The stages of flank ectoderm capable of responding to ridge induction in the chick embryo

By JILL L. CARRINGTON AND JOHN F. FALLON[1]

Department of Anatomy, The University of Wisconsin,
1300 University Avenue, Madison,
Wisconsin 53706, U.S.A.

SUMMARY

Reports on the stages when chick flank ectoderm can respond to ridge induction are contradictory. Different results have been obtained using presumptive wing or leg bud mesoderm as the inducing tissue with flank ectoderm as the responding tissue. In addition, although incomplete outgrowths have been obtained from recombinants with stage-19 flank ectoderm in a small percentage of cases, no complete outgrowths have been obtained from recombinants with ectoderm older than stage 18. We reinvestigated when chick flank ectoderm can respond to ridge induction and promote outgrowth of complete limbs. To do this, we combined flank ectoderm with *in situ* chick presumptive wing bud mesoderm using a pre-limb bud recombinant technique. When presumptive wing bud ectoderm was removed from the host and not replaced, wing development was suppressed. When host ectoderm was replaced with stage-15 through -18 chick flank ectoderm, limbs grew out in all cases; 86·4% of these recombinant limbs were distally complete. Stage-19 flank ectoderm formed a ridge and promoted limb outgrowth in 80·9% of recombinants; 52·9% of these were distally complete limbs. Recombinants made by grafting early stage-20 (40-somite donor) flank ectoderm to stage-15 hosts resulted in outgrowths in 60% of the cases and 33·3% of these were distally complete. Graft ectoderm from older donors did not respond to inductive mesoderm.

Our results demonstrate that chick flank ectoderm from stage-15 through early stage-20 donors can respond to inductive signals from presumptive wing bud mesoderm to form an apical ridge. This ridge can promote outgrowth of distally complete wings in a substantial proportion of recombinants. This is two stages beyond when the ability to promote outgrowth of distally complete wings appeared to be lost using other methods.

INTRODUCTION

The avian limb bud initially consists of a core of mesoderm capped by ectoderm. A region of specialized pseudostratified columnar epithelium, the apical ectodermal ridge, first becomes apparent in the wing bud ectoderm during stage 18 (Todt & Fallon, 1984). The ridge arises as the result of an inductive action of limb bud mesoderm on the overlying ectoderm (Kieny, 1960, 1968; Saunders & Reuss, 1974). Saunders (1948) and others (Summerbell, 1974; Rowe & Fallon, 1982) have shown that the presence of the apical

[1]Author for reprint requests

ectodermal ridge is necessary for outgrowth of chick limb bud mesoderm and the formation of limb parts. In addition, removal of the ridge between stages 18 and 20 results in death of the underlying limb bud mesoderm cells (Rowe, Cairns & Fallon, 1982). In turn, the mesoderm is responsible for maintaining the ridge in its morphological (Saunders, 1949) and functional states (Zwilling & Hansborough, 1956). Therefore, the outgrowth of the limb is dependent upon a series of interactions between limb bud ectoderm and mesoderm.

The cellular and molecular events which are responsible for each of the tissue interactions are only now beginning to be explored (see for example, Kosher, Savage & Walker, 1981; Solursh, Singley & Reiter, 1981; Reiter & Solursh, 1982). One difficulty in planning studies of limb development at the cellular and molecular level is that the timing of the interactions at the tissue level is not yet clearly delineated. Therefore, we do not know exactly when particular molecular or cellular changes should occur in tests of their involvement in limb development.

Kieny (1968) and Saunders & Reuss (1974) addressed the problem of timing of interactions by studying the stages when limb bud mesoderm was capable of inducing a ridge and when flank ectoderm was capable of responding to induction and subsequently promoting outgrowth of a limb. The general procedure involved implanting different stages of wing or leg bud mesoderm in the flank region of a host so that it came into contact with host flank ectoderm. A ridge was induced in the flank ectoderm (Saunders & Reuss, 1974) and recombinant limbs grew out. Kieny's (1968) data showed that when inductive wing bud mesoderm was implanted in the flank of various stage hosts, the flank ectoderm was responsive to induction only through stage 16. Saunders & Reuss (1974) also implanted wing bud mesoderm and concluded that flank ectoderm lost its capacity to respond to induction by the end of stage 17. The results from these laboratories using wing bud mesoderm are in general agreement.

When Kieny (1968) implanted stage-14 and -15 presumptive leg bud mesoderm (the stages at which the mesoderm was most highly inductive) beneath stage-18 host flank ectoderm, outgrowths were obtained in 50% of the cases. In further tests, she placed presumptive leg bud mesoderm from stage-14 and -15 chicks beneath the flank ectoderm of stage-19 hosts and rudimentary outgrowths developed in 3 of 22 cases. These appeared to be distal foot elements. Fraser & Abbott (1971) made similar presumptive leg bud mesoderm recombinants with stage-19 and stage-20 flank ectoderm and were able to obtain a partial outgrowth in one of 11 cases using stage-19 ectoderm and in one of eight cases with stage-20 ectoderm. These studies indicated that flank ectoderm may be responsive through stage 20 to presumptive leg bud mesoderm in a low percentage of cases. However, the capacity of the ectoderm to produce distally complete limbs apparently declined with age such that stage-17 to -19 flank ectoderm, in combination with leg bud mesoderm, only

permitted the formation of partial limbs (Kieny, 1968). In view of the low number of outgrowths formed, the incomplete nature of the outgrowths, and the apparent discrepancy between the response of flank ectoderm to presumptive leg bud or wing bud mesoderm, the significance of these data is not clear.

We have reinvestigated the question of which stages of flank ectoderm can respond to inductive wing bud mesoderm by forming a ridge and promoting outgrowth of a limb. To do this we have used a pre-limb bud technique for making recombinants of various stages of flank ectoderm with *in situ* presumptive limb bud mesoderm. Our results show the following. First, removal of presumptive wing bud ectoderm at stage 15 suppresses wing development. Second, flank ectoderm retains ridge-forming capacity and the subsequent ability to promote outgrowth of distally complete wings through early stage 20.

MATERIALS AND METHODS

Fertile White Leghorn chicken eggs were obtained from Sunnyside Poultry Farm, Inc. at Oregon, Wisconsin. Fertile quail (*Coturnix coturnix japonica*) eggs were obtained from flocks maintained at the University of Wisconsin. Eggs were incubated for 2 to 4 days in a humidified incubator at 38°C. They were then opened using standard techniques.

To prepare pieces of donor ectoderm, stage-15 through -22 quail or chick embryos were placed in Tyrode's solution. The number of pairs of somites was counted in order to determine the stage of each embryo according to Hamburger & Hamilton (1951). Then the flank regions of both right and left sides opposite somites 21 through 26 were removed and treated with 1% ethylene diamine tetraacetate (EDTA) (Errick & Saunders, 1976) in double-strength calcium-magnesium-free Tyrode's solution, to shorten the time required for EDTA treatment (Kato, 1969). In all cases, ectoderm from limb bud levels was excluded from the graft. Each piece was then placed in cold 10% horse serum in Tyrode's solution and the ectoderm was dissected away as a single sheet using sharpened tungsten needles. The resulting sheets of ectoderm were stained very lightly with Nile Blue A and held in cold serum solution until they were transferred to the host.

Stage-15 chicken embryos were used as hosts. The vitelline membrane was removed over the right side of the embryo and several drops of Tyrode's solution were pipetted over the embryo. The presumptive right wing region was lightly stained with Nile Blue A according to the technique of Saunders & Reuss (1974). The ectoderm was then peeled away to expose bare mesoderm extending from at least the level of somite 14 through the level of somite 21 and from the middle of the somites laterally into the extraembryonic ectoderm on the right side. A minimum area of 1550μm by 500μm of ectoderm was removed. Two pieces of donor flank ectoderm were then pipetted to each host egg and positioned over the presumptive right wing mesoderm of the host.

Care was taken that the two pieces were flat and overlapped slightly to cover all of the presumptive wing region.

In order to confirm that the grafted ectoderm formed the ridge in recombinant limb buds, two types of experiment were done. First, the host embryo was prepared as usual but no donor ectoderm was grafted over the bare mesoderm of the host. Second, stage-15 quail flank ectoderm was grafted to chick hosts and the recombinant bud was stained with the Feulgen reagent and sectioned so that the ridge could be viewed in cross section. With this procedure, the grafted quail ectoderm was easily distinguished from chick ectoderm and mesoderm in the recombinant due to darkly staining clumps of heterochromatin in the quail cell nuclei (Le Douarin & Barq, 1969).

After each operation the host egg was sealed with Scotch-brand 'magic' transparent tape and allowed to develop to stages suitable for sectioning or to 10 to 12 days for examination of cartilage structures. Each operation was checked within one hour of operating and any cases in which the grafted ectoderm did not heal as originally placed were eliminated.

In 47 operations using stage-18 to -22 donors, the ectoderm was marked with carbon particles prior to its isolation and the orientation of each piece on the host after grafting was noted. The orientation of grafted ectoderm on stage-15 presumptive wing bud mesoderm *in situ* had no apparent effect on the polarity of the recombinant limb bud or its ability to grow out. Therefore, subsequent operations were done without regard to orientation of the graft.

Quail–chick recombinants to be sectioned were fixed overnight in a mixture of 70% ethanol, 40% formaldehyde, and glacial acetic acid in a ratio of 17:2:1, and stained *en bloc* with the Feulgen reagent after acid hydrolysis. This method was introduced by Schmuck & Metz (1931) and we have modified it as follows. After fixation, embryos or limb buds were rinsed in 70% ethanol and rehydrated through a series of ethanols to water. They were placed in 5 N HCl for 60 min at room temperature, then stained in Feulgen reagent for 60 min at room temperature. They were rinsed three times for 5 min in a solution of 5 ml 10% sodium metabisulfite, 5 ml 1 N HCl, and 100 ml water, rinsed in water, and subsequently dehydrated, and embedded in paraffin or plastic. Longitudinal sections of the limb bud 5–7μm thick were cut on steel knives so that the ridge was seen in cross section. Chick–chick recombinants to be sectioned were fixed in a mixture of formaldehyde (2%), glutaraldehyde (2·5%), and picric acid (0·02%), buffered with 0·075 M-phosphate buffer to pH 7·2 (Fallon & Kelley, 1977). They were postfixed in osmium tetroxide, dehydrated and embedded in plastic. Sections 1–2μm thick were cut and stained with azure II-methylene blue (0·5%). For examination of whole cartilage structures, embryos were fixed in 10% formalin and stained with Victoria blue. Some were cleared using methyl salicylate.

RESULTS

Initial experiments

In twenty operations, no graft of ectoderm was placed on the host mesoderm after the host ectoderm was removed from the right side. In most cases there was no limb bud on the right side by 40 h after the operation. By 10–12 days none of the twenty had formed a distally complete right wing. Four of the 20 had formed a distally incomplete outgrowth while 16 of 20 formed no right wing outgrowth at all (Table 1). Therefore, in only 20% of the cases was the host ectoderm able to heal over the graft site, respond to the inductive wing bud mesoderm, and promote wing bud outgrowth.

It is possible that at some point host ectoderm could replace the grafted chick ectoderm in our recombinants. Since we would be unable to detect this, we used the 20% outgrowths obtained through simple healing over of host ectoderm as a baseline for comparison of the number of outgrowths obtained with grafted ectoderm. When the proportion of outgrowths obtained from recombinant buds fell to 20% or below, the outgrowths were considered to be the possible result of such a replacement and the capacity of the test flank ectoderm to form a ridge was assumed to be lost.

In order to learn more about why limbs failed to grow out in the initial experiments, ectoderm was removed from 23 embryos and the embryos were fixed at 4·5 h (two embryos), 6 h (four embryos), 7·5 h (three embryos), 10 h (three embryos), 12 h (three embryos), 18 h (three embryos), 22 h (two embryos), and 24 h (three embryos) after operating, stained, embedded in

Table 1. *Grafts of chick flank ectoderm*
to stage-15 chick hosts

Ectoderm donor stage (No. of somites)	N	Distally complete*	Proximal elements formed	No outgrowth	% outgrowths
15 (24–27)	5	5	–	–	100
16–17 (26–32)	6	5	1	–	100
18 (33–36)	11	9	2	–	100
19 (37)	10	5	5	–	100
19 (38)	20	10	5	5	75
19 (39)	12	3	6	3	75
19–20 (40)	10	2	4	4	60
20 (41)	6	–	1	5	16·7
20 (42)	5	–	–	5	0
21–22 (43–46)	13	–	2	11	15·4
No graft (control)	20	–	4	16	20

*A recombinant limb was counted as distally complete if at least two complete digits were formed in addition to proximal elements.
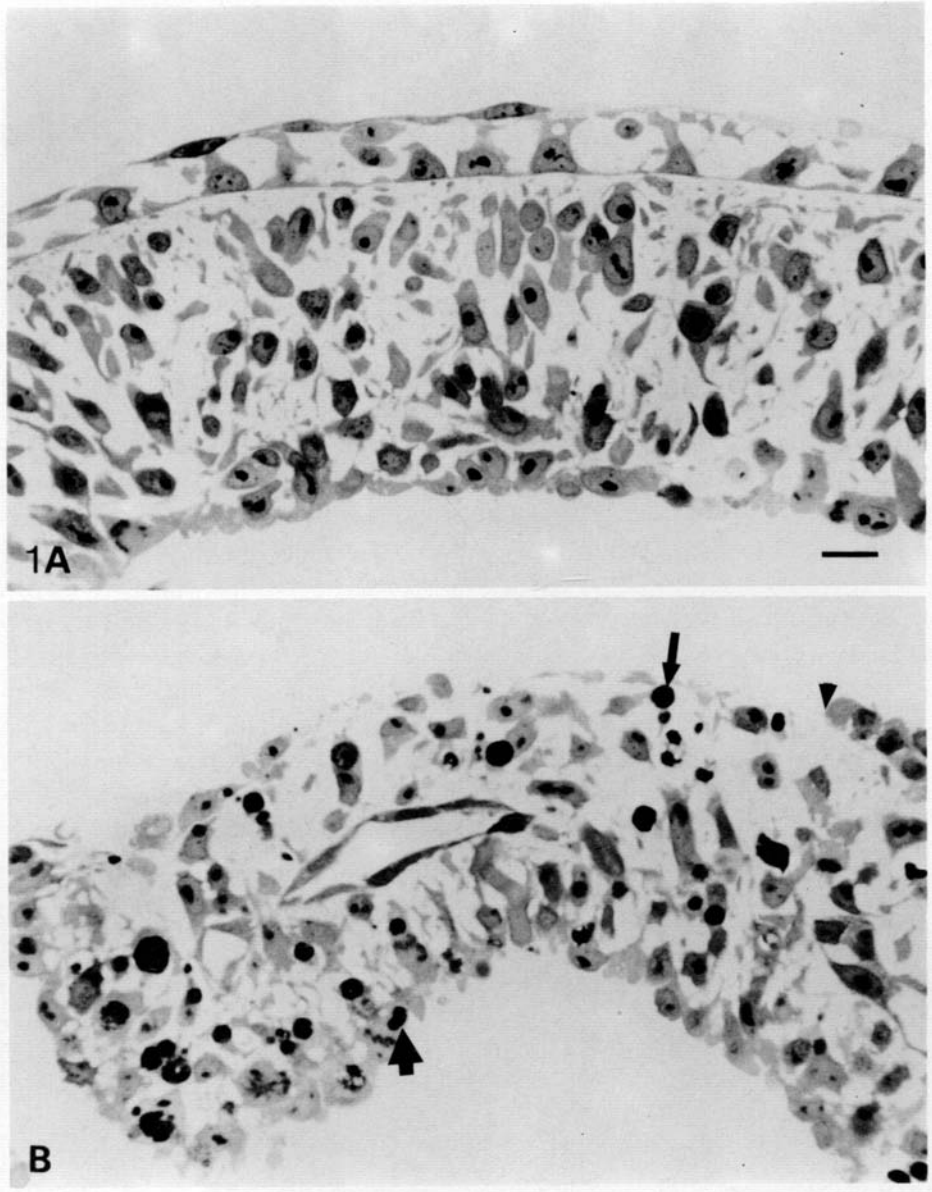
Fig. 1. Left and right presumptive wing bud mesoderm 6 h after ectoderm removal from the right side. (A) Section through the unoperated left somatopleure at the presumptive wing bud level. The ectoderm is intact and the mesoderm appears healthy. (B) Section through the right somatopleure at a level comparable to Fig. 1A. The ectoderm has not healed over the wound. The cells of the mesoderm are dying (arrows) both at the wound surface (small arrow) and in the region which will line the future coelom (large arrow). Blood vessels are prominent. The edge of the healing ectoderm is to the right (arrowhead). Scale bar = 10 μm.

paraffin or plastic, and serially sectioned. By 4·5 h, the cells of the mesoderm in the area of ectoderm removal appeared to be more densely packed than in comparable areas on the left side from which ectoderm had not been removed. The most striking finding was that there was a small amount of cell death in the mesoderm which increased so that there was massive cell death at 6 h (Fig. 1). Necrosis at this time was generally confined to the area denuded of ectoderm. The mesoderm was still massively necrotic at 7·5 h and the ectoderm had not yet healed over the wound. The cells in the healing ectoderm at the edges of the wound were very densely packed and frequently the ectoderm was very thick and irregular. Healing appeared to be nearly complete by 10·5 h and the wound was healed by 12 h. Frequently, healing took place so that the underlying mesoderm was folded back on itself and very distorted. Between 7·5 h and 18 h, cell death continued and extended medially through the mesoderm to involve part of the adjacent somite. It also appeared to involve cells more deeply located in the somatopleure, but fewer cells near the healing ectodermal surface. At 18 h, the cells of the mesoderm were sparsely distributed beneath the ectoderm when compared with the unoperated left side. By this time a limb bud with a developing ridge had formed on the unoperated left side but not on the right side. At 22 to 24 h, the healed ectoderm had a more normal appearance, being composed of mostly cuboidal to columnar cells, and mesodermal cell death was generally sharply reduced. None of the five specimens examined histologically at 22 to 24 h had a limb bud on the operated side but the left limb bud was well developed.

Ectoderm grafts

Observations on the experimental procedure.

Ectoderm which was fixed immediately after EDTA and 10% serum treatments was sectioned and stained. The morphology was somewhat altered. Most of the intercellular space was obliterated, and rounded cells filled with vacuoles were present. In addition, there were large clumps of heterochromatin in the nuclei (Fig. 2A). After grafting, pieces of ectoderm generally adhered to the host mesoderm almost immediately and stayed flat on the host as placed. In most cases, the ectoderm had healed in place by 30 minutes after grafting. Two recombinants were fixed and sectioned at this time. Both ectoderm and mesoderm appeared healthy in these recombinants. The ectoderm was thick but had a nearly normal appearance with cuboidal to columnar cells and large intercellular spaces (Fig. 2B). Additional recombinants were fixed at 6 h (two embryos) and 10 h (one embryo). There was no cell death in the mesoderm under the grafted ectoderm in any of these recombinants. By 40 h after the grafts were made, recombinant buds had formed in most cases. Recombinant buds were usually normally shaped and did not noticeably lag in development behind the stages expected for the total incubation time.
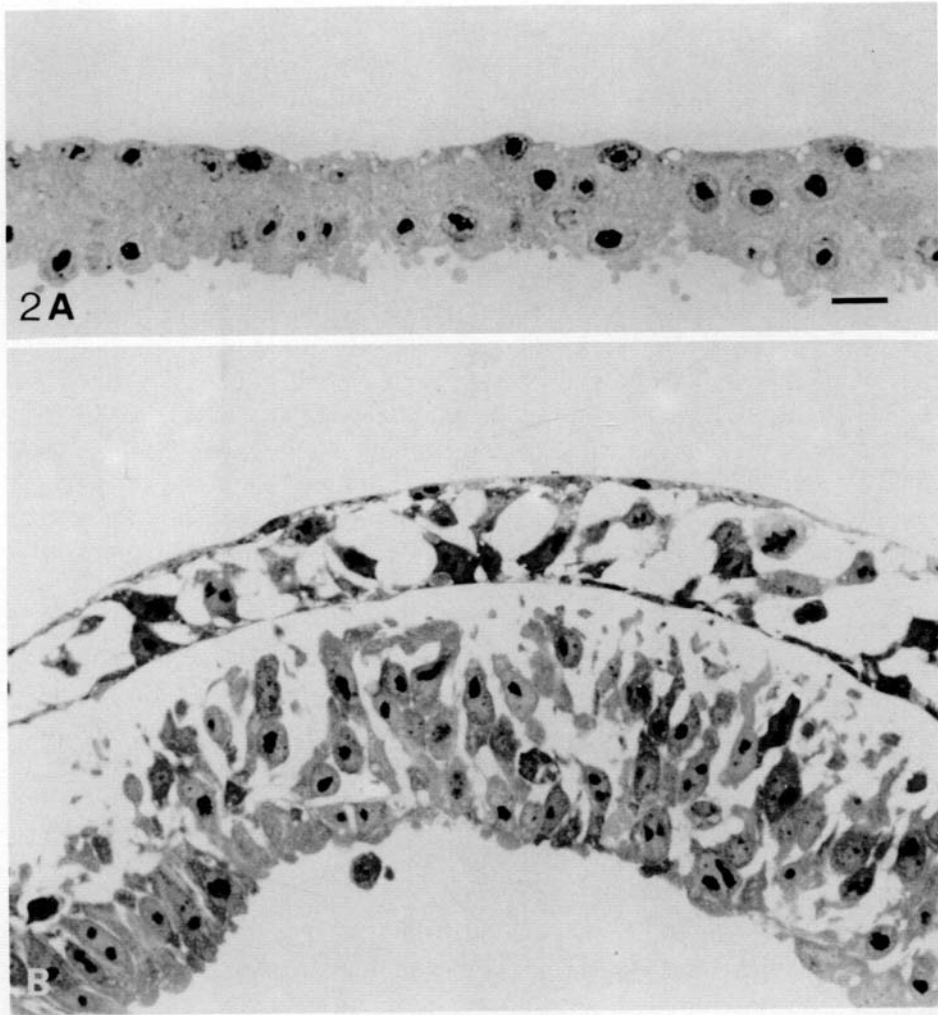
Fig. 2. (A) Flank ectoderm immediately after EDTA treatment and separation from underlying mesoderm. Most of the intercellular space has been obliterated. Large clumps of heterochromatin have formed in the nuclei and cells have vacuoles in them (cf. ectoderm in Fig. 1A). (B) Flank ectoderm on presumptive wing bud mesoderm 30 minutes after grafting. The ectoderm has a more normal morphology with cuboidal to columnar cells and large intercellular spaces. The large clumps of heterochromatin are no longer seen in the nuclei of cells in the ectoderm. Scale bar = 10 μm.

The graft ectoderm always appeared to be free of adhering mesoderm when viewed in the dissecting microscope. This was confirmed by sectioning nine quail–chick recombinant wing buds and examining every fifth section to determine the location of the grafted quail cells. No quail cells were seen in the mesoderm in any of the nine buds. In all nine recombinant buds the grafted
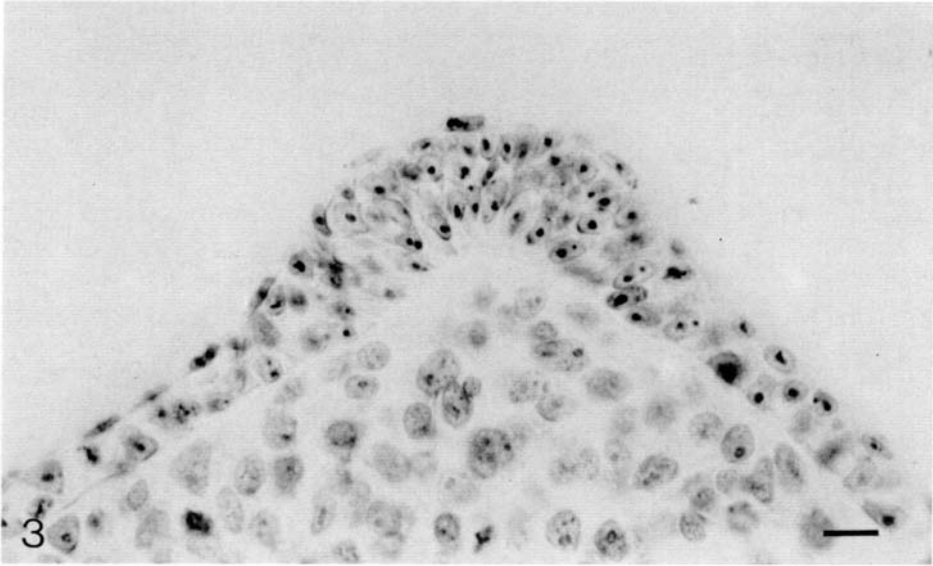
Fig 3. Section of a quail flank ectoderm-chick host recombinant wing bud fixed two days after grafting and stained with the Feulgen reagent. Quail cells can be easily distinguished due to the large clumps of heterochromatin in their nuclei. The grafted quail ectoderm has formed the entire ridge and no quail cells are found in the mesoderm. Scale bar = 10 μm.

quail ectoderm formed the entire ridge and also made up at least the distal dorsal and ventral ectoderm (Fig. 3). Therefore, ectoderm grafted according to our procedure forms the apical ectodermal ridge in the recombinant limb bud.

Grafts of chick flank ectoderm to chick hosts.

We made a series of grafts of chick flank ectoderm to stage-15 presumptive wing bud mesoderm of chick hosts to determine which stages of flank ectoderm were competent to promote wing bud outgrowth in response to the inductive signal of presumptive wing bud mesoderm. For data analysis we described wings with humerus, zeugopodium (forearm) elements, and at least two complete digits as distally complete because they contained representative elements of all proximodistal levels of the wing. Most wings which formed complete digits had either two or three digits. Two of the 41 outgrowths with a complete digit had only a single complete digit and both of these digits were poorly formed. Twenty-eight outgrowths had all three digits and eleven had two digits. Digit **2** was most frequently absent; seven of the outgrowths with two digits were missing digit **2**. The tendency for a single digit to be missing did not increase with age of the grafted ectoderm.

Twenty-six recombinant wings were distally incomplete. Eight of these were composed of a partial or complete humerus. Eleven distally incomplete wings

had a humerus and partial or whole zeugopodium (forearm) elements, and seven distally incomplete wings had proximal elements and partial digits or one digit. There was no tendency to form a particular digit. With regard to stage of the ectoderm graft and the level of truncation of the resulting recombinant wing, the three incomplete wings formed after grafts of stage-16 to -18 ectoderm each had at least one partial digit. In contrast, using stage-19 ectoderm, four of sixteen distally incomplete outgrowths formed partial digits. Incomplete outgrowths obtained after older ectoderm grafts were truncated at random levels proximal to the manus. We stress there were no cases of distal elements which formed without the formation of more proximal elements.
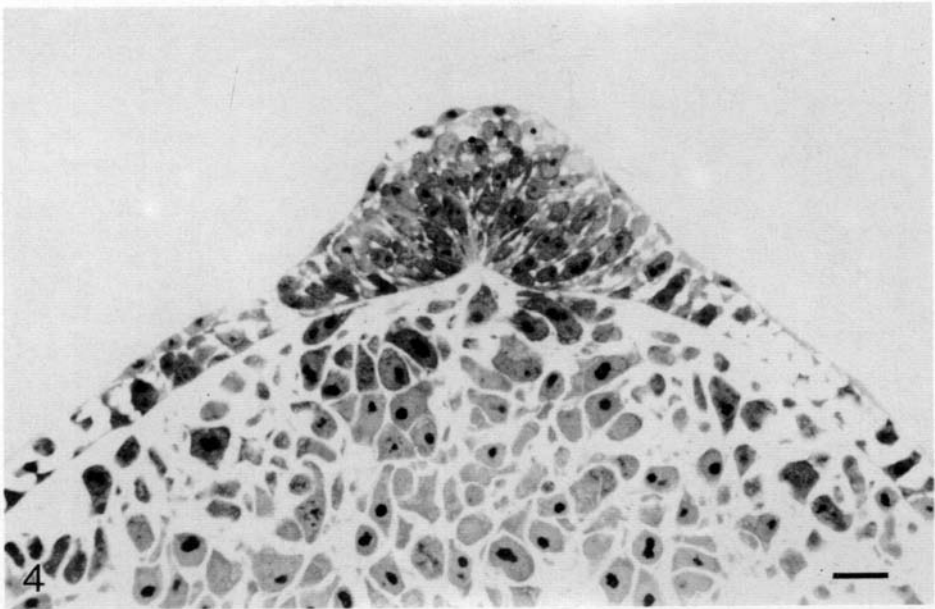

Fig. 4. Section of a recombinant wing bud made with stage-19 (38-somite) chick donor flank ectoderm on a stage-15 host. A normal ridge has formed and the limb bud mesoderm appears healthy. Scale bar = 10 μm.

Flank ectoderm from stage-15, -16, -17, and -18 chick donors was capable of forming an outgrowth in combination with stage-15 right wing bud mesoderm in 100% of the cases (Table 1). Most of these (19 of 22) were distally complete wing outgrowths.

Stage-19 flank ectoderm was capable of forming a normal ridge on an outgrowth of healthy wing bud mesenchyme (Fig. 4). Early stage-19 (37 somite) ectoderm, when combined with stage-15 presumptive wing bud mesoderm, still had the capacity to respond to mesodermal induction and promote outgrowth in 100% of the cases. Five of ten recombinant wings made with ectoderm at this stage were distally complete. Because the response of flank

ectoderm appeared to diminish during stage 19, four groups of operations were done with ectoderm from donors with 37, 38, 39, and 40 somites. The proportion of outgrowths obtained using ectoderm from stage-19 donors declined with age so that with late stage-19 (39 somites) flank ectoderm, outgrowths were obtained from 75% (nine of twelve) of the recombinants (Table 1) and three of the nine outgrowths were distally complete (Fig. 5).

Late stage-19 to early stage-20 (40 somites) ectoderm formed an outgrowth in combination with wing bud mesoderm in 60% of the cases (six of ten). Two of the six outgrowths were distally complete. During the rest of stage 20 and at all later stages tested, the grafted ectoderm was unable to promote wing bud outgrowth. In fact, the number of outgrowths using flank ectoderm at these stages was below the number (20%) which could be accounted for by rapid healing over of host ectoderm. When ectoderm from donors with 41 through 46 somites was used, only three of 24 recombinants formed an outgrowth (Table 1). In these three cases only the humerus and part of the radius or ulna formed.
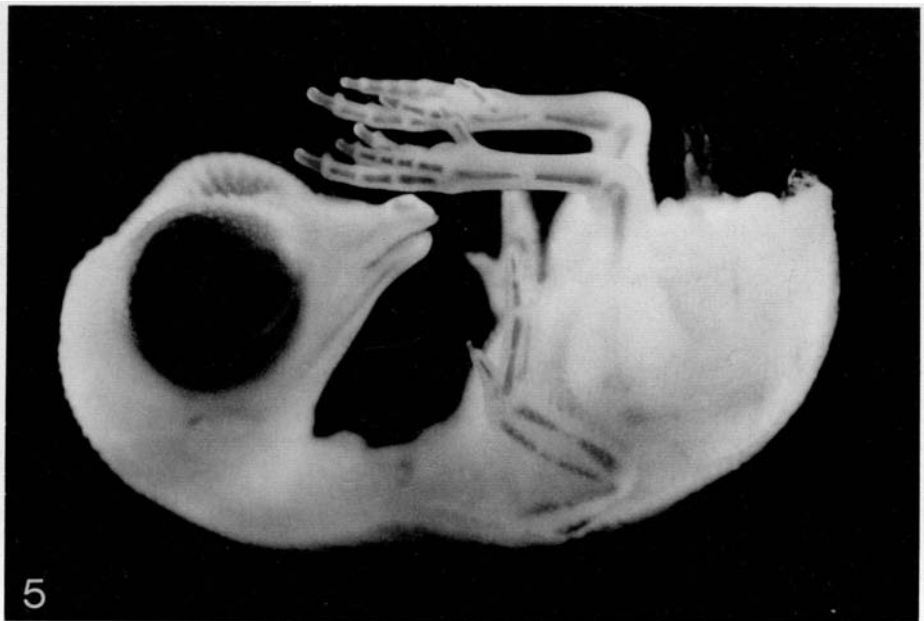

Fig. 5. Chick host with a recombinant right wing resulting from a graft of stage-19 chick flank ectoderm (39-somite donor). This wing is distally complete.

DISCUSSION

By making early recombinants, we have shown that flank ectoderm can respond to presumptive wing bud mesoderm and form a ridge through early stage 20. In fact, the capacity of flank ectoderm to respond to ridge induction

remains high (100 outgrowths) into early stage 19 (37 somites). Stage 19 appears to be a time of transition during which a declining number of grafts of flank ectoderm are capable of responding to inductive presumptive wing bud mesoderm by forming a ridge and promoting outgrowth of a limb. After early stage 20 (40 somites), the capacity of flank ectoderm to form a ridge appears to be lost or completely suppressed.

The ability of stage-18, -19, and early stage-20 flank ectoderm to form a ridge in response to wing bud mesoderm using our recombinant technique contrasts with results obtained by both Kieny (1968) and Saunders & Reuss (1974). Saunders & Reuss (1974) concluded "The competence of the flank or wing bud ectoderm to respond to induction by wing bud mesoderm is . . . lost at stage 17, according to results obtained by means of our technique" (p.48). Thus, the loss of ridge-forming capacity of limb and flank ectoderm was thought to be coincident and to occur within a few hours of limb bud formation (Saunders, 1977). Our data show that flank ectoderm does not lose ridge-forming capacity at this time. Therefore, flank and wing bud ectoderm may differ with regard to the times of ridge-forming capacity.

There may be a simple explanation of why our results differ from those obtained when presumptive wing bud mesoderm was grafted to the host flank (Kieny, 1968; Saunders & Reuss, 1974). Saunders & Reuss (1974) point out that healing of host flank ectoderm over the graft of limb mesoderm was necessary for subsequent ridge induction; the healing time was between 9 and 19 hours. In their experiments there appears to have been a lag time before the grafted mesoderm could interact with host flank ectoderm. It is likely that a stage-18 host could be at stage 20 before the flank ectoderm healed in place. Certainly a stage-19 host would be well into stage 20 before possible limb-mesoderm–flank-ectoderm interactions could occur. Therefore, in a host after stage 17, the healing of host flank ectoderm might not have been completed until a time when our data indicate the host ectoderm does not respond to ridge induction, namely during stage 20 (a later than 40-somite embryo). This would result in the conclusion that after stage 17 the flank ectoderm is no longer competent to form a ridge. Of course, two factors are assumed in this analysis. One is that the healing of the flank ectoderm must be completed before it can respond to the grafted mesoderm. The second is that the healing ectoderm ages in a similar fashion to the rest of the ectoderm. An alternative explanation for the different results between the two methods is that manipulation of the prospective limb mesoderm may have resulted in less than full expression of its inductive capacities.

In our experiments the grafted ectoderm appears nearly normal 30 minutes after placement, suggesting that any healing lag time is very short. However, we do not know when the presumptive limb mesoderm gives the inducing signal, and it is possible that some time elapses between placement of the graft and actual induction of a ridge by the mesoderm. A reasonable test of this

would be to use an older host. However, due to the developing curvatures of the host embryo after stage 15, and increased adherence of host ectoderm to the underlying mesoderm, this is a difficult set of experiments. Nevertheless, the precise timing of ridge induction in the embryo should be investigated.

Some of our recombinant operations resulted in the outgrowth of incomplete limbs. Rubin & Saunders (1972) showed that a stage-28 apical ectodermal ridge could promote outgrowth of complete limbs when combined with early mesoblasts. Therefore, if a ridge formed by flank ectoderm is comparable to that formed by presumptive limb bud ectoderm, any ridge which formed from the stages of ectoderm used in our experiments should have been able to promote formation of complete limbs. However, some limbs formed which were truncated at various proximal–distal levels. This result is consistent with the observations of previous investigators (Kieny, 1968; Fraser & Abbott, 1971; Saunders & Reuss, 1974) on recombinants of flank ectoderm and presumptive limb bud mesoderm. At present it is not clear why partial limbs are formed in recombinant experiments. There was no correlation between the level of truncation of a recombinant wing and the stage of the grafted ectoderm. However, our data show a tendency for recombinants made with stage-19 ectoderm to form fewer complete limbs than recombinants made with younger ectoderm. In addition, there is a general trend towards elimination of digit formation in recombinants made with progressively older ectoderm. Therefore, the phenomenon of partial limb formation may be age related. The fact remains that the formation of any limb structures from a recombinant made with stage-15 presumptive limb bud mesoderm and flank ectoderm indicates that a ridge was induced in that ectoderm.

In recent preliminary work reported by MacCabe *et al.*, (1983) when pre-limb-bud-stage recombinants were made according to the method of Zwilling (1955) using presumptive leg bud mesoderm and ectoderm, the orientation of the ectoderm influenced the dorsoventral polarity of the resulting leg. This method involved isolating both mesoderm and ectoderm, recombining them, and grafting the recombinant to a host. Although we did not do an extensive study, we detected no influence of the orientation of flank ectoderm on the polarity of recombinant limbs. However, as MacCabe *et al.*, (1983) discuss, there can be considerable differences in results generated using different methods. For example, when flank ectoderm healed over dorsoventrally reversed implanted mesoderm, the resulting limb had the polarity of the mesoderm (Saunders & Reuss, 1974). The presumptive wing bud mesoderm in our studies was left *in situ* and combined with flank ectoderm which was in the form of intact sheets. Using this method, the polarity of our recombinants matched that of the host mesoderm.

We found that when ectoderm was removed from the presumptive wing region of chick embryos at stage 15, the wing failed to grow out in the majority of cases. It is of interest to note that when Kieny (1968) removed the

presumptive limb bud ectoderm from the leg region of stage 14 through 17 embryos, she saw healing of the ectoderm in 6 h and outgrowth of normal limbs. Because we removed the ectoderm from a larger area (approximately 1550μm by 500μm as opposed to 1000μm by 400μm), healing was not complete until 12 h in our experiments. By this time (late stage 17) the mesoderm was approaching the limit of its capability for ridge induction (Saunders, 1977). In addition, we saw extensive cell death in the mesoderm over which ectoderm had not healed. This indicates that stage-15 presumptive wing bud mesoderm may require an overlying ectoderm for its survival and subsequent outgrowth. Early limb bud mesoderm (stage 18–20) has been shown to be dependent on the presence of an apical ectodermal ridge for survival (Rowe *et al.*, 1982). The cell death we observed may have been due to a similar mesoderm–ectoderm dependence. Further experiments are needed to determine whether this is the case.

The technique which we have used allows us to make limb recombinants by grafting ectoderm to *in situ* pre-limb-bud-stage mesoderm. This method yields 100% distally complete outgrowths using stage-15 chick flank ectoderm on stage-15 presumptive wing bud mesoderm. The recombinant limbs develop from cleanly prepared ectoderm and mesoderm as shown by quail–chick recombinants. The method is readily applicable to recombinant work with mutants or other problems involving heterotopic or heterotypic ectoderm grafts.

We have demonstrated that the capacity of chick flank ectoderm to form a ridge which promotes outgrowth of complete wings lasts beyond stage 17 to stage 20. This information is valuable for tests of ridge-forming capacity in limb mutants whose phenotype is not detectable until after early limb bud stages. In addition, comparisons between ectoderms with different ridge-forming capacities may aid in future searches for the molecular or cellular mechanisms involved in ridge formation. We hope the more complete knowledge of the timing of ridge-forming capacity in flank ectoderm may aid in such comparisons.

REFERENCES

ERRICK, J. E. SAUNDERS, J. W., JR. (1976). Limb outgrowth in the chick embryo induced by dissociated and reaggregated cells of the apical ectodermal ridge. *Devl Biol.* **50**, 26–34.

FALLON, J. F. & KELLEY, R. O. (1977). Ultrastructural analysis of the apical ectodermal ridge during vertebrate limb morphogenesis. II. Gap junctions as distinctive ridge structures common to birds and mammals. *J. Embryol. exp. Morph.* **41**, 223–232.

FRASER, R. A. & ABBOTT, U. K. (1971). Studies on limb morphogenesis. VI. Experiments with early stages of the polydactylous mutant eudiplopodia. *J. exp. Zool.* **176**, 237–248.

HAMBURGER, V. & HAMILTON, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.

KATO, Y. (1969). Epithelial metaplasia induced on extraembryonic membranes. I. Induction of epidermis from chick chorionic epithelium. *J. exp. Zool.* **170**, 229–252.

KIENY, M. (1960). Rôle inducteur du mésoderme dans la différenciation précoce du bourgeon de membre chez l'embryon de Poulet. *J. Embryol. exp. Morph.* **8**, 457–467.

KIENY M. (1968). Variation de la capacité inductrice du mésoderme et de la compétence de l'ectoderme au cours de l'induction primaire du bourgeon de membre, chez l'embryon de Poulet. *Archs Anat. Microsc. Morph.* **57**, 401–418.

KOSHER, R. A., SAVAGE, M. P. & WALKER, K. H. (1981). A gradation of hyaluronate accumulation along the proximodistal axis of the embryonic chick limb. *J. Embryol. exp. Morph.* **63**, 85–98.

LE DOUARIN, N. & BARQ, G. (1969). Sur l'utilisation des cellules de la Caille japonaise comme 'marquerurs biologiques' en embryologie experimentale. *C.r. hebd. Séanc. Acad. Sci., Paris D* **269**, 1543–1546.

MACCABE, J. A., LEAL, K. W. & LEAL, C. W. (1983). The control of axial polarity: A. Low molecular weight morphogen affecting the ectodermal ridge. B. Ectodermal control of the dorsoventral axis. In *Limb Development and Regeneration, Part A* (eds., J. F. Fallon & A. I. Caplan), pp. 237–244. New York: Alan R. Liss, Inc.

REITER, R. S. & SOLURSH, M. (1982). Mitogenic property of the apical ectodermal ridge. *Devl Biol.* **93**, 28–35.

ROWE, D. A. & FALLON, J. F. (1982). The proximodistal determination of skeletal parts in the developing chick leg. *J. Embryol. exp. Morph.* **68**, 1–7.

ROWE, D. A., CAIRNS, J. M. & FALLON, J. F. (1982). Spatial and temporal patterns of cell death in limb bud mesoderm after apical ectodermal ridge removal. *Devl Biol.* **93**, 83–91.

RUBIN, L. & SAUNDERS, J. W., JR. (1972). Ectodermal–mesodermal interactions in the growth of limb buds in the chick embryo: constancy and temporal limits of the ectodermal induction. *Devl Biol.* **28**, 94–112.

SAUNDERS, J. W., JR. (1948). The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. *J. exp. Zool.* **108**, 363–404.

SAUNDERS, J. W., JR. (1949). An analysis of the role of the apical ridge of ectoderm in the development of the limb bud in the chick. *Anat. Rec.* **105**, 567–568.

SAUNDERS, J. W., JR. (1977). The experimental analysis of chick limb bud development. In *Vertebrate Limb and Somite Morphogenesis* (eds., D.A. Ede, Jr., R. Hinchliffe & M. Balls), pp. 1–24. Cambridge: Cambridge University Press.

SAUNDERS, J. W., JR. & REUSS, C. (1974). Inductive and axial properties of prospective wing-bud mesoderm in the chick embryo. *Devl Biol.* **38**, 41–50.

SCHMUCK, M. L. & METZ, L. W. (1931). A method for the study of chromosomes in entire insect eggs. *Science* **74**, 600–601.

SOLURSH, M., SINGLEY, C. T. & REITER, R. S. (1981). The influence of epithelia on cartilage and loose connective tissue formation by limb mesenchyme cultures. *Devl Biol.* **86**, 471–482.

SUMMERBELL, D. (1974). A quantitative analysis of the effect of excision of the AER from the chick limb-bud. *J. Embryol. exp. Morph.* **32**, 651–660.

TODT, W. L. & FALLON, J. F. (1984). Development of the apical ectodermal ridge in the chick wing bud. *J. Embryol. exp. Morph.* **80**, 21–41.

ZWILLING, E. (1955). Ectoderm–mesoderm relationship in the development of the chick embryo limb bud. *J. exp. Zool.* **128**, 423–442.

ZWILLING, E.& HANSBOROUGH, L. A. (1956). Interaction between limb bud ectoderm and mesoderm in the chick embryo. III. Experiments with polydactylous limbs. *J. exp. Zool.* **132**, 219–240.