Morphological and Molecular Characterization of Retinoic Acid-Induced Limb Duplications in Mice

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This study reports a morphological, skeletal, and molecular characterization of the supernumerary limbs induced by systemic administration of all-*trans* retinoic acid to egg-cylinder stage mouse embryos. As initially described by Rutledge *et al.* (*Proc. Natl. Acad. Sci. USA* 91, 5436, 1994), we have found that oral administration of all-*trans* retinoic acid (70 mg/kg body weight) at 5.5 days postcoitum induced the formation of supernumerary limbs. Most often, these arose as a pair of extra buds located caudally and ventrally to the normal (orthotopic) hindlimb buds without duplication of the lower body axis. The resulting one or two supernumerary hindlimbs were connected to an imperfectly mirror-image-duplicated pelvic girdle. Variable truncations of the stylopodium and zeugopodium skeleton, as well as abnormal splitting of the distal skeleton, were frequently observed. The apical ectodermal ridge of the extra limb buds expressed expected growth factor genes. However, an ectopic anterior expression of *Sonic hedgehog* and *Hoxd-13* was seen in the supernumerary buds, suggesting that these buds would incorporate potential polarizing cells of the hindlimb or genital field and generate an ectopic polarizing zone. This is consistent with the reverse orientation of most supernumerary limbs at later stages. Some of the buds did not express limb-specific markers and were thus expected to degenerate or form nonlimb structures, as observed in an adult specimen. Less frequently, extra limb buds with normal polarity were associated to a duplicated lower body axis. Retinoic acid also generated a novel type of duplication in which ''twin'' hindlimbs with two parallel apical ectodermal ridges and zones of polarizing activity arose on one side of the embryo.   © 1996 Academic Press, Inc.

INTRODUCTION

Retinoic acid (RA), the active derivative of vitamin A, is believed to play an important role in vertebrate development. Fetuses of dams deprived of vitamin A present numerous abnormalities collectively known as the fetal vitamin A deficiency syndrome (Wilson *et al.,* 1953). Knockouts of retinoic acid nuclear receptors (RARs and RXRs) have demonstrated that RA is indeed required at many stages during early embryogenesis and organogenesis (Lohnes *et al.,* 1994; Mendelsohn *et al.,* 1994; Kastner *et al.,* 1994, 1995, and references therein). Administration of excess RA during embryogenesis has teratogenic effects which led to the proposal that RA plays key roles in pattern formation along the major body axis and vertebrate limb formation (for reviews, see Summerbell and Maden, 1990; Bryant and Gardiner, 1992; Hofmann and Eichele, 1993; Conlon, 1995). Local application of RA under the anterior margin of the developing chick wing bud results in mirror image duplication of the digits (Tickle *et al.,* 1982;

Summerbell, 1983). In these experiments, RA actually mimics the effect of the zone of polarizing activity (ZPA), a discrete region of the posterior mesenchyme involved in anteriorposte-
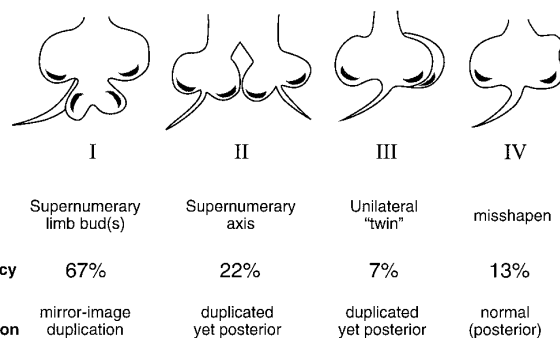

FIG. 1. Schematic representation of the morphology and polarity of RA-induced supernumerary limb buds at 11.5 dpc. The frequencies correspond to the percentage of a given type of abnormalities among a total of 44 embryos which showed detectable limb alterations of 224 embryos collected at 11.5 dpc.

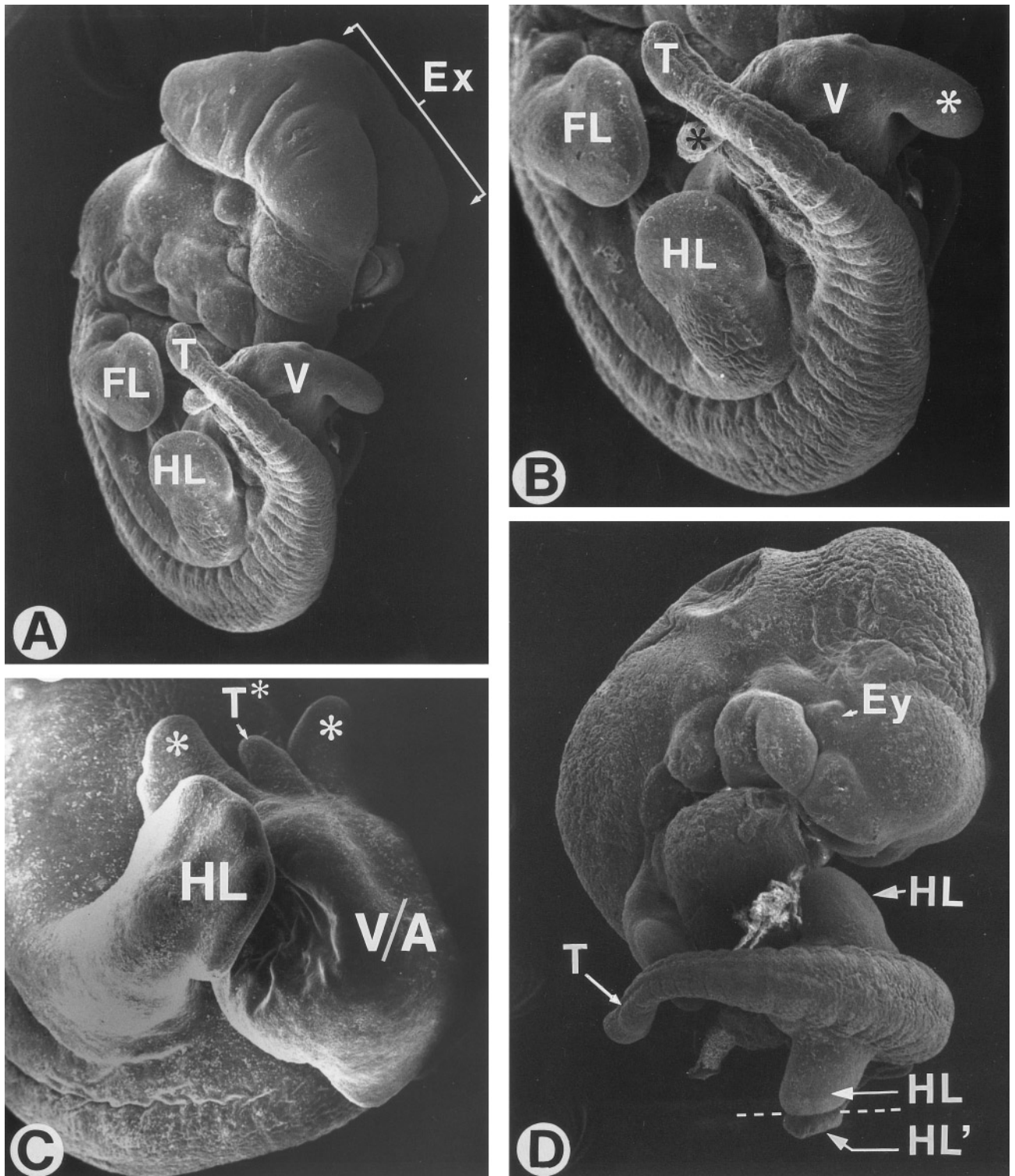[1] To whom correspondence should be addressed. Fax: (33) 88653203. e-mail IGBMC@IGBMC.U-STRASBG.FR.

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FIG. 2.   Scanning electron micrographs of RA-treated embryos with supernumerary limb buds. Embryos were collected at 11.5 dpc. (A and B) A specimen with paired supernumerary hindlimb buds (*) and severe exencephaly. (C) The caudal region of an embryo with two symmetrical supernumerary hindlimb buds (*) pointing caudally. The abnormal ventral protrusion (V/A) might in this case correspond to an aborted supernumerary axis with a truncated tail (T*). The ''main'' tail is hidden behind this protrusion. (D) shows an embryo with duplicated (''twin'') hindlimb buds (HL, HL′) on the righthand side and a severe abnormal twisting of the body axis arising at the level of the trunk. Other specimens showed abnormal twisting caudally to the hindlimbs. Abbreviations: Ex, exencephaly; Ey, eye; FL, forelimb; HL, hindlimb; T, tail; V, abnormal ventral mass; V/A, abnormal ventral mass/supernumerary axis.
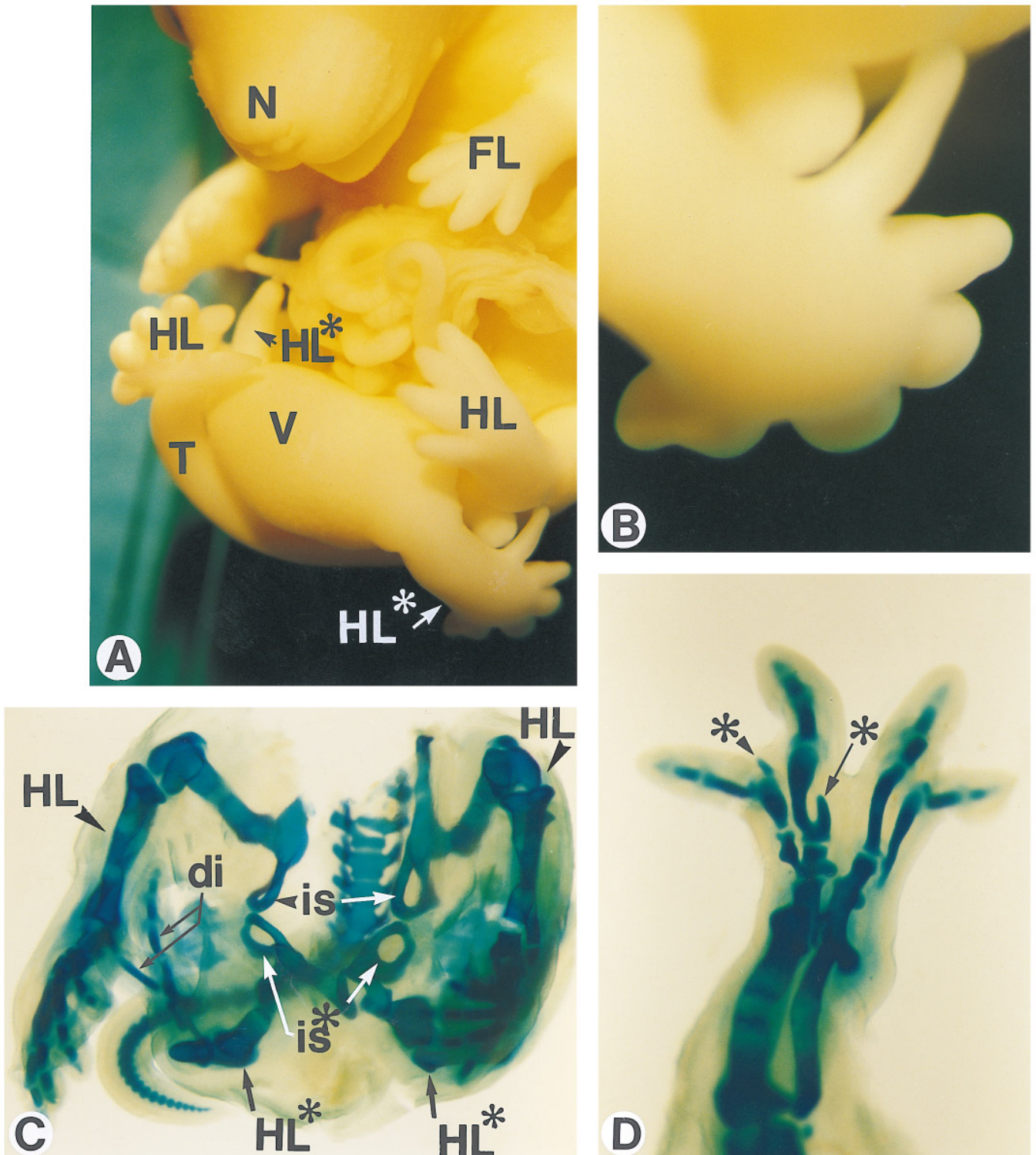
FIG. 3. (A) External view of a 15.5 dpc fetus with an abnormal ventral protrusion and a full supernumerary hindlimb (HL*) in opposite orientation to the orthotopic hindlimb. (B) Close-up of the supernumerary hindfoot showing polydactyly (six digits). (C) Alcian blue cartilage staining of the same specimen, showing the presence of a mirror-image-duplicated pelvic girdle (is*). The lefthand side supernumerary hindlimb (not shown, hidden by the normal hindlimb) displays a sixth digit rudiment, whereas the opposite supernumerary limb is severely truncated and has only two digits (di). (D) Alcian blue staining of another supernumerary hindlimb, showing abnormal splitting between tarsal cartilages and a four-digit pattern with two aborted digit rudiments (*) in the anterior (tibial) side. Abbreviations: di, digits; FL, forelimb; HL, hindlimb; HL*, supernumerary hindlimb; is, ischiopubic cartilage; N, nasal eminence; T, tail; V, abnormal ventral mass.

rior limb patterning (Saunders and Gasseling, 1958). It was recently reported that RA may induce an ectopic anterior ZPA through local induction of *Sonic hedgehog* (*Shh*), a secreted factor believed to mediate the signaling properties of this region (Riddle *et al.,* 1993). *Shh* acts in concert with growth-promoting factors which are specifically produced by the apical ectodermal ridge (AER) of the limb buds. These include fibroblast growth factor-8 (FGF-8), which is expressed throughout the AER, and FGF-4, which is expressed mainly in the posterior part of the AER (Niswander and Martin, 1992; Crossley and Martin, 1995; Heikinheimo *et al.,* 1994). Such growth factors control proximal–distal outgrowth of the buds by maintaining the distal "progress zone" cells in a proliferative state (Summerbell *et al.,* 1973). Patterning information is believed to be provided to the progress zone cells through the sequential activation of various regulatory gene products such as certain Hoxd homeoproteins. The coordinate activity of all these factors is required for normal growth and patterning of the limbs (reviewed in Tickle, 1995).

Systemic administration of RA to rodent embryos can lead to a wide range of developmental defects according to the stage of treatment. RA-induced limb malformations were classically described as a consequence of RA treatments between 10 and 12.5 days postcoitum (dpc) and consisted of various truncations of skeletal elements and a reduction in the number of digits (Kochhar, 1973, 1980; Kistler, 1981). More recent studies have shown that amphibian tadpoles exposed to RA during the process of tail regeneration displayed variable numbers of supernumerary limbs (Mohanty-Hejmal *et al.,* 1992; Maden, 1993). Rutledge *et al.* (1994) then made the startling observation that very early RA treatment of mouse conceptuses at the egg-cylinder stage (4.5 to 5.5 dpc) results in the appearance of supernumerary limbs and lower body duplications. These types of duplication events are unique among teratogenic drug effects in mammals (Sanders and Stephens, 1991). Complete supernumerary wings or legs have been produced in chick embryos following the local administration of FGF-1, FGF-2, or FGF-4 in the flank mesenchyme prior to limb outgrowth using a bead vehicle (Cohn *et al.,* 1995) or through retroviral infection (Ohushi *et al.,* 1995). In these experiments, the competence for supernumerary limb development appeared to be restricted to the flank and not present in the lateral plate mesoderm rostral to the wing field or caudal to the leg field (Cohn *et al.,* 1995).

In this study, we describe the morphological, skeletal, and molecular (gene expression) patterns of supernumerary limbs induced by early RA treatment of mouse embryos. Several types of supernumerary limb buds were observed. In the most common form, the extra limb buds were induced in a region caudal and ventral to the normal hindlimbs. The resulting supernumerary hindlimbs developed a reverse anterior–posterior polarity through the establishment of an ectopic anterior *Shh* expression domain. A novel type of "twin" hindlimb duplication is also reported. Specific skeletal pattern alterations in supernumerary limbs are described both at fetal and adult stages.

MATERIALS AND METHODS

Retinoic Acid Treatments

Our initial study was performed on CD1 females crossed with CBA/C57/BL 6J hybrid males; a similar range and frequency of effects were then found in CD1 × CD1 matings. Treatment of C57/BL 6J females crossed with F1 hybrid males and of C57/BL 6J inbred crosses similarly resulted in limb duplications, although far fewer embryos resisted treatment (data not shown). Pregnant females were used from natural overnight matings (morning of vaginal plug was considered as 0.5 dpc). All-*trans* retinoic acid (Sigma) was prepared just prior to treatment by resuspending the contents of a freshly opened 50-mg vial in 1 ml ethanol and vortexing for 1 min. Nine milliliters of sunflower oil was then added and the mixture vortexed for 5 min while protected from light. This suspension was administered by oral gavage to pregnant females at a concentration of 70 mg/kg body weight between 10:30 and 12:00 hr on Day 5 after mating.

Whole-Mount in Situ Hybridization (ISH) and Skeletal Analysis of Mutant Mice

Whole-mount ISH with digoxigenin-labeled riboprobes was carried out as described in Décimo *et al.* (1995). Embryos were fixed for 2 to 4 hr (depending on developmental stage) at 4°C in 4% paraformaldehyde, washed twice in PBS containing 0.1% Tween 20 (PBT), dehydrated through a graded series of methanol/PBT (25, 50, 75, and 100%), and stored at −20°C. Depending on the developmental stage, the embryos were incubated for 5 to 15 min in proteinase K (10 mg/ml) prior to hybridization. The probes used in this study have been described in the following references: *Fgf-8* (Crossley and Martin, 1993); *Fgf-4* (Niswander and Martin, 1992); *Shh* (Echelard *et al.,* 1993), *Hox d-13* (Dollé *et al.,* 1991b).

Whole-mount fetal Alcian blue staining and alizarin red/Alcian blue staining of 18.5 dpc animals was carried out as described previously (Dollé *et al.,* 1993).

RESULTS

To establish the appropriate concentration and timing of treatment, a preliminary dose–response curve for RA administration was performed. RA given by oral gavage at doses of 70 mg/kg body weight between 10:30 and 12:00 hr on Day 5.5 dpc produced the highest incidence of limb duplications (see Materials and Methods). Under these conditions, the percentages of embryos that harbored distinct limb duplication(s) were 19.6% when examined on 11.5 dpc, 9% on 15.5 dpc, and 8.9% on 18.5 dpc. RA treatments of 70 mg/kg body weight given at 4.5 and 5.0 dpc also resulted in complete limb duplications, although at a lower percentage (data not shown). We have performed histological analysis of several embryos at the time of RA administration. All of the 5.5 dpc embryos (*n* = 18) consisted of a double-layered egg-cylinder with a small proamniotic cavity but no posterior amniotic fold and no sign of mesoderm formation (see Fig. 12.8 in Snell and Stevens, 1966, or plate 2 in Kaufman, 1992). When RA administration was performed after 13:00 hr on 5.5 dpc, no duplications resulted.

Morphological Study of RA-Induced Supernumerary Limbs

The early appearance of supernumerary limb buds was assessed at 11.5 dpc in a series of scanning electron micrographs as well as in specimens processed for whole-mount *in situ* hybridization. The affected embryos could be classified in four distinct groups according to the morphology and location of their supernumerary limb buds (depicted in Fig. 1). These were: (I) a set of paired symmetrical limb buds arising on each side of an abnormal ventral mass located caudally to the orthotopic hindlimb buds (Figs. 2A–2C). The extra limb buds, sometimes of unequal size, were always more developmentally delayed than the "normal" hindlimbs and pointed in a rostral (Figs. 2A and 2B) or, less often, caudal (Fig. 2C) direction. (II) A duplication, or sometimes triplication (one case), of the lower body axis and tail, each of the axes harboring a pair of hindlimb buds (not shown; described in Rutledge *et al.,* 1994). Note that we never observed this malformation at later stages. (III) Unilaterally, two "twin" hindlimb buds joined along their rostral–caudal plane (Figs. 2D and 7E: HL and HL′). (IV) Misshapen hindlimb buds (generally unilaterally), which harbored more or less pronounced abnormal anterior protrusions (Fig. 7F and 7H). Only two specimens harbored unilaterally duplicated (Y-shaped) forelimb buds (not shown).

Other defects were observed in a subset of 11.5 dpc affected embryos. These included marked exencephaly (Fig. 2A) and spina bifida (Fig. 7H), facial cleft (not shown), abdominoschistis (lack of closure of the abdominal body wall—not shown), and an abnormal spiral twisting of the body axis (Fig. 2D and data not shown). These defects were never seen in 15.5 and 18.5 dpc specimens, clearly suggesting that severely affected embryos died and were resorbed after mid-gestation. This is consistent with the lower frequencies of limb duplications observed at these later stages.

External examination of 15.5 and 18.5 dpc fetuses showed several cases of entirely developed supernumerary limbs with digits (Fig. 3A and data not shown). An abnormal ventral mass was still visible at these stages, from which supernumerary limbs projected either in an opposite orientation (Fig. 3A) or in the same orientation as the orthotopic hindlimbs (not shown; described in Rutledge *et al.,* 1994). Whereas some of the extra limbs were polydactylous (Fig. 3B shows the presence of a sixth digit rudiment), others appeared truncated and/or oligodactylous (see below).

Skeletal Patterns of RA-Induced Supernumerary Limbs

The skeletal patterns of RA-treated fetuses were analyzed at 15.5 and 18.5 dpc by whole-mount Alcian blue and aliza-rin red/Alcian blue stainings, respectively. It appeared that none of these fetuses displayed a duplication of the vertebral column (as would be expected for the group II embryos described above). Most of the supernumerary limbs were connected to duplicated pelvic elements. Figure 3C shows a rare example where the supernumerary pelvis consisted of two perfectly mirror image-duplicated ischium cartilages. Typically though, the duplicated pelvis consisted of two rudimentary ischiums and very rudimentary putative iliums. The right side ischium was often connected to its orthotopic counterpart (Fig. 4A). Other animals had a symmetrical pelvic rudiment completely floating (Fig. 4C). None of these supernumerary pelvic elements were connected to the vertebral column. Interestingly, the specimen of Fig. 5B displayed a fused supernumerary ischium with a single acetabulum (articular cavity for the femur) and two mirror-image ilium rudiments.

All the supernumerary limbs displayed distinct stylopodium (femur), zeugopodium (tibia/fibula), and autopodium (tarsus and digits) elements. Most of the supernumerary limbs showed more or less severe truncations of the stylopodium and/or zeugopodium elements (see the left supernumerary limb in Fig. 4A, the very truncated femur in Fig. 4B, and the truncations of various elements in Fig. 4C). By contrast, the autopods were not truncated in size but displayed patterning alterations (see below).

Several supernumerary limbs showed abnormal splitting of their skeleton arising at various proximal–distal levels, with or without partial duplications of distal elements. One specimen showed abnormal splitting between the tibia and fibula. This lead to a split tarsus which was connected to four and two digits, respectively (Fig. 4B). In Fig. 3D, splitting occurred within the tarsus, leading to a four-digit autopod with two aborted digit rudiments on the tibial side (*). The "lobster-claw" of Fig. 5G (two digits) may be viewed as an abnormal splitting event that occurred distally to the tarsus. In contrast, the contralateral extra limb was polydactylous, with a highly abnormal "digit 1" which branched a supernumerary terminal phalanx (Fig. 5I). Figures 5A–5D show one of the most interesting supernumerary limb patterns. A single femur articulated to the duplicated pelvic element (Figs. 5A and 5B). It was followed by a single tibia and two symmetrical fibulae (Fig. 5D, compare to the control orthotopic hindlimb of Fig. 5C). Thus, two distinct autopods developed, one (articulated to the tibia + fibula) with a full tarsus and six digits, the other (articulated to one fibula) with only posterior tarsal elements and three digits (Fig. 5D).

The cases described above had apparently normal or-

FIG. 4. Skeletal patterns of RA-induced supernumerary limbs. (A) Lower body skeleton of a 18.5 dpc specimen with two supernumerary hindlimbs linked to imperfectly duplicated pelvic elements connected to the righthand side orthotopic ischium bone. (B) Skeleton of a dissected supernumerary limb split at the level of the tibia/fibula and tarsus (dashed arrow). (C) Skeleton of a supernumerary pair of limbs joined by a symmetrical ischium rudiment (the dashed line indicated the plane of symmetry) which was floating caudally to the orthotopic hindlimbs. Alcian blue and alizarin red staining. Abbreviations: Fe, femur; Fi, fibula; HL, hindlimb; il, ilium bone or rudiment; is, ischiopubic bone or rudiment; T, tail. The asterisks indicate supernumerary structures.
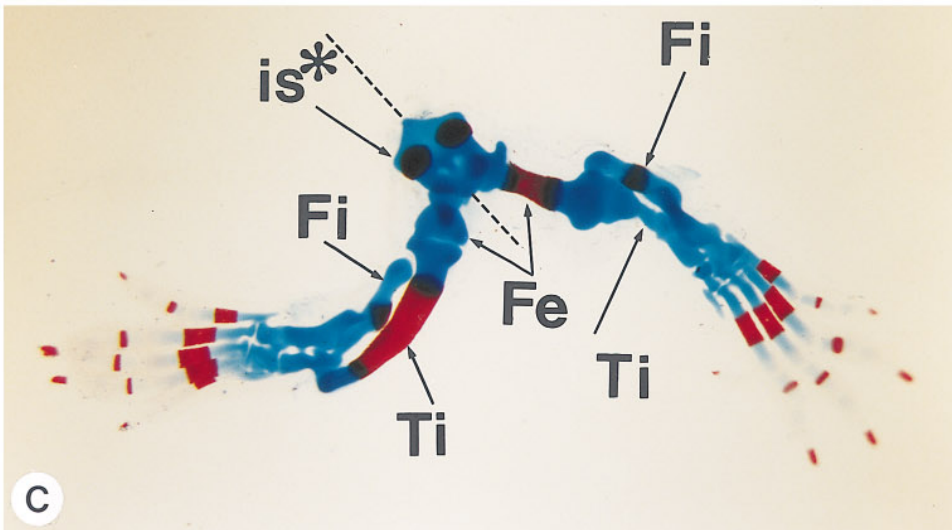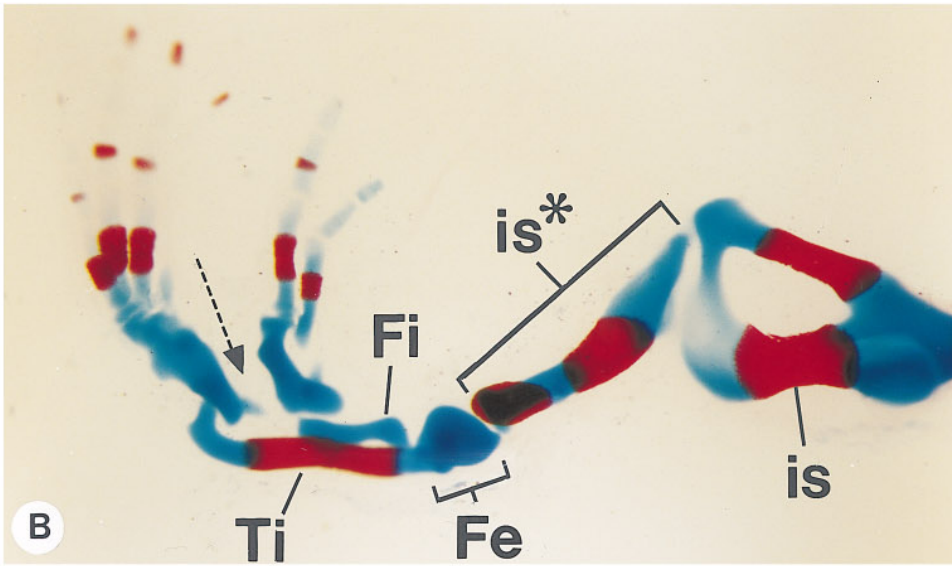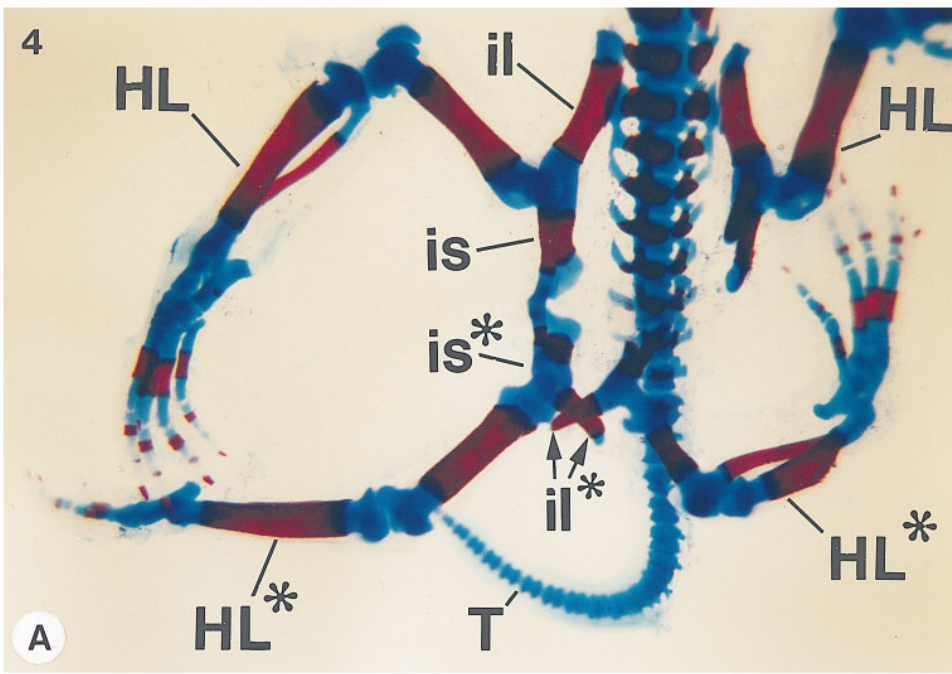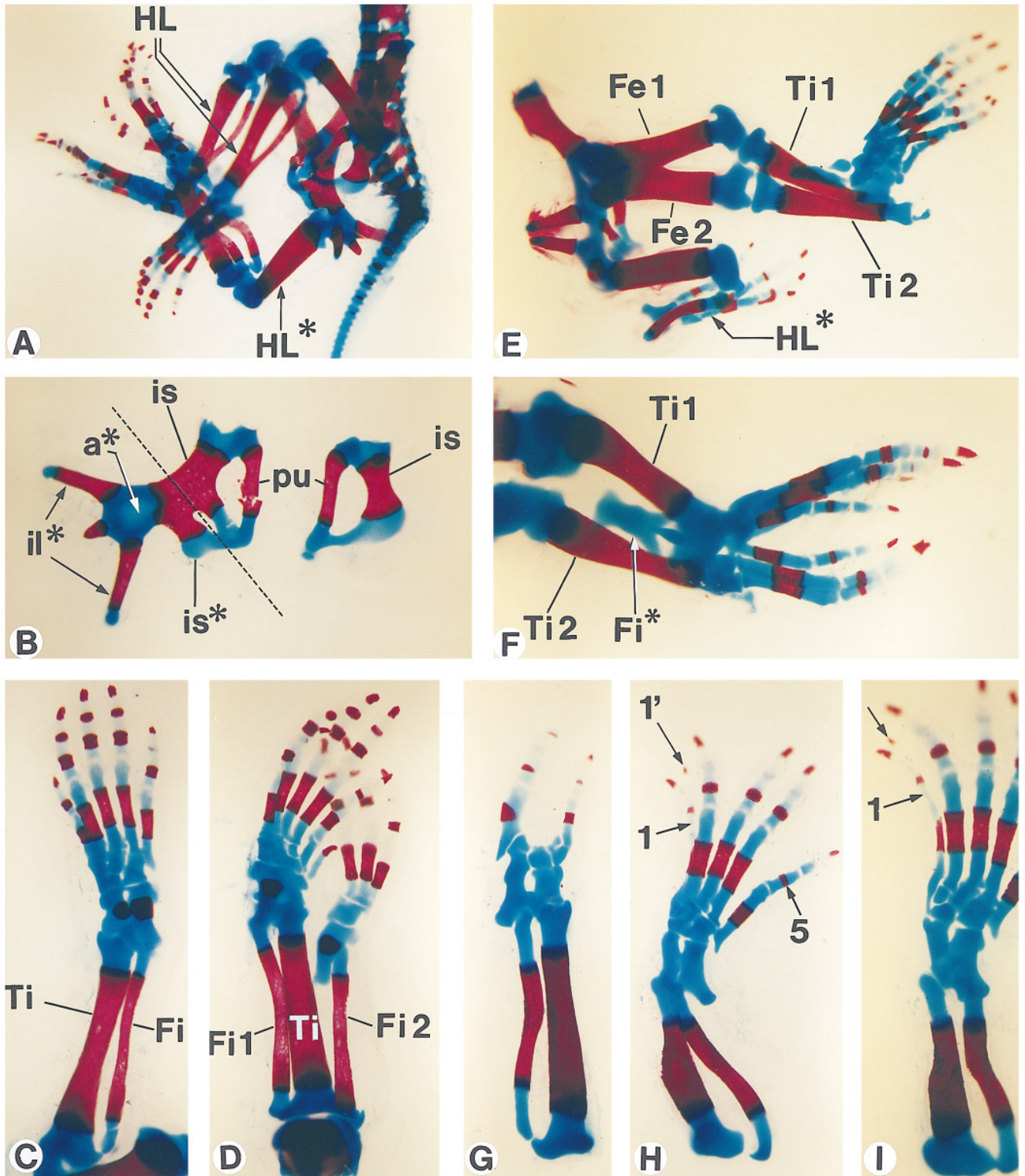
FIG. 5. Skeletal patterns of RA-induced supernumerary limbs. All skeletons are from 18.5 dpc fetuses. (A) Lower body skeleton of a specimen with a single symmetrical supernumerary limb linked to a complex duplicated pelvic element fused to the righthand side orthotopic ischium bone. (B) Dissected ischiopubic bones of the same specimen. The right side bone (shown on the left) harbors the duplicated pelvic element (the dashed line indicates the plane of fusion), the left side bone is unaltered. (C) Dorsal view of the right

thotopic hindlimbs. In contrast, three specimens displayed duplication patterns, while there was no ''normal'' hindlimb on one side of the fetus. One of these had two femurs fused at the level of their diaphyses (but with two separate heads—Fig. 5E), two tibiae, and a single, mirror-image symmetrical fibula rudiment (Figs. 5E and 5F). These were followed by two autopods with five and three digits, facing each other along their ventral sides (Fig. 5F). Note that this animal had another supernumerary hindlimb with a single zeugopodium bone and four digits (Fig. 5E). Two other specimens displayed very similar ''mirror-image-duplicated'' limbs, one of them with two fully separated femurs articulating to a single pelvic bone (5 + 4 digits), the other with two independent pelvic bones (4 + 4 digits) (not shown). These patterns are likely to result from the ''twin'' (group III) limb bud phenotypes described above.

Supernumerary Limbs in Adult Mice

Seven newborn mice with visible supernumerary limbs were obtained from RA-treated dams which were allowed to deliver. Of these, five survived until 7 weeks postpartum and were sacrificed for analysis. One specimen showed a fully developed six-digit supernumerary hindfoot, facing the ''normal'' hindfoot in an opposite dorsal–ventral orientation (Fig. 6A). Histological analysis showed that the extra hindlimb had well-developed vasculature and muscles, although it did not appear motile (not shown). Three other animals displayed a severely atrophic supernumerary hindlimb (Fig. 6B and data not shown). Nevertheless, these limbs displayed footpads, indicative of a dorsal–ventral polarity, and nails (Fig. 6B). Another animal showed an elongated mass consisting of fur-covered soft tissue which was connected by a fibrous stem to the base of the penis (Fig. 6C).

Skeletal analysis revealed that the example of Fig. 6B corresponds to a single supernumerary limb with a very truncated and curved zeugopodium bone, this limb being split at the tarsal level into one digit (below) and two fully fused digits (above). Another animal displayed an altered hindlimb pattern similar to that of Figs. 5E and 5F, with an interesting duplication of the terminal phalanges and nails in two of its digits (Fig. 6G). Another specimen only had a polydactylous hindlimb with no associated supernumerary limb (Fig. 6D). Skeleton analysis showed the presence of nine digits, two of them being terminally fused (digits 4 and

5 in Figs. 6E and 6F). As the two extreme digits corresponded anatomically to normal digits 1 and 5, we conclude that the polydactylous autopodium had a normal anteroposterior polarity and a reiteration of some central digits (Fig. 6E). This example corresponds to a predictable phenotype for group IV affected limb buds (see above).

Gene Expression Patterns in Supernumerary Limbs

We have examined the expression patterns of the *Fgf-4, Fgf-8, Shh,* and *Hoxd-13* genes by whole-mount ISH analysis of RA-treated embryos collected at 11.5 dpc. *Fgf-4* and *Fgf-8* are believed to regulate limb bud outgrowth and are expressed, respectively, in the posterior region and throughout the AER (Crossley and Martin, 1995; Niswander and Martin, 1992). We found that *Fgf-8* was specifically expressed along the distal margin of some of the supernumerary limb buds (Fig. 7A). In some instances, however, one of the extra buds was clearly devoid of *Fgf-8* transcripts, suggesting that such a bud would not develop into a limb (*, Fig. 7A). The extra bud shown in Fig. 7B displayed an interrupted pattern of *Fgf-8* expression in three patches of cells. Such an altered production of FGF-8 may be correlated to the fact that supernumerary buds often display variable truncations of their skeletons. *Fgf-4* expression was also detected along the distal margin of most supernumerary buds (Fig. 7C), thereby confirming that their AER produces the two major growth factors involved in normal limb outgrowth. In most cases, the *Fgf-4* expression domain was centered distally, with no clear anterior–posterior asymmetry (Fig. 7C and data not shown). Some extra buds showed a preferential anterior distribution (not shown), consistent with the *Shh* expression patterns described below. As indicated above for *Fgf-8,* several extra buds appeared devoid of *Fgf-4* transcripts (not shown).

Shh gene expression is restricted to the posterior margin of the normal limb buds, and its product is believed to mediate the anterior–posterior polarizing properties of this region, the ZPA (Riddle *et al.,* 1993). In most of the RA-induced supernumerary limb buds without tail duplication, *Shh* was expressed as a ''mirror image'' of the normal hindlimb, i.e., along the anterior margin of the extra bud (Fig. 7D). There were examples with anterior (reversed) *Shh* expression in both extra limb buds, anterior expression in one bud (HL*) and lack of expression in the contralateral one

orthotopic hindlimb (zeugopodium and autopodium) of the same specimen. This limb is not altered and is shown as a control. (D) Dorsal view of the supernumerary hindlimb from the same specimen showing a partial mirror-image duplication, the unique tibia encompassing the plane of symmetry. (E) Righthand side hindlimbs of an RA-affected fetus. Note the duplication of the orthotopic hindlimb at the level of the femur and the presence of another, abnormal, supernumerary limb (bottom). The lefthand side hindlimb of this specimen was normal. (F) Enlargement of the distal skeleton of the same hindlimbs, showing mirror-image duplication of the zeugopodium and autopodium. (G–I) Distal skeletons of two supernumerary limbs. (G) shows a two-digit split autopodium. (H, I) An abnormal digit 1 and a supernumerary terminal digit (1′, arrow). Alcian blue and alizarin red staining. Abbreviations: a*, supernumerary acetabulum; Fe1, Fe2, mirror-image duplicated femurs; Fi, fibula; Fi1, Fi2, mirror-image-duplicated fibulae; HL, hindlimb; Ti, tibia; Ti1, Ti2, mirror-image-duplicated tibiae; is, ischium bone or rudiment; il, ilium bone or rudiment; pu, pubis; 1–5, digit number. The asterisks indicate supernumerary or duplicated structures.

(*, Fig. 7E), or distally centered expression domains in very small supernumerary buds (not shown). By contrast, *Shh* transcripts had a ''normal'' posterior polarity in the extra limb buds associated to a duplicated tail (group II embryos—data not shown). We note that *Shh* also displayed a ''normal'' expression pattern along the notochord and floor plate of both duplicated tails (not shown). ''Twin'' (group III) hindlimb buds showed two symmetrical *Shh* transcript domains along the posterior margins of both limb buds (Fig. 7E, HL and HL'; a consistent duplication of the *Fgf-4* expression domain was observed in another specimen with the same phenotype). Finally, *Shh* expression was detected along the posterior margin of group IV altered hindlimbs (with abnormal anterior protrusions), but never in an ectopic anterior location, whatever the size of the deformation (Fig. 7F and data not shown).

Hoxd genes are expressed in sequential and nested domains in the limb bud mesenchyme, *Hoxd-13* displaying the most restricted domain toward the distal and posterior extremity of the bud (Dollé *et al.,* 1991b, 1993; see Fig. 7G). We found that *Hoxd-13* was specifically expressed in most, but not all, supernumerary limb buds (Fig. 7G and data not shown). The additional *Hoxd-13* domains were nested toward the distal and anterior margins of the buds and thus displayed a reversed polarity with respect to the normal hindlimbs (Fig. 7G), consistent with the patterns of *Shh* gene expression. Finally, *Hoxd-13* expression was detected in the distal region of the abnormal anterior protrusion of a group IV altered hindlimb (Fig. 7H).

DISCUSSION

Our study confirms the finding of Rutledge *et al.* (1994) that systemic administration of RA to mouse embryos at the egg-cylinder stage (4.5 to 5.5 dpc) can induce the formation of supernumerary limbs and occasional duplication of the lower body axis. It was shown earlier that exposure to vitamin A or RA can induce the generation of extra limbs at the site of tail amputation in frog tadpoles (Mohanty-Hejmadi *et al.,* 1992; Maden, 1993). Although both types of experiments have strikingly similar outcomes, they actually reveal distinct effects of retinoids on a regenerating tissue (in the frog) or in normal developing embryos (in the mouse). Some of the retinoid-treated frog tadpoles, however, developed a duplicated hindlimb proximal to the site of amputation, showing that such duplications can occur outside of the regenerating blastema (Mohanty-Heijmadi *et al.,* 1992).

We have further characterized RA-induced supernumerary limbs in mice, both morphologically and with the use of molecular markers. The majority of the present extra limb buds (group I embryos in Fig. 1) arose in pairs, ventrally and caudally to the orthopic hindlimbs and without tail duplication. Most of these buds expressed molecular markers of normal limb development (*Fgf-4, Fgf-8, Shh,* or *Hoxd-13*). The extra buds which did not express these molecular markers are likely to degenerate or form nonlimb structures. In this respect, we note that one adult mouse displayed a large protrusion consisting only of soft tissues.

Most of the extra limbs were orientated oppositely to the normal hindlimbs. Accordingly, we found that *Shh* was expressed in an ectopic location along the *anterior* margin of the extra buds. These ectopic expression domains were thus facing the posterior *Shh* domains of the orthotopic hindlimbs. *Hoxd-13* transcripts were also distributed anteriorly and distally in most supernumerary limb buds, further suggesting that these buds have a reverse anterior–posterior polarity. Similar cases of reverse polarity were observed in the supernumerary limb buds induced by FGFs in the flank of chick embryos (Cohn *et al.,* 1995; Ohuchi *et al.,* 1995). These additional buds developed as mirror images of the normal wing or leg and showed restricted *Shh* expression along their anterior margin, i.e., as a mirror image of *Shh* transcripts in the normal wing bud. This may be explained by the fact that, prior to budding, potential polarizing activity is not restricted to the wing field but extends in cells of the anterior and mid flank (Hornbruch and Wolpert, 1991). Cohn *et al.* (1995) suggested that the FGF-induced supernumerary buds may incorporate cells of the anterior flank with potential polarizing activity. Instead of remaining dormant as in normal embryos, these cells would eventually be converted into a true polarizing region that expresses *Shh* and sends a reversed signal in the supernumerary bud. The same explanation may apply to the ''group I'' RA-induced limb buds reported in this study. These extra limb buds do not arise in the flank, but instead arise caudally to the normal hindlimbs. Hence, they might incorporate potential polarizing cells from the hindlimb field (Hornbruch and Wolpert, 1991) or the genital bud area (Dollé *et al.,* 1991a) and convert them into a polarizing region which expresses *Shh* at their anterior margin. Failure of some of the supernumerary buds to successfully produce a polarizing region expressing *Shh* would explain why they do not develop into a limb.

FIG. 6. RA-induced supernumerary limbs in adult mice. All the mice were sacrificed at ∼7 weeks postpartum. (A) Unilateral mirror-image supernumerary hindlimb (HL*). (B) Unilateral atrophic supernumerary hindlimb (HL*) with a severe split between two of its three digits. (C) Distended outgrowth of soft tissue connected to the penis. (D) Ventral view of a polydactylous hindpaw. The ventral supernumerary claw (4 & 5) actually consists of two terminally fused digits (see F). (E, F) Skeletal staining of the corresponding autopodium: dorsal view (E) and close-up of two supernumerary digits on the ventral side (F) fused at the level of their terminal phalanges (4 & 5). Note that the extreme digits (1 and 9) have the morphology and the phalangeal count of normal digits 1 and 5, respectively. (G) Close-up of a digit duplication event arising at the level of a first phalanx in another specimen. (E–G), Alcian blue and alizarin red staining. Abbreviations: 1–9, digit number; HL, hindlimb; P1, first phalanx; P2, second phalanx.
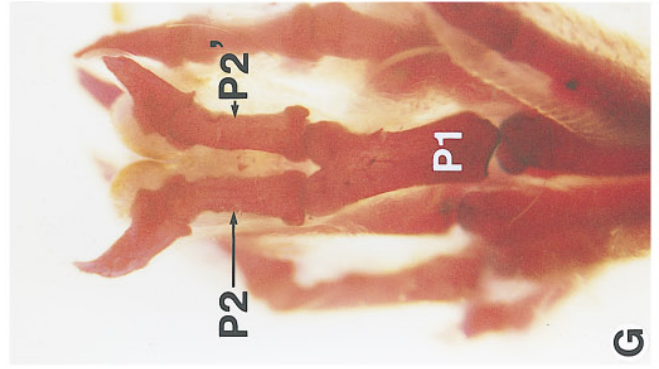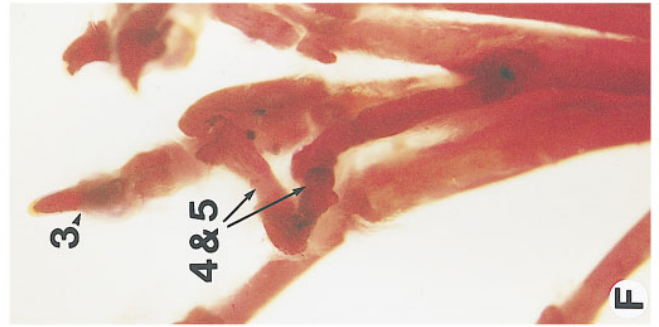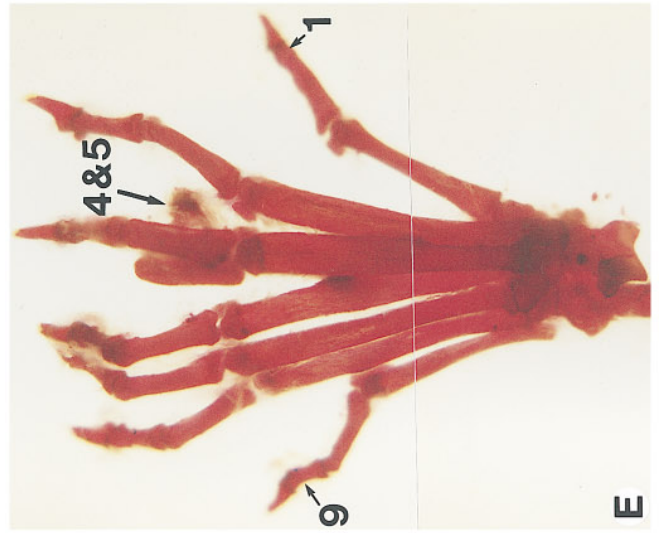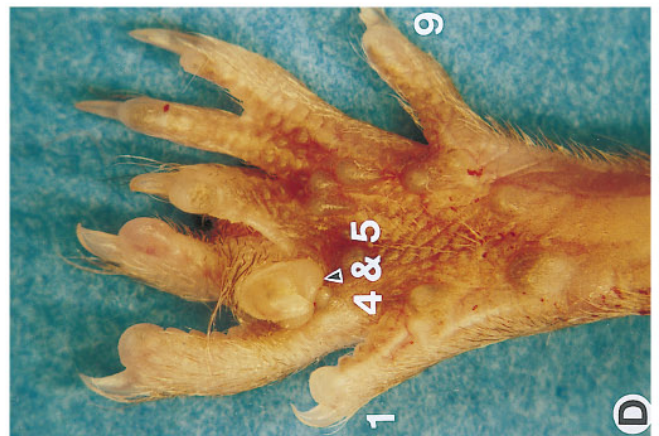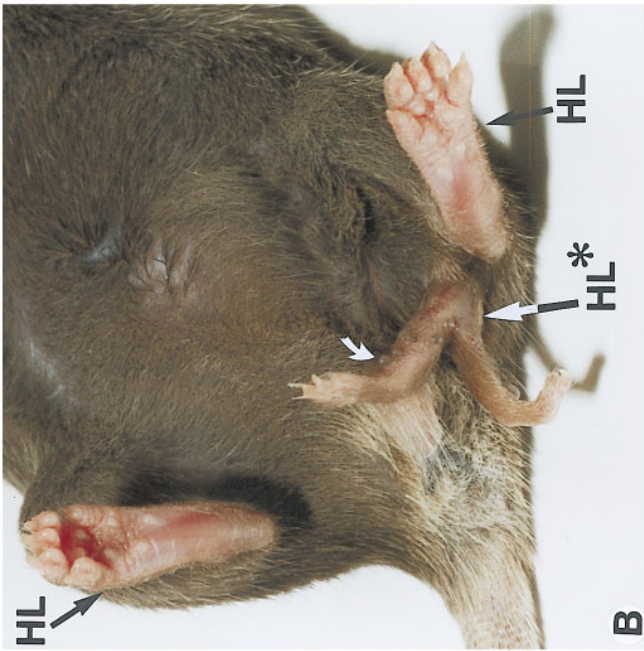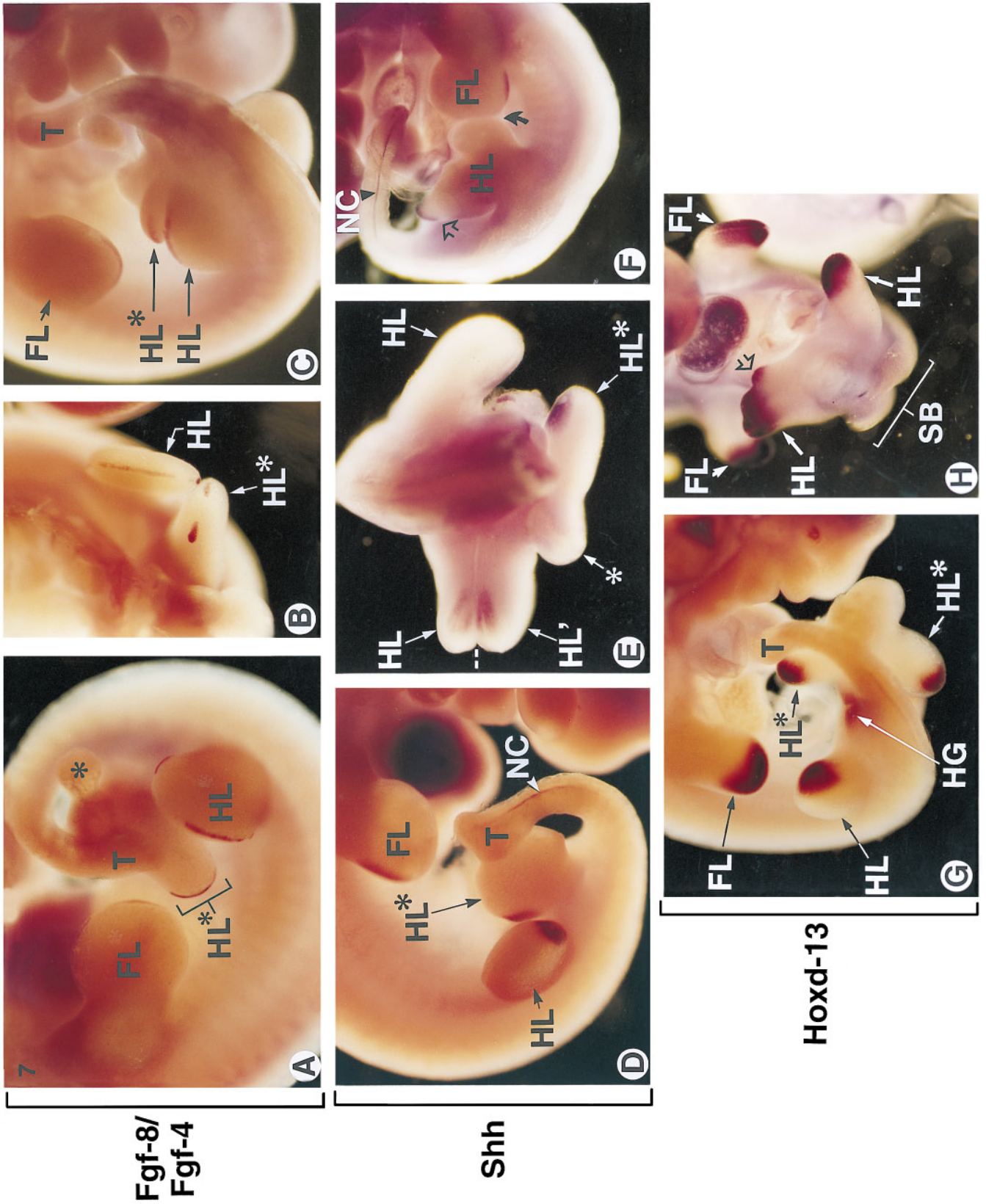
Other types of supernumerary limb buds were observed less frequently (groups II to IV in Fig. 1). The variability in outcomes of RA treatment might be explained by variations in the concentration of RA that reaches each embryo combined with variations in the developmental stage of the embryo at the time of treatment. Group II corresponds to an extra pair of hindlimb buds associated to a duplicated (or exceptionally triplicated) lower body axis and tail. We found that these extra limbs always displayed a normal anterior–posterior polarity, as revealed by the normal (posterior) expression patterns of *Shh*. In contrast to the study of Rutledge *et al.* (1994), we never observed such duplications at 18.5 dpc, possibly because such embryos had been eliminated earlier due to additional RA-induced alterations (see Results).

Some specimens (group III) showed two partly duplicated (twin) hindlimb buds. This phenotype is consistent with some of the 18.5 dpc skeletal patterns showing mirror-image duplication of a hindlimb arising at various levels of the femur. Such duplicated hindlimb buds displayed two parallel AERs and duplicated *Shh* transcript domains with a normal (posterior) polarity. This phenotype, which had not been previously described in the mouse, is somewhat similar to that of the *eudiplopodia* chick mutant which has the property of forming a second AER along the dorsal surface of the four limb buds, giving rise to duplications of the distal parts of the limbs (Goetinck, 1964). Such a phenotype had never been described in the mouse.

Other specimens (group IV) had an altered hindlimb bud with more or less pronounced anterior protrusion, but no true supernumerary limb buds. The fact that *Hoxd-13* is expressed in the altered region suggests that it has the potential of forming distal structures. The fact that systemic administration of RA resulted in an anterior duplication of the *Shh* expression pattern in the *pectoral* fin buds of zebrafish embryos (Akimenko and Ekker, 1995) suggests that group IV altered hindlimbs might be the result of an RA-induced ectopic ZPA. We never detected an ectopic anterior *Shh* expression domain in such altered hindlimb buds. However, a possible ectopic expression of *Shh* which would be below the detection threshold cannot be ruled out. Note that the digit pattern of an RA-generated adult polydac-

tylous hindlimb supports the idea that there was no ectopic anterior ZPA. Despite the fact that this specimen had nine digits, its most anterior digit was anatomically a digit 1, and its most posterior digit a digit 5. The supernumerary digits actually corresponded to additional central digits. Thus, it appears that there is no duplication of the ZPA in such RA-affected hindlimbs, in contrast to what was observed in the polydactylous mouse mutants *Strong's luxoid* (Chan *et al.,* 1995), *Rim4, Hemimelic extra toes,* and *Extra toes* (Masuya *et al.,* 1995) or in transgenic mice overexpressing *Hoxb-8* (Charité *et al.,* 1994).

How can RA, when administered at the egg-cylinder stage, direct nonlimb cells into the pathway of limb formation? The primary effect of such RA treatment is likely to be restricted to pre-gastrulation stages, as excess RA is believed to be cleared from the embryo in less than 12 hr (Wang *et al.,* 1980; Ward *et al.,* 1995). It seems unlikely that a limb field (cells which have already been determined to form limbs) exists at that stage. Early limb fields have not been studied in the mouse. However, Rudnick (1945) found limb-forming areas shortly after gastrulation in chick embryos at stages 6–12, which would be equivalent to Day 7– 8 mouse embryos. Excess RA may alter the regulation of secondary signaling molecules which are involved in the process of normal limb induction during or after gastrulation. This alteration seems to be in a limited time window and/or within a defined region of the embryo) such that the supernumerary limbs appear in a restricted territory and almost exclusively develop as hindlimbs.

Fibroblast growth factors (FGFs) have been postulated to be endogenous limb-inducing factors. It was shown recently that local administration of FGFs in the flank of chick embryos can induce the formation of supernumerary limbs (Cohn *et al.,* 1995; Ohushi *et al.,* 1995). Furthermore, overexpression of FGF-4 in chimeric mice leads to the outgrowth of multiple limb bud-like structures in the embryonic flank (Abud *et al.,* personal communication). Cohn *et al.* (1995) suggested that experimentally administered FGFs would mimic the effect of an endogenous FGF produced in local sources to determine limb position along the body axis. The nature of the limb-inducing FGF, as well as its exact location and source of production, remain to be eluci-

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FIG. 7. Whole-mount *in situ* hybridization study of RA-induced limb buds in 11.5 dpc embryos. (A) Ectopic expression of *Fgf-8* along the distal margin of a supernumerary limb bud (HL*). Note that the contralateral bud (*) does not express *Fgf-8*. (B) *Fgf-8* expression was detected in three distinct small patches in this supernumerary limb bud (two of them are visible on this view). Note that the contralateral supernumerary bud (not shown) showed continuous expression of *Fgf-8*. (C) Ectopic expression of *Fgf-4* along the distal margin of a supernumerary hindlimb bud (HL*). (D) Ectopic expression of *Shh* along the anterior margin of a supernumerary hindlimb bud (HL*). (E) Duplication of the *Shh* expression domain along the posterior margin of "twin" hindlimb buds (HL, HL′). Note that this specimen also harbors two ventral supernumerary buds (* and HL*), only one of them (righthand side) showing *Shh* expression in a reverse polarity. (F) *Shh* expression in a specimen with abnormal (group IV) hindlimb bud. A single domain of expression is detected at the posterior margin of this highly deformed limb (open arrow). The filled arrow points to the anterior margin of the abnormal hindlimb which shows no *Shh* labeling. (G) Ectopic expression of Hoxd-13 in two supernumerary hindlimb buds (HL*). Note the reverse ("mirror-image") polarity of the ectopic domains compared to the normal hindlimb. The normal expression of *Hoxd-13* in the hindgut region is not affected. (H) *Hoxd-13* expression in an embryo with an altered (group IV) hindlimb bud (lefthand side). Note that *Hoxd-13* transcripts extend distally in the abnormal anterior protrusion. The tail of this specimen has been sectioned in order to show both hindlimbs. Abbreviations: HL, hindlimb bud; HL*, supernumerary hindlimb bud; FL, forelimb bud; HG, hindgut region; NC, notochord; SB, spina bifida; T, tail.

dated. In this context, it is tempting to speculate that early RA treatment might locally alter an FGF-induced signaling pathway to generate ectopic limb outgrowth. Future experiments will investigate whether this effect of RA might involve the ectopic activation of a gene(s) encoding a given FGF or its receptor(s). It will also be of interest to investigate whether the limb-inducing effect of RA is mediated by a specific nuclear receptor by testing various RAR or RXR mutant embryos (Lohnes *et al.,* 1994; Mendelsohn *et al.,* 1994; Kastner *et al.,* 1994, 1995, and references therein) or receptor-specific synthetic retinoids.

ACKNOWLEDGMENTS

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