done following the protocol given by Ausubel *et al.* (1987).

Cells, transfection and detection of expression

Transient assays for lacZ expression upon induction by all-*trans* RA in P19 EC cells were carried out as described earlier (Shen *et al.*, 1991).

ES cells (E14, Handyside *et al.*, 1989) were maintained in BRL-CM containing 20% (v/v) fetal calf serum. Approximately 10^7 cells were electroporated with 40 µg linearized DNA at 240 V, 500 µF in 0.8 ml Ca^{2+}- and Mg^{2+}-free PBS. Subsequently, cells were plated on gelatin-coated 6-well dishes (Costar, USA) and G418 was applied at a concentration of 200 µg/ml two days later. Resistant clones were isolated three weeks after selection when no control cells electroporated in the absence of DNA had survived.

The X-gal staining procedure for ES cells was identical to that described for EC cells (Shen *et al.*, 1991).

Manipulation of embryos

Embryo manipulations were carried out as described by Hogan *et al.* (1986). Blastocysts were obtained from superovulated MF1 females at 3.5 days of pregnancy and 20-30 transfected ES cells were introduced per embryo by blastocyst injection (Shen, 1990).

For lacZ staining, embryos were fixed in PBS containing 1% (v/v) formaldehyde, 0.2% (v/v) glutaraldehyde and 0.02% (v/v) NP-40 at 4°C for 30 min and washed twice in PBS at room temperature for 20 min. Staining was carried out overnight at 30°C in PBS containing 1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$ and 5 mM $K_3Fe(CN)_6$.

Sectioning of the embryos was performed according to the standard paraffin section procedure after fixation of stained embryos in 4% (w/v) paraformaldehyde in PBS at 4°C overnight. Photographs were taken from the dark field view.

RA administration

All-trans RA (30 µl) at a concentration of 0.1 M in DMSO was diluted to 0.2 ml with rape oil and applied into the pharynx of a mother at 8.5 days of pregnancy. Control mothers were received 0.2 ml of oil only. After 20 h, RA-treated embryos and control embryos were recovered and stained with X-gal as described above.

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