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Positional signalling by Hensen's node when grafted to the chick limb bud

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SUMMARY

Hensen's node from stage 4 to stage 10 shows polarizing activity when grafted to the anterior margin of the chick limb bud. It can specify additional digits though its action is somewhat attenuated when compared with the effect of a grafted polarizing region. At stage 10 the activity disappears from the node and is found both posterior to the node and in the future wing region of the flank. The ability of Hensen's node to generate a positional signal suggests that the signal in the limb and early embryo may be similar. The results support the view of the polarizing region as a discrete signalling region.

INTRODUCTION

The polarizing region of the chick limb bud was discovered by Saunders & Gasseling (1968). This region is at the posterior margin of the limb bud and when grafted to a more anterior region can specify the formation of additional structures. For example, if the graft is placed at the anterior margin of the wing bud the typical pattern of digits that forms is **432234** instead of the normal **234**. Results of this type have been interpreted in terms of a signal from the polarizing region specifying positional information along the anteroposterior axis (Tickle, Summerbell & Wolpert, 1975). There is also reason to believe the signal could be a diffusible morphogen. It has been shown that the activity of the polarizing region can be attenuated by a variety of treatments, the most direct being to reduce the number of polarizing cells grafted (Tickle, 1981). A rather different view has been taken by Iten & Murphy (1980), who have interpreted the patterns resulting from grafts of polarizing region in terms of intercalation resulting from regions of differing positional values being placed in apposition along the lines of the polar coordinate model of French, Bryant & Bryant (1976). This interpretation is unlikely in view of the experiments of Honig (1981), who showed that a signal from the polarizing region could be transmitted across leg tissue, and Wolpert & Hornbruch (1981), who showed that an intercalation model was not consistent with the effect of grafting two polarizing regions.

The polarizing region of the limb bud is not the only region with polarizing activity. Saunders (1977) briefly reported that other tissues such as flank, mesonephros and somites from the limb region had polarizing activity, if the tissues

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were placed directly beneath the apical ridge. Tail bud mesoderm has since been found to be a particularly potent source by Saunders & Gasseling (1983), who have suggested that 'posterior positional information may be introduced into the limb field from non-limb sources and can be as effective in eliciting pattern regulation as can the so-called ZPA'.

We have investigated regions of the early embryo for polarizing activity and its development in the very early limb field. Here we report the polarizing activity of Hensen's node. We initially tested Hensen's node because we had shown that it is capable of specifying additional somites when grafted into the early blastoderm (Hornbruch, Summerbell & Wolpert, 1979), and in the belief that positional signalling in different parts of the embryo may have a common basis, we tested it for polarizing activity.

MATERIALS AND METHODS

Host embryos

Fertilized chicken eggs were purchased from a local breeder (Poyndon Farm, Waltham Cross, UK) and incubated at 38 ± 1°C on stationary shelves. Relative humidity was maintained between 50 and 70 %. Eggs were windowed and staged (Hamburger & Hamilton, 1951) on the third day of incubation. Embryos were operated on when they had reached stage 20. Operations were performed using a slightly modified method, first introduced by Saunders (1977), which differs from the usual type of polarizing region graft in which a cube of tissue is removed to make room for the graft. The graft was placed directly under the apical ectodermal ridge. The site for the graft was prepared opposite somite 17 along the anterior margin of the limb. The apical ectodermal ridge was loosened from the underlying mesenchyme without removing any of it. The graft was then pulled through the loop of the ridge. A few drops of Hanks BSS with additional 20 mM-Hepes and 1 % antibiotic–antimycotic solution, containing 10 000 units penicillin, 10 000 mg streptomycin and 25 mg fungizone per ml (Gibco) were added before sealing the egg with Sellotape and returning it to the incubator. Incubation was terminated on the 10th day. Both wings were dissected from the trunk, fixed in 5 % TCA, stained in 0·1 % alcian green, dehydrated and cleared in methyl salicylate to examine the skeletal pattern (Summerbell & Wolpert, 1973).

Donor embryos

Fertilized chicken eggs were incubated for 18–60 h. The eggs were windowed in the usual way and the blastoderms cut from the yolk and placed in sterile phosphate-buffered saline (PBS) Dulbecco 'A' from Oxoid, to remove excess yolk. Blastoderms of the desired stage were pinned out, dorsal side facing the operator under Hanks BSS in a small Petri dish lined with a transparent silicon elastomer, Sylgard 180, from Dow Corning Corporation, Midland, Michigan, USA. The nodes, excised with fine tungsten needles, measured 200 μm in cranial–caudal dimension and 100 μm in medial–lateral direction. These oblong strips of node tissue were then threaded under the loosened apical ectodermal ridge of host embryos at stage 20. The sites from which tissue was taken are shown in Fig. 1.

Assay of polarizing activity

To quantify positional signalling, termed 'polarizing activity', we employed the method described by Honig, Smith, Hornbruch & Wolpert (1981). An extra digit **4** was scored as 100 %, digit **3** as 67 %, and digit **2** as 33 %. Only the highest digit was scored for each graft. The activity for a particular set was taken as the average. A digital plate **43234** would score 100 %. If another graft of the same set gave **234** it would score 0: the average would be 50 %.

RESULTS

The polarizing region of a stage-21 embryo served as a control and had an activity of 87 %. Eight of the ten grafts gave an extra digit **4** (Fig. 2B). The main results of grafts from Hensen's node and adjacent regions from stage 4 to 12 are summarized in Table 1. Table 2 shows the patterns of digits obtained from grafts of Hensen's node. On occasion the grafted node tissue could be seen as a discrete structure in a more proximal position lying anteriorly to the radius (Fig. 2C).

Stage 4

Hensen's node from embryos at the definitive streak stage grafted to a site on the anterior margin of the limb bud at stage 20 resulted in the specification of extra digits **2** and **3**. Most common digit patterns were **3234** or **23334** (Fig. 2G,H). The index of polarizing activity is 48 %. None of the 18 grafts induced a digit **4** next to the graft. Grafts of tissue immediately posterior to the node exhibited no polarizing activity, nor did tissue from other parts of the primitive streak or the area pellucida lateral to the node.

Stage 5, head process

Hensen's node from the head process stage gave similar results to stage 4 with an index of 52 % activity from twelve grafts. The majority of grafts displayed **2234** or **2334** digit patterns and one graft gave **22234**. Grafts from regions anterior to the advancing notochord namely the head process showed little activity, one extra digit **2** from seven grafts. Other regions of the blastoderm were not investigated.

Stage 6, head fold

The results for the head fold stage are in agreement with those of the previous two stages. Node grafts giving digit patterns of **2234**, **23234** (Fig. 2F) were predominant but several grafts showed no activity at all, which is reflected in the lower index for 42 % for twenty-three grafts. Tissue from immediately posterior to the node and lateral regions in the area pellucida were devoid of polarizing activity. Grafts of the head process, however, gave several wings with an additional digit **3** which was reduced in size.

Stage 7, one to three pairs of somites

Node grafts from blastoderms with one to three pairs of somites compare well with the results for stages 4–6 showing digit patterns of additional digits **2** and **3** with the index at 40 %. In accordance to the previous stage most grafts resulted in limbs with digit arrangements **32234** or **2334** and some had no effect. Tissue from the primitive streak adjacent to and posterior of Hensen's node was tested and found inactive. Single somites 1, 2 and 3 were also grafted to the anterior margin of the limb bud but failed to give rise to supernumerary digits.

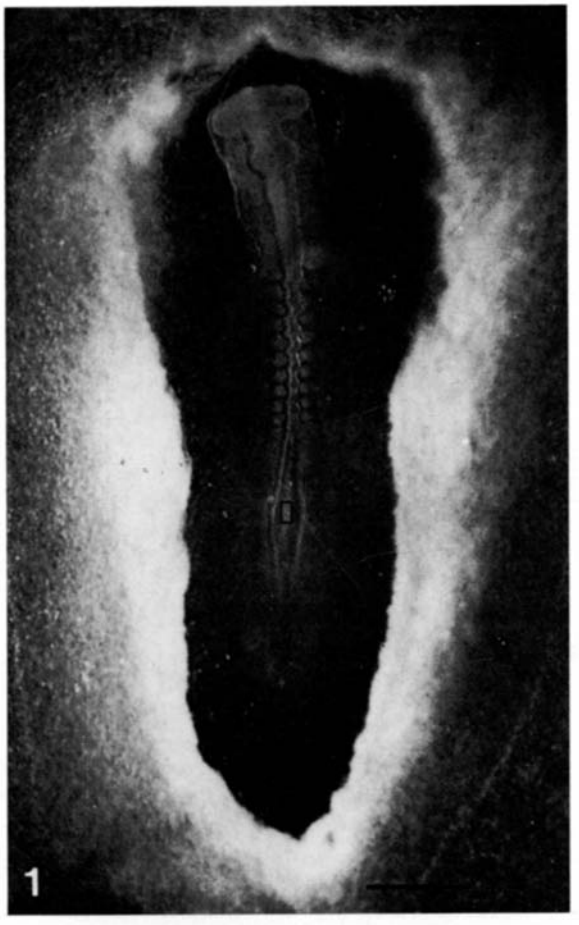
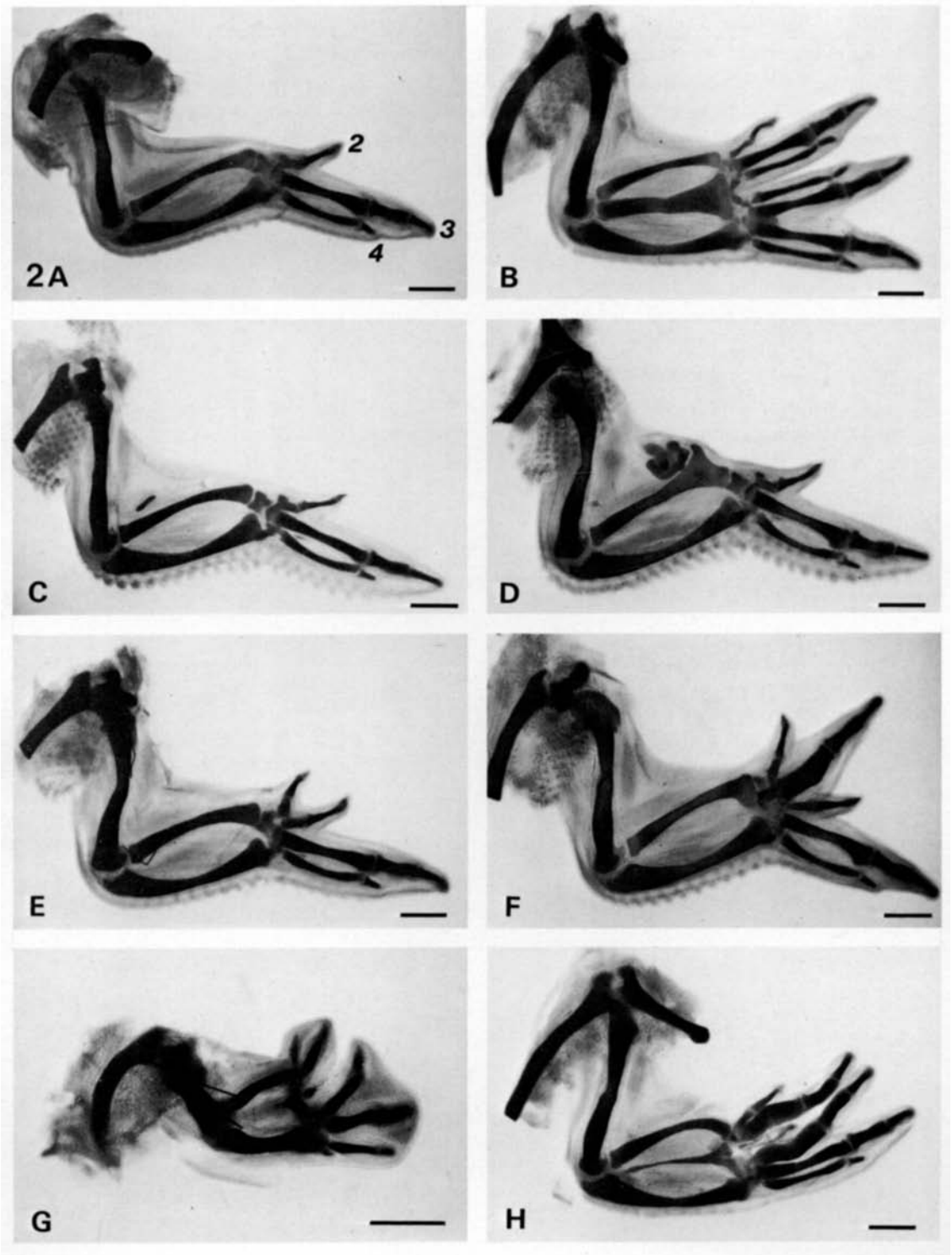


Fig. 1. Dorsal view of a stage-10 embryo with 10 pairs of somites. The graft material used throughout all stages of this experiment is the outlined oblong on the example. Scale bar, 1 mm.

Fig. 2. (A) Dorsal view of a whole mount stained with alcian green of a normal 10-day-old embryonic chick wing with digits **234**. Subsequent figures received the same treatment except when stated. (B) Reduplication pattern **2344334** resulting from a graft of polarizing region from a stage-21 donor and placed under the AER opposite somite 17 of a stage-20 host. (C) No reduplication resulted from this graft of Hensen's node stage 4; however, the graft tissue can be seen as a small cartilage rod anterior to the radius. (D) No reduplication resulted from this graft of somite 1 from a stage-8 blastoderm with four pairs of somites. The somite has undergone selfdifferentiation into a vertebra-like structure. (E) Reduplication of digit **2** following a graft of the node from a stage-8 embryo. (F) Unusual arrangement of additional digits **2** and **3** resulting from a graft of node tissue from a stage-6 blastoderm. (G) Grafts of Hensen's node stage 4 resulting in the digit combination of **23334**. The graft is still clearly visible in both whole mounts between the two anterior digits **3**. (G) was fixed 48 h after the operation to show the position of the graft. (H) was fixed the usual 6 days after the operation. The third element in the zeugopod is not a common feature in this set of results. Scale bar in (A–H), 1 mm.


2
3
4
2A
B
C
D
E
F
G
H

Stage 8, four to six pairs of somites

This stage shows the most consistent results. From a total of eighteen node grafts ten displayed the digit patterns **2234** (Fig. 2E), and only two grafts had an extra digit **3**. The remaining five grafts had no effect. The index was 29 %. Single somites had no activity: in several cases the grafted somite could be seen as a cartilaginous structure (Fig. 2D). No other tissue from the area pellucida was investigated.

Stage 9, seven to nine pairs of somites

Nodes from stage 9 show similar characteristics as described for stages 4–5 with the majority of cases exhibiting an extra digit **3** in the pattern **2334**. The polarizing activity index is 50 %. The notochord anterior to the node was also grafted and found to have a similar activity index of 48 %.

Tissue posterior to the node as well as lateral to it, the region of the prospective flank or wing anlagen, was tested. The activity was low with 25 % but consistent with only digits **2** forming from these grafts.

Stage 10, ten to twelve pairs of somites

Eleven grafts from node tissue were performed and none gave rise to an extra digit. The notochord anterior to the node was tested and gave a similar result.

Table 1. *Grafts of Hensen's node and adjacent regions*

Stage	Graft	No. of grafts	Activity %
4	Node	18	48
5	Node	12	52
	Head process	7	5
6	Node	23	42
	Adjacent node	8	0
	Head process	10	30
7	Node	14	40
	Somites 1–3	18	0
8	Node	18	29
	Somites 4–6	16	0
9	Node	10	50
	Anterior to node	7	48
	Posterior to node	4	16
	Limb region	8	25
10	Node	11	0
	Anterior to node	8	0
	Posterior to node	13	25
	Limb region	24	31
11	Node	8	0
	Posterior to node	8	25
	Limb region	12	20
12	Node	9	4
	Posterior to node	11	55

Table 2. *Pattern of digits resulting from graft of Hensen's node*

Stage	**234**	**2234**	**23234**	**23334**	**3234**	**344334 or 2344334**	Number of grafts	Activity %
4	4	2	3	2	7		18	48
5	1	3		7	1		12	52
6	6	5	5	1	6		23	42
7	4	2		5	3		14	40
8	5	10		3			18	29
9	2	1		7			10	50
10	11						11	0
11	8						8	0
12	8	1					9	4
ZPA	1			1		8	10	87

Tissue posterior to the node showed polarizing activity of 25 %. The lateral prospective wing regions registered 31 % activity. Both regions specified mainly additional digits **2**.

Stage 11, thirteen to fifteen pairs of somites

Stage 11 shows no activity from node or notochord tissue. The area posterior to the node corresponds to the previous stage with 25 %. (The behaviour of the flank tissue will be considered in a later paper).

Stage 12, sixteen to eighteen pairs of somites

Grafts from node tissue fail to give rise to extra digits, except in one case where an additional **2** formed. But the tissue posterior to the node exhibits a polarizing activity of 55 %.

DISCUSSION

The striking result is that Hensen's node has polarizing activity when grafted to the anterior margin of a limb bud, and that this activity disappears with time. Polarizing activity is present in Hensen's node from stage 4, the definitive streak stage, till stage 10 (ten to twelve pairs of somites) at which time it disappears from the node. From stage 10 at least to stage 12 activity is found posterior to the node and in the future wing region of the flank. In the earlier stages the polarizing activity is confined to the node and is absent in adjacent regions with the exception of low levels in the head process. The digits specified by the node are similar to those specified by an attenuated polarizing region (Smith, Tickle & Wolpert, 1978). Only additional digits **2** or **3** were found and in no case did we observe an additional digit **4**. The reason for this could be that the activity in the node is low or that the signal is attenuated when node cells are confronted with limb mesenchyme cells. The pattern of the supernumerary digits is not always of the mirror-image type reduplication familiar with grafts of the polarizing region to the anterior margin opposite somite 16, which typically gives **432234**. The morphology of the

ridge at stage 20 dictated the position for the graft: the ridge has to be strong enough to support the graft, and this was best achieved opposite somite 17 in a stage-20 limb bud. As a result of this there are limbs with digit patterns such as **23234** (Fig. 2F) or **23334** (Fig. 2H) as might be expected.

At stage 10, when activity disappears, the node has regressed to the posterior end of the primitive streak. Although the morphology still suggests the structure of a bulb, it becomes increasingly more difficult to establish the location of the node. Grafts demonstrate no or very low levels of activity in the bulbous region where the notochord has not yet formed. But tissue posterior, as well as lateral, to that exhibits polarizing activity between 25% and 35%. This can be taken to be tissue of the prospective flank and wing region, for the following reasons. Meier & Jacobson (1982) have suggested that the unsegmented somitic mesoderm is already preprogrammed as far as the regressing node into ten to twelve somitomeres, only just visible in three-dimensional scanning electron micrographs. Simple measurements with an eye-piece graticule of posterior somites and relating their size to the total distance of the preceding unsegmented somitic mesoderm of each individual embryo suggests very similar results. Not so reliable, but comparable, results can be achieved with carbon particles placed at recorded distances on the unsegmented somitic mesoderm and then following the segmentation pattern. This brings the region around the node of a stage-9 embryo with seven to nine pairs of somites right to the centre of the prospective wing, namely somites 17 to 21. It is as if the polarizing activity of the node now moves to the future wing region. Its detailed distribution in early limb development is being investigated.

The presence of polarizing activity in the node suggests that the signal from the node that is capable of organizing additional rows of somites (Hornbruch *et al.* 1979) is similar to the signal from the polarizing region. Perhaps embryos are conservative in the number and type of signals they use. Grafts of the polarizing region to the early blastoderm have, however, given negative results.

It is very hard to reconcile our results with a model based on intercalation. Such a model would require that intercalation occurs between posterior graft tissue and the anterior tissue at the graft site. The node in normal development gives rise to the notochord and it is difficult to see how early nodes could be regarded as having posterior positional values. But the most convincing evidence against such a view is that tissue anterior to an early node can signal. Our results thus support the idea of the polarizing region as a discrete source of a signal.

The presence of polarizing activity in the early embryo is consistent with the results of Rudnick (1945). She grafted regions of the early embryo to the coelom and found that limb structures developed from as early as stage 8 or 9, provided that Hensen's node was included in the graft. From stage 9 to 11, if the graft included node and anterior tissue, wing structures developed, but if posterior tissue was taken with the node they gave rise to leg structures.

In a subsequent paper we will describe the polarizing activity of the tissues in and around the presumptive limb field.

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