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in the Absence of the Mesonephros

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With rapid progress in understanding the genes that control limb development and patterning interest is becoming focused on the factors that permit the emergence of the limb bud. The current hypothesis is that FGF-8 from the mesonephros induces limb initiation. To test this, the inductive interaction between the Wolffian duct and intermediate mesoderm was blocked rostral to the limb field, preventing mesonephric differentiation while maintaining the integrity of the limb field. The experimental outcome was monitored by following expression of *cSim1* and *Lmx1,* molecular markers for the duct and the mesonephros, respectively. Evidence is presented that the intermediate mesoderm undergoes apoptosis when the inductive interaction with the Wolffian duct is blocked. *fgf-8* expression was undetectable in the mesonephric area of embryos with confirmed absence of mesonephros; nevertheless, limb buds formed and limb development was normal. The mesonephros in general, and specifically its *fgf-8* expression, was shown to be unnecessary for limb initiation and development; the hypothesis linking the mesonephros and limb development is not supported. Further studies of axial influences on limb initiation should now concentrate on medial structures such as Hensen's node and paraxial mesoderm; the alternative that no axial influences are required should also be examined.

INTRODUCTION

Recently great progress has been made in our understanding of how inductive cellular interactions and gene expressions permit development of the primary embryonic axis and subsequently the central nervous system (Rubenstein and Puelles, 1994). Similar progress has been made in other organ systems; limb development is in the forefront of this rapid progress (Tickle and Eichele, 1994). This very progress, however, has brought into sharp focus an area where there is little or no understanding, namely, how the cells that will give rise to the limb bud take on their identity as the limb field. The hypothesis that the mesonephros may be involved in limb development in the chick was proposed by Stephens (Stephens and McNulty, 1981; Strecker and Stephens 1983; Stephens *et al.*, 1991) based on the development of defective or absent wings when communication between the mesonephros and lateral plate mesoderm was blocked by a barrier. Mesonephros ablation also caused reduction defects in wing development, further implicating a critical role for the mesonephros in limb development (Geduspan and Solursh, 1992a). Very recently a possible molecular correlate to these experimental results was reported, namely, that *fgf-8* (*Fibroblast growth factor-8*) mRNA was expressed in a rostrocaudal sequence by the mesonephros. The provocative observation was that *fgf-8* mRNA was expressed by the mesonephros at the prospective wing bud level during late stage 13 through stage 15 at the very time when the limb field is probably determined. It was also noted that *fgf-8* mRNA was expressed by the wing field ectoderm during stage 15/16 and in the apical ridge later in limb development. This has resulted in the formal hypothesis that FGF-8 from the mesonephros induces wing budding in the chick (Crossley *et al.*, 1996). When considered in the context of the ability of the FGFs, including FGF-2, 4, and 8 (Crossley *et al.*, 1996; Cohn *et al.*, 1995; Vogel *et al.*, 1996), to cause ectopic limbs to form in the flank lateral plate, this hypothesis has taken center stage in limb development research.

The mesonephros isolation and ablation experiments are not easily controlled and are open to criticism, mainly because the operation may disrupt limb field integrity. How-

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ever, the sequence of kidney development permits a relatively clean test of the hypothesis involving *fgf-8*. The intermediate mesoderm at trunk levels remains as an undifferentiated mesenchyme until, under the inductive influence of the Wolffian duct, it develops into the mesonephros. The Wolffian duct is the excretory canal of the pronephros (the primitive kidney at the level of the neck) and progressively descends along the anteroposterior axis of the embryo, inducing the differentiation of the mesonephros as it passes by. It has been demonstrated that in the chick there is no self-differentiation of the intermediate mesoderm without the presence of the Wolffian duct; if caudal elongation of the duct is blocked the differentiation of the mesonephros is prevented (Bishop-Calame, 1965; Wolff, 1970). In the present work we have mechanically arrested the caudal elongation of the Wolffian duct by the placement of a barrier, following the method of Calame (1961) and LeDouarin and Fontaine (1970). This procedure prevents the differentiation of the mesonephros caudal to the barrier. The barrier was placed rostral to the wing field, so that no mesonephros developed at the wing level. The advantage of this design is that there is no interference with the integrity of the prospective limb field.

The presence of the barrier did not disturb gross development of the embryo. Experimental success in arresting elongation of the Wolffian duct was evaluated using the expression of *cSim1* as a marker of the duct. We found that expression of *fgf-8* was undetectable in the undifferentiated mesonephric area at any stage after the operation; however, wings developed normally. Blockage of mesonephric differentiation in *limbless* mutant embryos demonstrate that initial outgrowth of the limb occurs with undetectable expression of *fgf-8* at both the mesonephros and limb ectodermal levels. Our data indicate that the mesonephros and in particular its *fgf-8* expression are not required for limb initiation or development.

MATERIALS AND METHODS

Implantation of Barriers

Fertilized chicken embryos were purchased from Ibertec Farm, Valladolid, Spain. Eggs were routinely incubated, opened, and staged according to Hamburger and Hamilton (1951). *Limbless* mutant embryos and normal embryos were obtained from a heterozygous mating flock maintained at the University of Wisconsin Poultry Science Department (Madison, WI). Barriers were implanted transversally in embryos from stage 9 through stage 11 following the technique described by Calame (1961) and Le Douarin and Fontaine (1970). We have used three kinds of barriers: the internal eggshell membrane, Millipore filter (0.45-μm pore, 25-μm-thick size), and tantalum foil. Preliminary experiments demonstrated that the three types of barriers were effective in the blockage of the caudal elongation of the duct and subsequently we used the internal eggshell membrane and the Millipore filter because they were much lighter and easier to use than the tantalum foil. The procedure outlined by Le Douarin and Fontaine (1970) was followed. Briefly, the vitelline membrane of embryos stages 9–11 was opened and the barrier introduced through a transverse slit made with a sharpened tungsten needle. Because the caudal end of the Wolffian duct lies slightly caudal to the last formed somite (Fig. 2D), we implanted the barrier at a distance equivalent to two or three somites caudal to the last formed somite (Fig. 1A). In order to assure good survival and to prevent abnormal development, it was essential that after the operation the air space on top of the embryo was filled with saline and the egg rotated 180° and returned to the incubator (Fisher and Schoenwolf, 1983).

Whole Mount in Situ Hybridization

The embryos were fixed at 16, 18, 24, 36, and 48 hr after the operation in 4% paraformaldehyde and processed into methanol for whole mount *in situ* hybridization. Preparation of digoxigenin-labeled antisense riboprobes, pretreatment of embryos, hybridization, and posthybridization washes were performed according to the procedure of Nieto *et al.* (1996), except that the time of proteinase K treatment was varied from 10 to 20 min. After visualization of the reaction product the embryos were photographed without clearing. Some of the stained embryos were embedded in Paraplast and processed for histology.

Cell Death Analysis

Cell death was studied in embryos at 24, 30, and 44 hr after the operation. *In situ* detection of DNA fragmentation was performed using terminal transferase to incorporate fluorescein-dUTP (''*In situ* cell death detection kit, Fluorescein'', Boehringer-Mannheim), following manufacturer's directions.

Histological and Skeletal Analysis

For histology the embryos were sequentially fixed in 4% paraformaldehyde or in Bouin's solution, embedded in Paraplast, and serially sectioned (6 μm). The sections were routinely stained in hematoxylin–eosin.

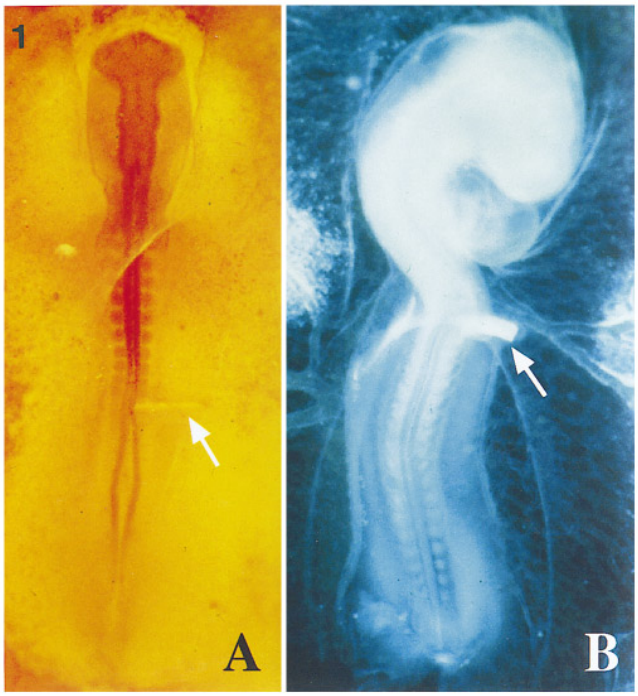
Some embryos were fixed at day 10 of development to analyze the skeletal pattern of the limbs. These embryos were dissected out, fixed in 10% Formalin, stained with Victoria blue, and cleared in methyl salicylate, and the pattern of the digits was analyzed.

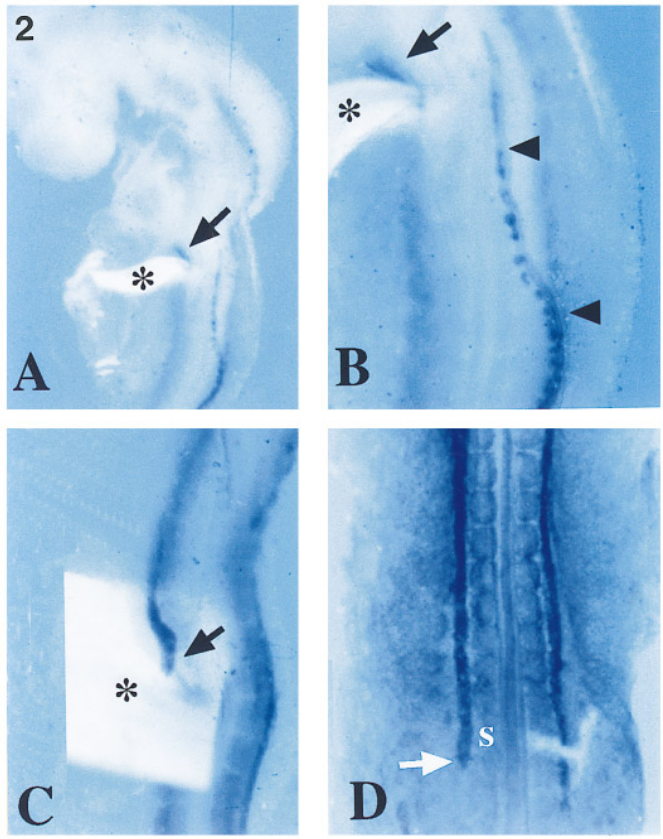
RESULTS

Expression of cSim1 Confirms the Blockage of the Caudal Elongation of the Wolffian Duct

Most barriers were placed on the right side of embryos at stages 9–11HH (Hamburger and Hamilton, 1951) (Fig. 1A). There was no obvious disturbance of subsequent development in the experimental embryos (Fig. 1B), except that in some cases the curvature of the embryos was abnormal. Occasionally, double barriers were placed, one at each side of the embryo, without affecting the gross development of the embryo.

To confirm that the caudal migration of the Wolffian duct had been blocked after the operation, we monitored the expression of *cSim1*, an avian homologue of the *Drosophila*





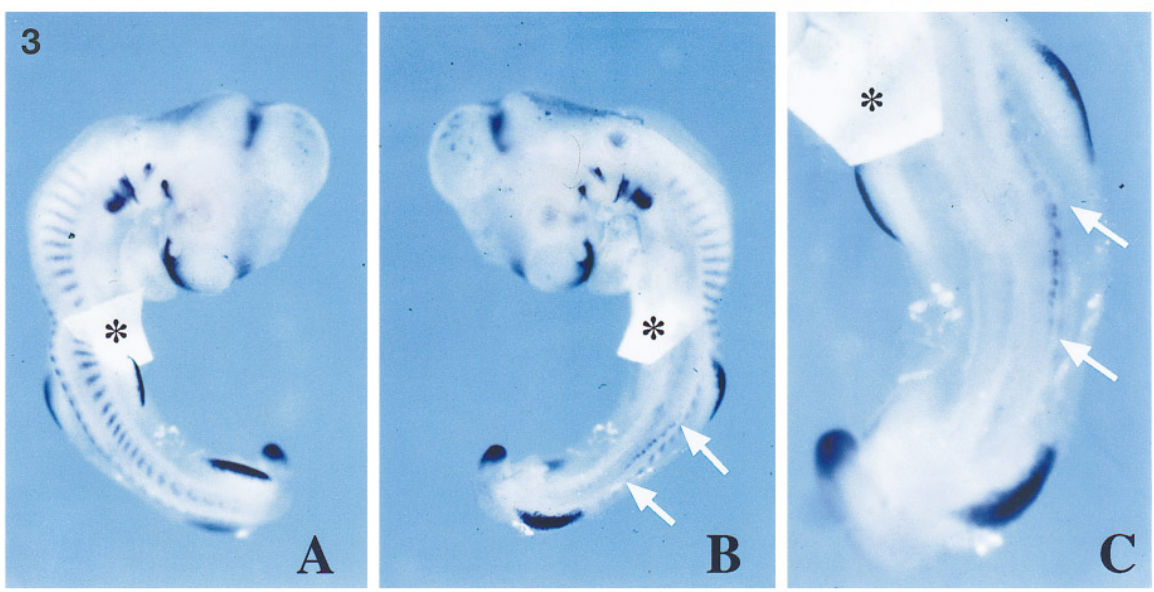


FIG. 1. Implantation of a transverse barrier does not alter further gross development of the embryos. (A) Stage 10 embryo immediately after the placement of the barrier (white arrow). (B) The same embryo shown in (A) fixed 36 hr after the operation. The gross development of the embryo appears normal. Note the level of the barrier (white arrow).

FIG. 2. Expression of *cSim1* shows that the barrier effectively blocks the caudal elongation of the Wolffian duct, preventing mesonephric differentiation. (A) Ventral view of a stage 16 embryo showing the blockage of the Wolffian duct by the barrier. The left Wolffian duct is clearly distinguished by its strong expression of *cSim1*. In the higher magnification shown in (B) mesonephric differentiation on the operated side is clearly prevented when compared with the normal Wolffian duct and mesonephric tubulogenesis in the contralateral side (arrowheads). (C) The blockage of the Wolffian duct in a stage 15 embryo. Note how the Wolffian duct ends in a terminal ampulla at the

single minded gene implicated in somite patterning that is strongly expressed in the Wolffian duct (Pourquié *et al.*, 1996; Fig. 2D). Whole mount hybridization performed 16 to 48 hr after the placement of the barrier confirmed that in 50% of the cases (10 of 20) the caudal elongation of the Wolffian duct was effectively blocked by the barrier. On the operated side the Wolffian duct was detectable by its *cSim1* expression only rostral to the level of the barrier, while a complete descending duct was clearly observed on the contralateral side (Figs. 2A–2C). As can be seen in Figs. 2B and 2C the duct generally ended with a dilated terminal ampulla at the level of the barrier (Bishop-Calame, 1965; Wolff, 1970). In the remaining 50% of the cases the blockage of the duct was not efficient, either because the continuity of the duct was unaffected by the barrier (10%; probably due to lateral displacement of the barrier) or because although the Wolffian duct was interrupted, one or two small portions of the duct (30 to 300 μm in size) were observed caudal to the obstacle and not in continuity with the cranial part of the duct (not shown). These isolated fragments of the Wolffian duct were always observed in the pathway that should have followed the duct. Our observations are in agreement with findings already reported for mesonephros development (Bishop-Calame, 1965; Le Douarin and Fontaine, 1970).

Normal Wings Form in the Absence of Mesonephric fgf-8 Expression

FGF-8 produced by the mesonephros is proposed as the endogenous molecule for limb bud induction (Crossley *et al.*, 1996, Vogel *et al.*, 1996). *fgf-8* expression is detected in the mesonephric mesenchyme close to the Wolffian duct probably in association with the beginning of tubulogenesis (Crossley *et al.*, 1996). We have analyzed *fgf-8* expression in embryos with blocked Wolffian duct elongation and found that *fgf-8* was not detectable in the intermediate mesoderm of the operated side, while the contralateral mesonephros expressed *fgf-8* normally. Figure 3 shows a stage 20 embryo 2 days after the operation. The barrier lies immediately cranial to the wing and is indicated by an asterisk. The right wing bud has emerged and developed normally compared to the contralateral side (Fig. 3A). Observation from the ventral aspect of the same embryo demonstrates the absence of *fgf-8* expression in the operated mesonephric area (Figs. 3B and 3C) while expression in the contralateral nonoperated side is normal. This result indicates that *fgf-8* is expressed by the mesonephric mesenchyme in response to the induction of the Wolffian duct.

It is important to note that *fgf-8* was undetectable in the intermediate mesoderm of the operated side at all times analyzed after the placement of the barrier (16 to 48 hr, Fig. 4). In particular, during stages 13 through 15 when the inductive action of the mesonephros is thought to take place (Stephens *et al.*, 1991; Grieshammer *et al.*, 1996), the undifferentiated intermediate mesoderm below the barrier did not show detectable expression of *fgf-8*. Figure 4 shows embryos of different stages with absence of *fgf-8* expression at the mesonephric area of the operated side.

The percentage of embryos lacking expression of *fgf-8* at the mesonephric area in the operated side was 60% (29 of 51). This percentage corresponded to the percentage of embryos with complete blockage of the Wolffian duct elongation documented with *cSim1* expression. The rest of the embryos showed one or several patches of *fgf-8* expressing cells along the operated mesonephric area, in correlation with the embryos that showed one or some isolated fragments of the Wolffian duct under the barrier.

Despite the lack of detectable mesonephric *fgf-8* expression, initiation and development of the ipsilateral limb bud was normal when compared with the contralateral limb in all the embryos analyzed (Figs. 3 and 4). An important observation was that embryos with undetectable mesonephric *fgf-8* expression on the operated side had normal *fgf-8* expression at the preridge and ridge ectoderm (Figs. 3C, 4D, and 7A), demonstrating that *fgf-8* expression by wing ectoderm is independent of earlier mesonephric *fgf-8* expression (cf. Crossley *et al.*, 1996). Our results indicate that expression of *fgf-8* by the mesonephros is not required for limb bud initiation or development.

We have demonstrated previously that *fgf-8* expression by preridge ectoderm is not required for initiation of the limb, as the amelic chick mutant *limbless* normally initiates limb development (Carrington and Fallon, 1988) without detectable *fgf-8* expression by the prelimb ectoderm (Ros *et al.*, 1996; see also Noramly *et al.*, 1996; Grieshammer *et al.*, 1996). However, mesonephric *fgf-8* expression in *limbless* homozygous embryos is normal. It remains possible that *fgf-8* expression by either the developing mesonephros alone or the limb field ectoderm alone would be sufficient to stabilize the limb field and permit wing budding. To test this we placed barriers as before in stages 9–11 embryos from *limbless* heterozygous crosses. Homozygous *limbless* embryos could be

level of the barrier (arrows in B and C). (D) Caudal region of a stage 13/14 embryo with 20 somites hybridized with the *cSim1* probe. Note that the caudal end of the Wolffian duct (white arrow) extends slightly caudal to the last formed somite (s). This is the reason the barrier was implanted at a distance equivalent to 2 or 3 somites caudal to the last formed somite. The barrier is labeled with an asterisk.

FIG. 3. Normal wing development without mesonephric *fgf-8* expression. Embryo fixed 48 hr after placement of the barrier and examined for *fgf-8* expression, shown in dorsal (A), ventral (B), and higher magnification (C). The right wing bud has normally developed and shows normal expression of *fgf-8* in the apical ridge. The ventral and the higher magnification views demonstrate lack of *fgf-8* expression in the right mesonephric area compared to normal expression in the left mesonephros (arrows). The barrier is labeled with an asterisk.

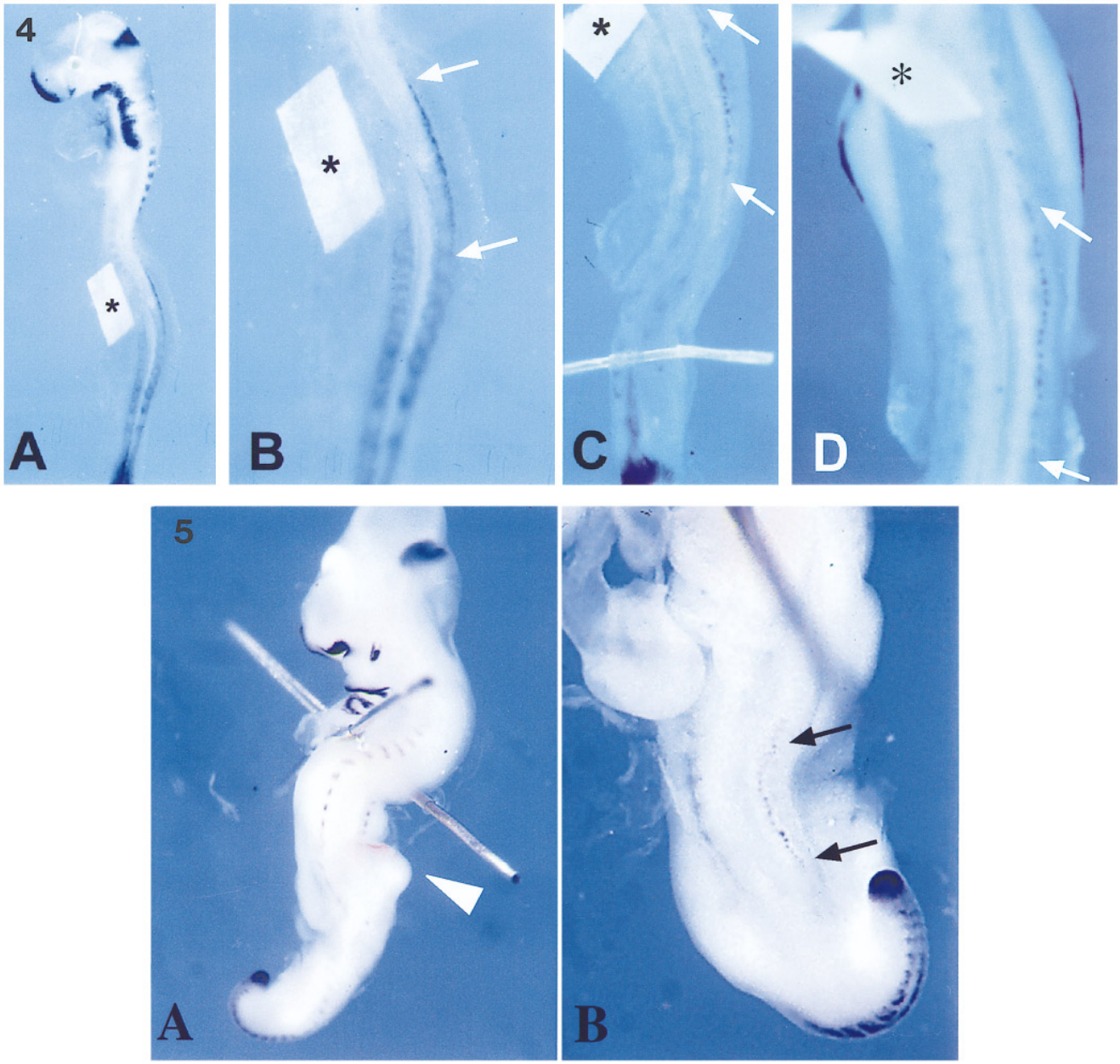


FIG. 4. Undifferentiated intermediate mesoderm does not express *fgf-8* at any stage. (A) Stage 15 embryo showing normal mesonephric expression of *fgf-8* on the left side, while it is undetectable on the operated right side. A magnification view of the same embryo is shown in (B). Mesonephric *fgf-8* expression is at the wing level at stage 15 but is progressively displaced caudally. (C) and (D) show, respectively, stage 17- and stage 19-operated embryos with absence of mesonephric *fgf-8* expression in the operated side.

FIG. 5. Limb initiation does not require *fgf-8* expression by the mesonephros, nor by the limb ectoderm. (A) *fgf-8* expression in a *limbless* embryo that received a barrier to the right side. The dorsal view shows development of both the right and left forelimbs. Note the lack of *fgf-8* expression at the apex of the limb (white arrowhead). (B) Ventral view showing that mesonephric *fgf-8* expression is detectable only on the left side (arrows). The barrier is just anterior to the right forelimb.

distinguished after stage 16 by whole mount *in situ* hybridization with *fgf-8*. We found that *fgf-8* expression was undetectable in the intermediate mesoderm region on the side with the barrier; however, wing budding was comparable on both sides of the embryo (Fig. 5). Serial sections through an embryo similar to that in Fig. 5

showed the lack of the mesonephros on the right side with a typical *limbless* limb bud (not shown).

fgf8 expression in the developing mesonephros occurs in a rostral to caudal wave. When the wing bud develops at stage 17, *fgf8* expression has passed onto the flank–interlimb segment making it difficult, using *fgf8,* to assess whether there is a mesonephros present at the wing bud level (Fig. 4). To distinguish the mesonephros at the level of the wing during later stages of limb development we have chosen the *Lmx1* gene as a marker. *Lmx1* is a LIM-homeodomain gene that is very highly expressed by the differentiating mesonephric mesenchyme. Expression of *Lmx1* was studied sequentially from 16 to 48 hr after the operation and the results obtained were very similar to those reported for *fgf-8*. In about 50% of the cases mesonephric *Lmx-1* expression was undetectable caudal to the barrier (Fig. 6A). In the remainder of the cases one or several small clumps of mesonephric mesenchymal cells expressing *Lmx1* were detected caudal to the barrier (arrowhead in Fig. 6B). *Lmx1* is also expressed in the dorsal limb bud mesenchyme starting at stage 16 (Riddle *et al.*, 1995; Vogel *et al.*, 1995). When *Lmx1* was first detectable in the contralateral control limb, it was also detected in a normal dorsal pattern in the wing of the operated side (Figs. 6A and 6B).

Another set of experimental embryos was analyzed for expression of *Hoxd-13, Shh,* and *Msx-1*. The expression of these genes was normal in the limb of the operated side (Nelson *et al.*, 1996), further confirming the normal development of the limb without mesonephros (not shown).

Histological Confirmation of the Absence of Mesonephros

Experimental embryos were sequentially fixed and serially sectioned to confirm histologically the absence of mesonephric differentiation. In 40% (6 of 15) of the specimens the mesonephros was completely absent in the operated side (Fig. 7A). Another 40% of the embryos showed small clumps of poorly developed mesonephric tissue at localized levels (not shown). In many cases mesonephric tissue at the operated side was detected only for about 30 μm. In general these clumps of mesonephric tissue ranked in size from 30 to 300 μm. Whenever mesonephric differentiation was detected at the operated side, the Wolffian duct was also present. The percentages obtained in the histological study correlate with the percentages obtained in the whole mount hybridizations. Taken together these data indicate the great inductive capabilities of the Wolffian duct, as small isolated fragments induce localized mesonephric differentiation.

Death of Uninduced Intermediate Mesoderm

When the Wolffian duct was blocked, the intermediate mesoderm remained undifferentiated, did not activate detectable expression of *fgf-8,* and showed pycnotic nuclei from 30 hr after the operation. *In situ* detection of DNA fragmentation by TUNEL (terminal deoxynucleotide transferase nick end labeling) confirmed the presence of apoptotic cells in the mesonephric area peaking at about 44 hr after the operation (Fig. 7B), suggesting that the intermediate mesoderm dies when its differentiation is prevented. Cell death in the contralateral mesonephric area was not observed at these stages.

Normal Skeletal Pattern in the Absence of Mesonephros

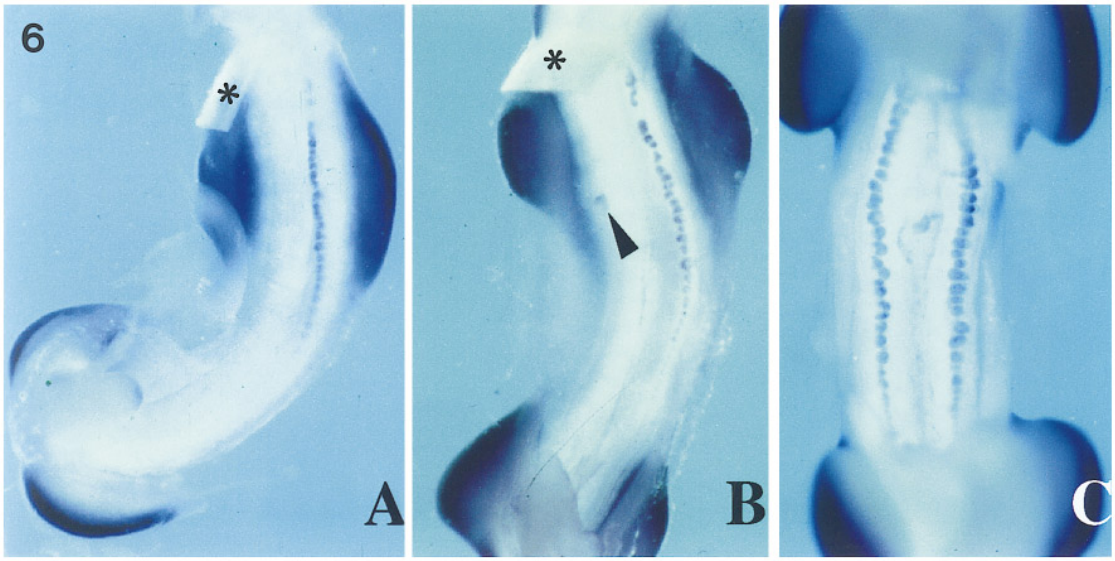
Some of the operated embryos were allowed to develop up to 10 days to analyze the skeletal pattern of the formed limbs. The wings of the operated side showed a normal skeletal pattern in 80% of the cases (10 of 12; Fig. 7C). Two specimens with abnormal skeletal pattern had missing anterior elements (radius and digit 2; not shown). We interpreted these deletions to be a consequence of the barrier localized at mid wing level isolating anterior wing tissues from posterior wing bud influence. It has been reported that the anterior half of the wing bud cannot survive when isolated from the posterior part (Todt and Fallon 1987; Wilson and Hinchliffe, 1987). In our experiment some of the barriers ended up at the level of somite 16–17, reproducing the experiment in which a barrier is placed in the middle of the wing bud and resulting in a limb that lacks anterior elements (Todt and Fallon, 1987; Wilson and Hinchliffe, 1987). Histological serial sections confirmed total absence of the right mesonephros in 40% of the experimental embryos with normal skeletal pattern (Fig. 7D).

DISCUSSION

In this study we have analyzed limb development in association with blockage of mesonephric differentiation. An important advantage of our experimental approach is that it maintains the integrity of the prospective limb field preventing interference due to the microsurgery itself. If a transverse barrier is placed caudal to the tip of the Wolffian duct, its caudal elongation is blocked and the induction of the mesonephros does not occur (Bishop-Calame, 1965; Wolff, 1970). We found that *fgf-8* expression is undetectable in the mesonephric area of operated embryos; however, limb development is initiated and proceeds normally. Our data call into question the proposed link between mesonephros and limb development and indicate that FGF-8 is not required for the initial outgrowth of the limb.

FGF-8 Is Not the Limb-Inducing Signal

Proliferation studies demonstrated that the limb bud emerges because the limb field mesoderm maintains a high labeling index while the flank region shows a reduction (Searls and Janners, 1971). The signal responsible for this differential rate of labeling index between limb-forming and nonforming regions of the lateral plate is presently un-



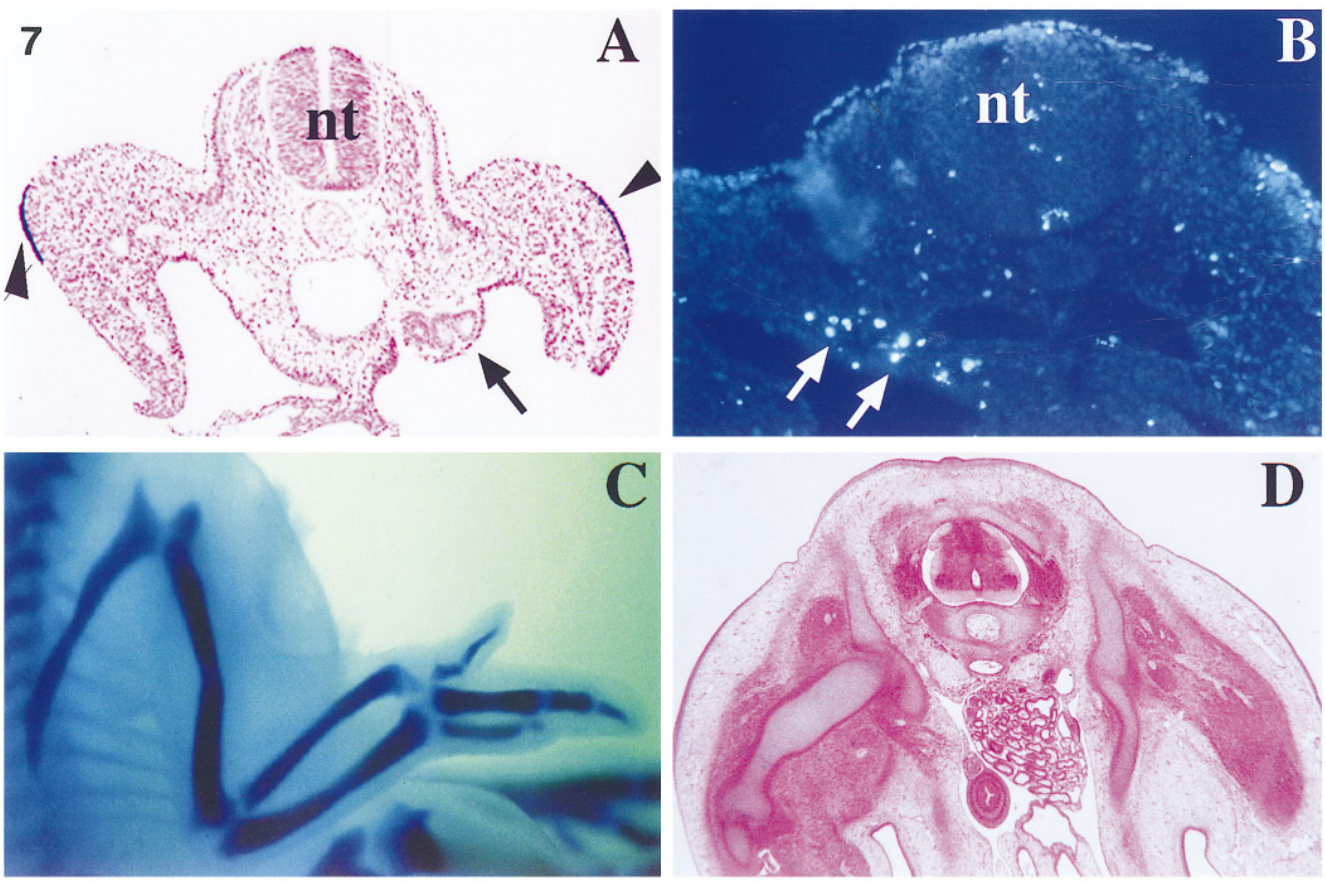


FIG. 6. *Lmx1* expression in the operated embryos. (A) Ventral view of an experimental embryo analyzed for *Lmx1* expression 2 days after the operation. Note that *Lmx1* expression is undetectable in the right mesonephric area, while its expression is normal in the nonoperated left side. (B) This experimental embryo shows a small clump of mesonephric cells expressing *Lmx1* in the operated side at caudal wing level (arrowhead). (C) Shows a ventral view of a control embryo hybridized with the Lmx1 probe for comparison. The barrier is labeled with an asterisk.

FIG. 7. Histology. (A) Transverse section at mid wing level of stage 19 embryo hybridized in whole mount with *fgf-8*. Note the absence of right mesonephros compared with normal on the left side (arrow). Expression of *fgf-8* is normal at both apical ridges, as can be seen

known. Recently, FGF-8 was proposed as the limb-initiation signal (Crossley *et al.*, 1996; Vogel *et al.*, 1996). *fgf-8* is expressed by the mesonephros at the wing level in a pattern spatially and temporally compatible with it being the limb-inducing signal. Furthermore, FGF-8, like other members of the FGF family (Cohn *et al.*, 1995; Mima *et al.*, 1995; Ohuchi *et al.*, 1995), is sufficient to stimulate the development of an extra limb when ectopically applied to the flank region (Crossley *et al.*, 1996; Vogel *et al.*, 1996).

It is known that mesonephric differentiation depends on the inductive interaction of the Wolffian duct (Bishop-Calame, 1965; LeDouarin and Fontaine, 1970). When this interaction is blocked, as in the experiments presented here, *fgf-8* expression is undetectable in the undifferentiated mesonephric mesenchyme. It remains possible that *fgf-8* expression occurs at an extremely low level, under the level of detection of the whole mount hybridization technique, but a more likely explanation is that the activation of *fgf-8* expression by the mesonephric mesenchyme requires the induction of the Wolffian duct. This hypothesis fits very well with the observation that *fgf-8* expression within the normal mesonephric cord occurs in cells close to the duct (Crossley *et al.*, 1996, and unpublished observations).

Our results show that budding and further development of the limbs proceed normally in the absence of mesonephric expression of *fgf-8*. This demonstrates that the mesonephric domain of *fgf-8* expression is not required for the initial outgrowth of the limb and that very likely, FGF-8 is not the postulated limb-inducing signal. It is important to note that in limbs formed in the absence of mesonephric expression of *fgf-8* there is a normal expression of *fgf-8* in the preridge and the ridge ectoderm. This demonstrates that FGF-8 from the mesonephros is not the signal that induces *fgf-8* expression in the limb field ectoderm (cf. Crossley *et al.*, 1996).

We have previously shown that *fgf-8* expression is undetectable in the *limbless* limb ectoderm indicating that this domain of *fgf-8* expression is dispensable for budding (Ros *et al.*, 1996; see also Noramly *et al.*, 1996; Grieshammer *et al.*, 1996). Because *fgf-8* expression in the mesonephros is normal in *limbless* (Ros *et al.*, 1996), it is possible that only one source of FGF-8 (mesonephros or limb field ectoderm) is sufficient for budding. Our data show that *limbless* limb buds emerge in the absence of mesonephric and limb ectoderm *fgf-8* expression. Taken together, this demonstrates that expression of *fgf-8* at the level of the mesonephros and at the level of the limb or prelimb ectoderm are not interdependent and that both domains of *fgf-8* expression appear to be dispensable for the normal emergence of the limb bud.

Our results indicate that FGF-8 is not the limb-inducing signal. It could be argued that *fgf-8* expression by the somitic mesoderm could account for the normal limb development observed in this study. However, it is difficult to assess such a role for *fgf-8* as it, and all FGFs, avidly binds to heparin and they are unlikely to diffuse long distances (Basilico and Moscatelli, 1992). The same line of reasoning would apply for the contralateral mesonephros influencing the limb field on the barrier side as well. However, this possibility is rejected as the initiation of the wing buds is normal in embryos with bilateral blockage of mesonephric differentiation (data not shown).

If limb initiation and development can occur in the absence of a local adjacent source of FGF-8, as we show here, it remains to be explained how a local application of FGF-8 to the flank is sufficient to initiate the whole cascade of events that leads to the development of an extra limb (Crossley *et al.*, 1996; Vogel *et al.*, 1996). It is important to note that several members of the FGF family, including FGF-1, FGF-2, and FGF-4, are also capable of inducing a limb (Cohn *et al.*, 1995), and accordingly another member of the family could be implicated in the induction of the limb. However, to date an appropriate pattern of expression has not been described for any of these molecules. It is worth noting that normal mesonephric *fgf-8* expression occurs at the flank level during periods (i.e., stage 17, see Fig. 4C) in which FGF-8 beads can induce extra limbs in this region. It is possible that the FGF beads induce or mimic the expression of the limb-inducing molecule in a way similar to retinoic acid with the zone of polarizing activity.

It seems reasonable to assume that the same mechanisms would apply for the induction of both (fore and hind) limbs. During the stages before and coincident with the emergence of the leg bud, mesonephric *fgf-8* expression is not detectable at leg levels. FGF-8 from Hensen's node has been suggested for the initiation of the leg bud (Crossley *et al.*, 1996). However, we believe this is unlikely because of the amount of time between the putative induction and emergence of the leg bud. We suggest the initiation of the leg bud also occurs in the absence of a localized source of FGF-8.

We have found that after the barrier experiment a small proportion of the limbs developed with abnormal skeletal pattern. The abnormality obtained was missing anterior cartilage elements (radius and digit 2). Proximal and posterior development of the limb was normal, indicating that limb initiation had not been affected. We interpreted this alteration to be due to a barrier ending up too caudally, at mid wing level, isolating anterior tissues from posterior (Todt and Fallon, 1987; Wilson and Hinchliffe, 1987). At the very least, limb field integrity was disturbed. We have observed that the segmental plate caudal to the formed somites expands considerably as development proceeds. Because of this it is not possible to plot the level or position of a specific

by the blue color (arrowheads). (B) *In situ* detection of DNA breaks by the TUNEL method detected an area of apoptotic cells at the level of the right undifferentiated intermediate mesoderm (white arrow). (C) Normal wing skeletal pattern (Victoria blue staining) of an experimental embryo with confirmed lack of right mesonephros, as illustrated in the transverse section shown in (D). nt, neural tube.

unformed somite using the formed somites as units of measure. A case in point is Smith *et al.* (1996), who blocked Wolffian duct expansion in 12 somite embryos by placing barriers at a level assumed to be prospective somite 14. We have found (unpublished results) that the length of the segmental plate that appears to correspond to two somites (using somite units of measure) at this stage actually develops into 4 to 5 somites. In our hands, a barrier placed as shown by Smith *et al.* (1996) in Fig 1, page 127, usually ends up at a level posterior to somite 14 and as caudal as somite 17–18. Because of this, while the barrier does block Wolffian duct expansion it also interferes directly with development of the limb bud, in some cases probably blocking communication between the anterior and posterior limb bud (Todt and Fallon, 1987; Wilson and Hinchliffe, 1987). This is a reasonable alternative explanation of the abnormal limbs reported by Smith *et al.* (1996) and fits with the data reported here.

Axial Influences in Limb Development

We have shown that normal limb initiation and development occurs without differentiation of the mesonephros. Specifically, limb development does not appear to require *fgf-8* expression at the mesonephric area. It also can be inferred that any molecule expressed by the intermediate mesoderm in response to the Wolffian duct induction is not required for limb development. Discrepancies between our results and those of previous studies (Stephens *et al.*, 1991; Geduspan and Solursh, 1992a) may be due to the different type of manipulation performed. We believe that physical disruption close to the limb field, however minor, as with a barrier or other surgery (cf. Geduspan and Solursh, 1992a), may lead to abnormal limb development. For example, a wound or physical disruption close or within the limb field could interfere with the extensive morphogenetic displacements that prospective limb ectoderm undergoes over the mesoderm (Geduspan and Solursh, 1992b). We stress that these abnormalities probably have nothing to do with the factors controlling limb initiation per se.

Our work does not eliminate the possibility that the putative molecule (other than FGF-8) responsible for the initiation of the limb is expressed by the intermediate mesoderm even when its differentiation is prevented. In our experiment, this hypothetical factor would be expressed before the uninduced intermediate mesoderm cells die. However, we do not favor this possibility because cell death starts during the period when the influence of the mesonephros is supposed to occur.

Whatever the endogenous signal to induce limb development is, it should account for the unique localization of the limbs at two precise levels in the lateral body wall. This could be explained by a localized production of the signal at the appropriate level (as proposed for FGF-8 from the mesonephros) or by a differential competence to respond to the signal by the somatopleure. The flank, although normally not receiving or not being competent to respond to the signal, is capable of forming a limb when provided with exogenous FGFs (Cohn *et al.*, 1995; Vogel *et al.*, 1996; Crossley *et al.*, 1996). Alternatively, both the signal and the competence to respond might be uniform along the anteroposterior axis of the embryo, supposing there is an inhibitory signal at the levels where no limbs develop. This fits well with the reduction in the proliferation index in the flank region during stage 16 (Searls and Janners, 1974) mentioned above.

It remains possible that there are axial influences in limb development (cf. Stephens *et al.*, 1991; Pinot, 1970; Kieny, 1971; see especially Michaud *et al.*, 1997) and the search for the localization and identification of the putative signal(s) should concentrate on medial structures (e.g., Hensen's node, neural tube, paraxial mesoderm) rather than the mesonephros. However, further studies should be designed so that the limb field integrity is not compromised. At the same time, the alternative that no axial influences are required for limb development should also be explored.

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Note added in proof. After this paper went to press, a paper appeared by Ohuchi *et al., Development* **124,** 2235–2244, indicating that FGF10 is the apical ridge inducing factor and probably the ridge maintenance factor.

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