Development 110, 1271–1284 (1990)
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Epithelial scatter factor and development of the chick embryonic axis

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Summary

Scatter factor, a recently characterised protein secreted by certain embryonic fibroblasts, affects cultured epithelia by increasing cell motility, the breakdown of cell junctions and cell scattering. The process of gastrulation in higher vertebrate embryos, during which the primitive streak forms, involves an epithelial-to-mesenchymal transformation resembling the effects of the factor on cultured cells. The factor was applied locally to chick embryos, using both scatter-factor-secreting cell lines and inert carriers. We found that scatter factor can generate local supernumerary axial structures resembling primitive streak and/or neural plate and conclude that it may have primitive-streak- and/or neural-inducing activity in chick embryos.

Key words: scatter factor, epithelial–mesenchymal interactions, primitive streak, mesoderm, neural induction, embryonic axis, motility factors.

Introduction

Scatter factor is a recently discovered protein, of M_r 62×10^3, which is secreted by some cultured fibroblastic cells and which alters the locomotory behaviour of certain cultured epithelial cells (Stoker and Perryman, 1985; Stoker *et al.* 1987; Stoker, 1989; Gherardi *et al.* 1989). When added to cultured MDCK cells, it causes a rapid increase in spreading and motility, breakdown of existing intercellular junctions and a change from epithelial to a more fibroblastic morphology. It is found in amniotic fluid (Rosen and Goldberg, 1989) and in fetal calf serum, but to a much lesser extent in adult serum (Stoker and Gherardi, 1987), suggesting that its main role may be during embryonic development. It has generated considerable interest among cell biologists because its existence provides a possible molecular basis for processes such as wound healing and transitions between epithelium and mesenchyme during development. However, direct evidence that it plays a role during normal development is lacking.

Among the developmental processes in which scatter factor or similar substances could play a role are those in which epithelial–mesenchymal conversion takes place, such as the invasion of the uterine endometrium during implantation of the mammalian embryo, the dispersion of somites into a mesenchymal sclerotome, the migration of neural crest cells and the de-epithelialisation of the early epiblast to give rise to the mesoderm and the primitive streak. In this paper, we examine the effects of scatter factor on the latter processes in the chick embryo.

The formation of the mesoderm in the chick embryo, as in other higher vertebrates, involves the de-epithelialisation of the upper layer, or epiblast, during which certain cells ingress into the interior of the embryo and eventually coalesce in the posterior (caudal) midline to give rise to the first visible axial structure, the primitive streak (Vakaet, 1984; Bellairs, 1986; Harrisson, 1989). From the primitive streak arise the mesoderm (which gives rise to the skeleton and musculature, the circulatory system and most of the internal organs of the adult) and the definitive endoderm (which gives rise to the gut and contributes to associated organs). It has been known for a long time that the primitive streak is capable of 'homoiogenetic induction', that is, of stimulating its own formation (see Nieuwkoop *et al.* 1985 for review). Thus, if a piece of primitive streak is grafted elsewhere in a host embryo, the epiblast overlying the graft is 'induced' to become primitive streak tissue; this site becomes a site for ingression of cells and a second embryonic axis often results. Middle layer cells can therefore 'autocatalyse' their own formation; this concept was used as the basis of a simple model to account for the changes that occur during early morphogenesis in amniote embryos and to

explain the inhibition of formation of secondary embryonic axes during normal development (Stern, 1984).

The model made one specific prediction: that a local disruption in the continuity of the epiblast in chick embryos at appropriate stages of development should suffice to elicit the formation of a secondary embryonic axis. In this study, we set out to test this hypothesis using scatter factor as a means of disrupting the continuity of the epiblast in a local way. Although the results obtained do not provide conclusive evidence either for or against the prediction, scatter factor can generate supernumerary axial structures resembling a primitive streak and/or a neural plate. We speculate that scatter factor may have primitive-streak- and/or neural-inducing activity in the chick embryo.

Some of the results presented here have been published elsewhere in abstract form (Ireland *et al.* 1987).

Materials and methods

Embryo techniques

Fertile hens' eggs were obtained from H. A. Coppock's Poultry Farm, Carterton, Oxford, and incubated at 38°C until the embryos had reached between stage XI (pre-primitive streak stages in Roman numerals according to Eyal-Giladi and Kochav, 1976) and stage 4^+ (later stages in Arabic numerals according to Hamburger and Hamilton, 1951). The embryos were then explanted in Pannett-Compton saline for whole-embryo culture with the embryo (dorsal side down) attached to its vitelline membrane, which was stretched around a glass ring laid over a pool of thin egg albumen (New, 1955; with minor modifications described previously; Stern and Ireland, 1981).

Grafting operations (see below) were done under Pannett-Compton saline, using tungsten needles, entomological pins (A1) and iridectomy knives (Weck, 15° angle). Different sets of instruments were used for grafting control and experimental material to avoid cross-contamination. The pellet of cells or scatter-factor-containing carrier was inserted antero-laterally to the presumptive host axis, between the upper (epiblast) and lower (hypoblast) germ layers. Occasionally, the hypoblast could not be made to cover the graft completely; however, this did not appear to affect the results. After grafting, the excess saline was removed from the ventral side of the operated embryo and the assembly transferred to a 30 mm Petri dish for further incubation at 37°C in a humid atmosphere. Embryos were grown in these conditions for a further 10–24 h, but each experiment contained batches of control and experimental embryos, which were fixed after the same culture period. After this period, they were fixed in 4 % formaldehyde in phosphate-buffered saline (pH 7.0), transferred to 70 % alcohol, and then stained as whole mounts in 0.1 % Fast Green FCF in alcohol. After washing in absolute alcohol, they were cleared and stored in cedarwood oil. Each embryo was then photographed on PanF film (Ilford).

For histological observation, the stained and cleared embryos were transferred to xylene and embedded in Paraplast. 10 μm sections were cut and mounted on glass slides coated with gelatin-albumin. After air-drying, the sections were dewaxed in xylene, hydrated with a graded series of alcohols and stained in Harris's haematoxylin, washed in tap water, dehydrated with a series of alcohols to xylene and finally mounted using DePeX or Canada balsam. A total of 468 embryos were grafted (194 with cells and 274 with purified factor), of which 177 were processed for histology.

Cell lines

The cell lines used are listed in Table 1. Swiss 3T3 cells and SV40 transformed Swiss 3T3 cells were obtained from Dr C. O'Neill (ICRF, London), BHK-21 cells from Dr A. Brown (MRC Cell Biophysics Unit, London), human dermal fibroblasts from Dr G. Jones (Department of Anatomy, King's College, London), MRC-5 cells from Flow Laboratories, J2 cells from Dr F. Watt (ICRF, London) and *ras*-NIH3T3 cells from Dr C. Marshall (Institute of Cancer Research, London). The MDCK cells used for scatter factor assays were obtained from Dr A. Brown. Most of these cells were grown in Dulbecco's modification of Eagle's medium containing 5 or 10 % foetal calf serum as described before (Stoker *et al.* 1987). BHK cells were grown in Glasgow modified minimal essential medium. Conditioned media were obtained by incubating just confluent cells in serum-free medium for 3 days.

Grafting of cells into embryos

Embryos placed in modified New (1955) culture as described above were used as hosts for grafting a small pellet of cultured cells of various types, selected from the lists published by Stoker and Gherardi (1987) and Stoker *et al.* (1987). Cells to be grafted were washed in 5 ml of warm phosphate-buffered saline containing 0.02 % sodium EDTA (PBS EDTA). This was followed by incubation in PBS EDTA containing 0.05 % trypsin. Most of this solution was then removed, leaving approximately 0.5 ml in which the cells began to detach. 3 ml of fresh medium were then added to inactivate the trypsin and the cells suspended using a Pasteur pipette. The suspension was added to 20 ml of sterile Tyrode's saline in a plastic Universal tube and the cells pelleted by centrifugation in a bench top centrifuge at 800 g. The pellet was resuspended in 2 ml of fresh Tyrode's and an estimate of cell number was made using a haemacytometer (Fuchs-Rosenthal). The cell suspension was transferred to an Eppendorf tube which was centrifuged in a microcentrifuge (MSE) for 20 s at the 3000 revs min^{-1} setting to form a firm pellet which could be displaced with a tungsten needle. The pellet was transferred to fresh Tyrode's on ice until required for grafting. 95–99 % of the cells prepared in this way were found to be viable even after many hours on ice, as judged by their ability to spread when plated in Petri dishes containing culture medium. The pellet of cells was cut into small pieces, each containing 500–5000 cells.

Scatter factor assay

Scatter factor activity was assayed on MDCK cells grown in plates of 96 flat-bottomed tissue culture wells as described previously (Stoker and Perryman, 1985). Briefly, the solution to be tested was added to the first well of a row and then diluted serially in normal medium across the wells (i.e. doubling dilutions) in a volume of 150 μl. 3000 MDCK cells were then added per well in 150 μl of Eagle's medium containing 10 % fetal calf serum. After overnight incubation at 37°C in an atmosphere of 5 % CO_2, the plate was fixed in formol saline for 20 min and stained with Harris's haematoxylin. After washing with tap water and rinsing in distilled water, the plate was air dried and each well examined for scattering as described previously (Stoker and Perryman, 1985). The 'titre' of the scatter factor solution is the maximum

factor by which it can be diluted while retaining an observable effect on MDCK cells in this assay (e.g. 1:64, 1:128, etc.).

Cell shape assay

MDCK cells were dissociated as in the scatter factor assay and plated at a density of 9000 cells cm^{-2}. Dissociated *area pellucida* cells (the majority of which were epiblast cells) were obtained by cutting out the central region from embryos at stages XII–XIV. The pieces were washed in Ca^{2+}- and Mg^{2+}-free Tyrode's and then placed in cell dissociation medium (Sigma) at 37°C. After 15 min, 500 μl of DMEM containing 5 % Fetal Calf Serum (FCS) were added and the cells pipetted up and down to dissociate them. They were then plated at low density, left to spread overnight, and then fixed and stained as described for the scatter factor assay. Measurements of area (A), perimeter (P) and shape factor ($4\pi A/P^2$) were obtained for single cells by using a MOP Videoplan (Kontron). The shape factor gives an indication of the smoothness of the outline of the cell, where a value of 1.0 is a perfect circle and values less than 1.0 indicate a more irregular outline. An image of a single cell was obtained using a 20× objective and an inverted microscope. An overlay facility allowed the outline to be drawn and the parameters computed. Samples of 60–80 cells were used for each treatment. The mean value for shape factor was compared between treatments using the variable:

$$d = \frac{(m_1 - m_2)}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

for comparison of two normal samples (Bailey, 1974), where m_1, m_2=mean (shape factor) for each sample, with standard deviation (s_1, s_2) and n_1, n_2=number of cells constituting each sample.

Purification of scatter factor from ras*-NIH3T3 conditioned medium*

Purification of scatter factor was carried out from serum-free medium conditioned by *ras*-transformed NIH3T3 fibroblasts as described (Gherardi *et al.* 1989). Briefly, serum-free conditioned medium was concentrated about 50-fold on an Amicon P30 membrane, dialysed against 0.05 M MES, 0.25 M NaCl pH 6.0 (MES saline) and cleared by centrifugation and filtration. Up to 200 mg of protein were loaded on a Mono-S column (0.5×5.0 cm, Pharmacia-LKB) equilibrated in MES saline and eluted with a gradient of NaCl (0.25–1.0 M). Fractions containing scatter factor were identified in the MDCK assay, pooled, dialysed and used as a source of partially purified factor for grafting (see below). In several experiments, the Mono-S fraction was purified further by re-chromatography on the Mono-S column after dialysis against MES saline. Serum-free medium from non-secreting NIH3T3 cells (NIH/1, Stoker *et al.* 1987) was processed exactly as the one from the producer clone and used as a control for grafting experiments.

The protein composition of column fractions containing partially purified scatter factor was analysed by SDS–polyacrylamide electrophoresis (Laemmli, 1970) under both reducing and non-reducing conditions. Gels were stained using the Bio-Rad silver staining kit. Protein was measured by absorbance at 280 nm using bovine serum albumin (BSA) as standard.

Grafting of partially purified scatter factor into embryos

In preliminary experiments to test the effect of scatter factor on intact cultured embryos, medium conditioned by MRC5 cells was added to the embryo after it had been set up in New (1955) culture; this treatment did not cause detectable anomalies.

(a) Elvax

The cold-setting plastic, Elvax (kindly supplied by Dr Paul Martin), was used to prepare a polymer containing partially purified scatter factor as described elsewhere for EGF (Murray *et al.* 1983). Briefly, 17 mg BSA (which had been pulverised in a solution in methylene chloride using a Polytron and then dried by heating in a 37°C water bath in a fume cupboard for 30 min) were added to 300 μl of a 10 % solution of Elvax in methylene chloride (made by slowly rotating the plastic in a glass stoppered vial) and mixed thoroughly using a vortex mixer. Then 50 μl of either purified scatter factor in MES saline or 50 μl of this saline alone were added and the solution mixed. This solution was then made to flow by capillarity between a glass slide and a glass coverslip to produce a thin film, and the assembly placed at −20°C to set. The glass coverslip was then removed and the Elvax film dried and then cut with a sharp scalpel blade. Pieces of the plastic containing purified scatter factor or control buffer were then grafted into host chick embryos as described above. In many cases it proved difficult to perform these grafts because of a tendency of the Elvax to float within the saline covering the embryo.

(b) Agar or agarose

Conditioned medium or purified scatter factor was incorporated into a small volume of agar or agarose made with Pannett-Compton saline (pH 7.4); the factor was added to the agar or agarose (1:1 by volume; final concentration of agar or agarose, 1 %) when this had cooled down to about 40°C.

(c) Ion exchange beads other than Mono-S

In preliminary experiments, single ion exchange beads were soaked in medium conditioned by MRC5 cells, in purified scatter factor or in control buffer (see above, under 'Purification of scatter factor'). The factor or control buffer was diluted to working strength using Pannett-Compton saline (pH 7.4) containing a trace of phenol red. The following types of ion exchange beads (all obtained from BioRad) were tested: AG1-X2 formate 100–200 and 200–400 mesh [strongly basic anion exchanger]; AG1-X2 chloride 200–400 mesh; AG4-X4 chloride 100–200 mesh [weakly basic ion exchanger]; AG50W-X2 hydrogen 100–200 mesh [cation exchanger]; BioRex70 100–200 mesh [weakly acidic cation exchanger] and BioRex5 100–200 mesh [intermediate basic anion exchanger]. We found AG1-X2 formate (200–400 mesh) to be the most convenient and effective; these beads were therefore used in subsequent experiments.

(d) Mono-S beads

Mono-S beads, loaded with partially purified serum-free medium from either producer or non-producer cells (NIH/1; see above), were collected from the top of the FPLC column and used for grafting. The beads were washed briefly in MES saline and recovered by centrifugation at 12 000 revs min^{-1} for 5 min in a microcentrifuge. A small group of beads was grafted into a host embryo as described above either directly (the beads were collected and delivered to the embryo using a siliconised Pasteur pipette), or after adsorption onto a single

carrier AG1-X2 formate (200–400 mesh) ion exchange beads, or after incorporation of the Mono-S beads into agarose prepared as described above. In some cases, a trace of phenol red solution was added to the solution containing the control or experimental ion exchange carrier to make it more easily visible during grafting. Several of these experiments were carried out as double-blind trials: scatter factor and control samples were coded by an independent worker and the embryos scored for abnormalities before the code was broken.

Results

Grafting of cells

In initial experiments to assess the effects of scatter factor on early development of axial structures in young chick embryos, small pellets of cells derived from various cell lines (MRC-5 [n=72], J2 [n=33], *ras*-NIH3T3 [n=11], Swiss 3T3 [n=10], SV40-Swiss-3T3 [n=11], BHK21 [n=8], HDF [n=8]; Table 1) were grafted into chick embryos between stage XI and 4^+, and the host embryo cultured for a further 10–24 h. Initially, these cells were chosen as being producing or non-producing on the basis of published results (Stoker and Gherardi, 1987; Stoker *et al.* 1987). However, the cell types used were tested for secretion of scattering activity (Table 1) and retested at the same time as they were grafted.

After the period of further incubation, several unusual anomalies were seen in embryos that had been grafted with scatter-factor-secreting cells (MRC-5, J2, *ras*-NIH3T3). The results are summarised in Fig. 1. The anomalies seen included partial or complete duplications of the embryonic axis (Fig. 2A,B), dramatic bending of the axis (Fig. 2C,D), or failure of the embryonic axis to form ('exogastrulae' and 'other' in Fig. 1B,C). Although failure of development of the embryonic axis and double embryos are seen at low frequency in unoperated embryos and in those grafted with non-secreting cells, the dramatically sharp bending of the embryonic axis is never seen in control embryos.

Embryos grafted with different producing cell lines gave similar results (Fig. 1A), but a higher proportion (11/33; 33%) of those grafted with a mouse cell line (J2) died. The frequencies of the different classes of anomalies obtained with grafts of producing cells are significantly different (χ^2=43, 2 d.f., which corresponds to a probability P<0.001) from those obtained with non-producing cells.

Histological observation of grafted embryos (41 embryos grafted with producers and 14 grafted with non-producers; Figs 3, 4) revealed further details about the type of anomalies seen in the whole-mounted specimens. In those grafted with secreting cells, we observed an accumulation of middle layer cells around the graft even in regions normally devoid of mesoderm (Fig. 3A), while this was not seen in embryos grafted with non-secreting cells (Fig. 4). In some embryos grafted with secreting cells, the epiblast over the graft had morphological features of a primitive streak or of a neural plate (Fig. 3), while these structures were never observed in embryos grafted with non-secreting cells (Fig. 4). Primitive-streak-like structures were characterised by a groove in the upper layer that was continuous with an accumulation of middle layer tissue (Fig. 3A,B), while neural-plate-like structures were characterised by a distinct thickening of the epiblast, a palisade arrangement of cells (Fig. 3C–E) and sometimes a V-shaped morphology (Fig. 3E). In most embryos grafted with secreting cells that were allowed to develop until they had formed somites, the somites on the side that received the graft of secreting cells were more difficult to see (Fig. 2B,D; Fig. 3D) and had often lost their epithelial structure.

The types of anomalies observed varied according to the stage at which the embryos were grafted with producing cells. The results are summarised in Fig. 1C. Some anomalies were more common in embryos grafted at early stages (XII–XIV) of development (e.g. complete duplications of the embryonic axis), while others, such as somite disruption, were seen mainly in later embryos (stages 3^+–4^+).

In an attempt to establish the minimum duration of exposure required to cause the anomalies observed, we grafted a single pellet of MRC5 cells into chick embryos, incubated the grafted embryos for 3.5 h, and then removed the pellet, followed by further incubation of the embryo overnight. The results are shown in Fig. 1A. Three of the embryos operated in this way were processed for histological examination. No residual grafted cells were seen in these embryos. Since both the proportion of abnormal embryos and the nature of the abnormalities are not significantly different from those seen when the graft was not removed (comparison of MRC-5 cell grafts and the cells removed after 3.5 h yields χ^2=2.9, 2 d.f., which corresponds to a probability P>0.05), we conclude that 3.5 h of exposure are sufficient to cause the effects observed.

Table 1. *Cell lines used and the relative scatter factor activity of their conditioned medium*

Cell line/strain	Activity in scatter factor assay
Swiss 3T3K	<2
Human Dermal Fibroblast (HDF)	<2
SV40 Swiss 3T3	<2
BHK-21	<2
MRC-5	128
ras-NIH3T3 (clone D4)	256
Swiss 3T3 (clone J2)	128

Cell lines used to graft into embryos and the measured scatter factor in conditioned media taken from confluent dishes after 3 days.

Local application of purified scatter factor into embryos

The effectiveness of different carriers for delivering purified scatter factor in a local and continuous way to early chick embryos was evaluated. This was done by loading a carrier with medium conditioned by producing or non-producing cell lines and grafting the carrier

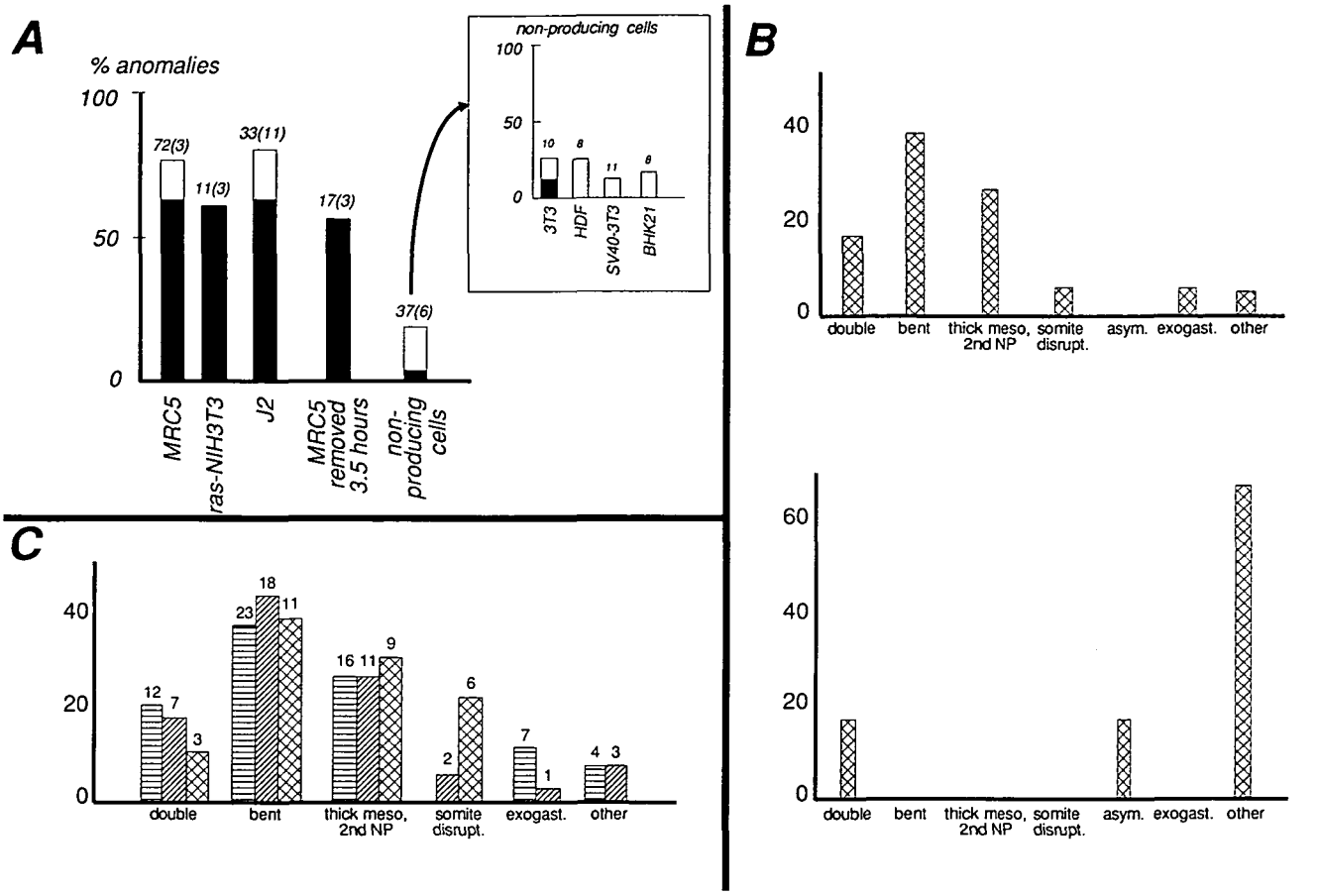

Fig. 1. Summary of results obtained after grafting pellets of different cell types into chick embryos. (A) The main histogram compares the results of pellets of scatter-factor-producing cell lines (MRC5, *ras*-NIH3T3, J2) with pellets of non-producing-cells. The abscissa shows the proportion (%) of surviving embryos displaying anomalies. The numbers above each bin correspond to the total number of embryos grafted and, in brackets, the number of embryos that died. The dark shaded part of each bin represents axial bending or duplication and secondary mesodermal or neural structures; the unshaded part of the bin corresponds to other anomalies. The inset gives further details of the results obtained with different non-producing cell lines. (B) Incidence of different types of anomalies, analysed by treatment type. The upper histogram corresponds to embryos grafted with producing cells, the lower one to control cells. The abscissa represents the frequency of each type of abnormality among the embryos; the sum of all the bins in each histogram gives 100% (all anomalies seen). Certain anomalies (bent axes [bent], secondary mesodermal or neural structures [thick meso, 2nd NP], disruption of somite structure [somite disrupt.] and exogastrulae [exogast.]) are only seen in embryos grafted with producing cells. Other anomalies (asymmetry in the shape of the *area pellucida* [asym.] and milder malformations such as small or 'thin' embryos [other]) constitute a larger proportion of anomalous embryos grafted with non-producing cells. (C) Incidence of different types of anomalies in embryos grafted with producing cells, according to the stage at which the grafting was performed. The bins with horizontal shading correspond to embryos grafted with producing cells at stages XII–XIV, the bins with diagonal shading are embryos grafted at stages 2–3 and the cross-hatched bins correspond to those grafted at stages 3^+–4^+. Abbreviations as in B. The figures above each bin represent the total number of cases of each type of anomaly observed in embryos grafted at the stages indicated. The frequency of some types of anomaly increases with stage at the time of grafting (e.g. somite disruption), while it decreases in others (e.g. double embryos, exogastrulae).

into chick embryos, which were then incubated and scored for the incidence of anomalies similar to those seen in embryos grafted with pellets of producing cells.

The carriers tested initially included: agar, agarose, the cold setting plastic Elvax and a variety of ion exchange beads (listed in the Methods). It was found that AG1-X2 formate beads loaded with scatter factor were the most effective at producing anomalies in embryos, although the proportion of embryos affected was not as high as that seen with grafts of producing cells. In an effort to identify a more effective carrier for scatter factor, we devised a method for using the same ion exchange beads that are used to purify the factor to deliver it to the embryo. The method consisted of extracting beads from a Mono-S FPLC column loaded with partially purified scatter factor (**SF+** in Fig. 5). As controls, we used either beads obtained after elution (**eluted beads** in Fig. 6) or beads from a column loaded with medium conditioned by a non-producing cell line (NIH/1; **SF−** in Figs 5–6).

Using the above methods to deliver scatter factor to chick embryos between stages XII and 4, we found that

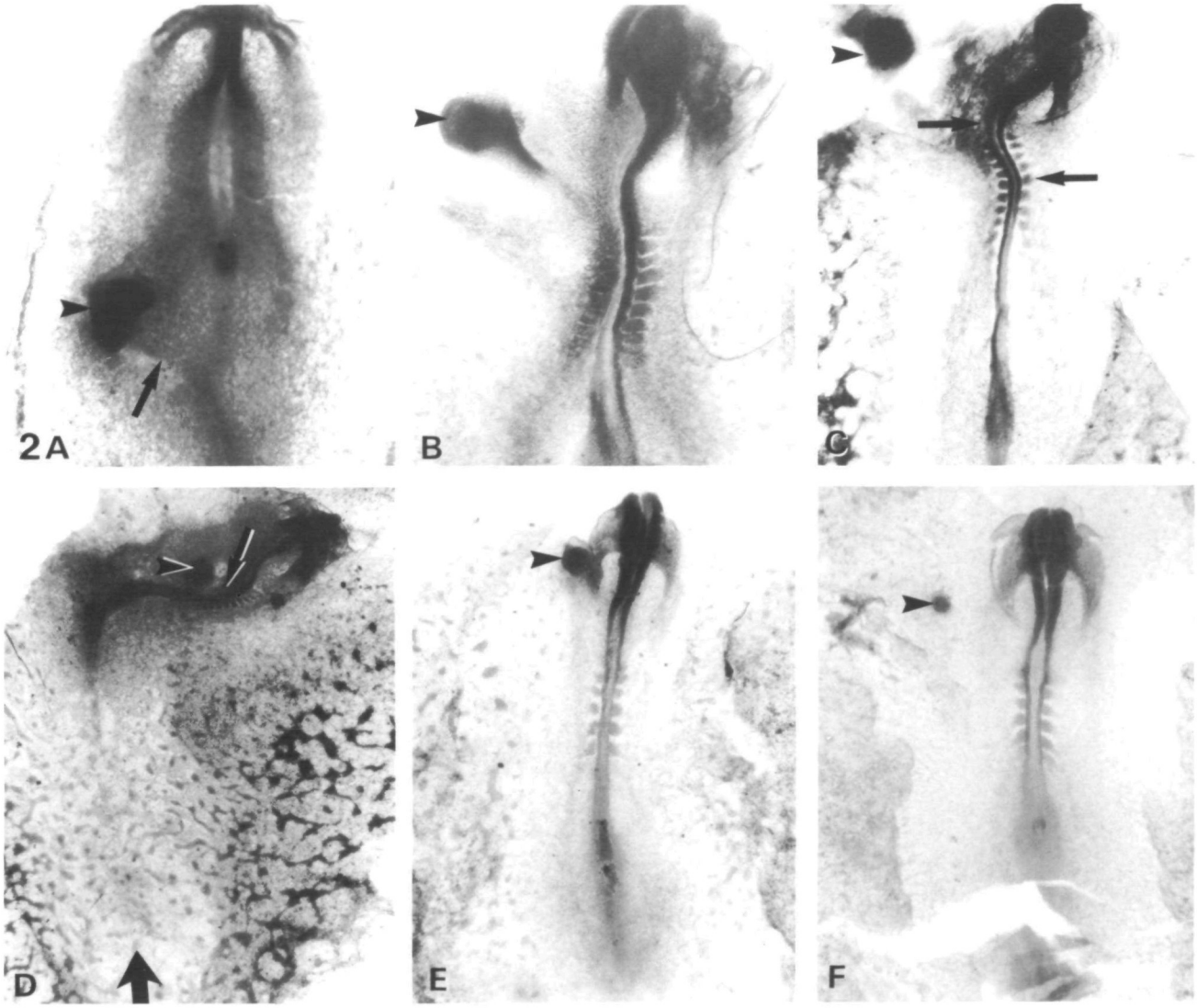

Fig. 2. Fixed and stained embryos grafted with cell pellets (arrowhead); A–D, MRC-5 (scatter factor producing cells); E, 3T3 (non-producing cells); F, HDF (non-producing cells). (A) Arrow indicates primitive-streak-like structure, branching off the normal primitive streak. (B) The somites on the side of the graft appear disrupted and a thickened 'bridge' spans the gap between the graft and the host axis. (C) Arrows indicate bends in the axis. (D) The embryo displays spectacular 'S'-shape bending of the axis: the posterior end of the *area pellucida* lies towards the bottom of the photograph (large arrow). The small arrow indicates a region where somites are missing next to the graft.

the proportion of abnormal embryos and the type of abnormalities seen closely resembled those obtained by grafting pellets of producing cells (Figs 6–8). With all the delivery methods used, only preparations containing the factor were effective at generating anomalies such as supernumerary neural plates and extra mesoderm, although some methods seemed better than others. Agar, AG1-X2 formate beads and Mono-S beads were found to be more effective as carriers than the others. Agar or beads alone, or beads containing proteins from conditioned medium of non-producer cells were all ineffective. The protein composition of the partially purified protein carried by Mono-S beads from producer (SF+) cells is similar to that carried by beads from non-producer cells (SF−) except for the presence of the band of $M_r=62\times10^3$, which contains scattering activity (Fig. 5). These results strongly suggest that the effects observed with Mono-S beads are due to scatter factor.

The frequencies of the different classes of anomalies obtained with SF containing beads were significantly different ($\chi^2=35.8$, 2 d.f., which corresponds to a probability $P<0.001$) from those obtained with control beads (eluted beads and SF−).

In whole mounts (Fig. 7), some of the abnormal embryos displayed bending of the embryonic axis (Fig. 7A), in others the axis failed to form, while in others the axis was duplicated (Fig. 7B), as found with grafts of producing cells. Grafts of control beads (Fig. 7C,D) showed few anomalies. Unlike embryos grafted with pellets of non-producing cells, some of those grafted with control beads displayed bending of

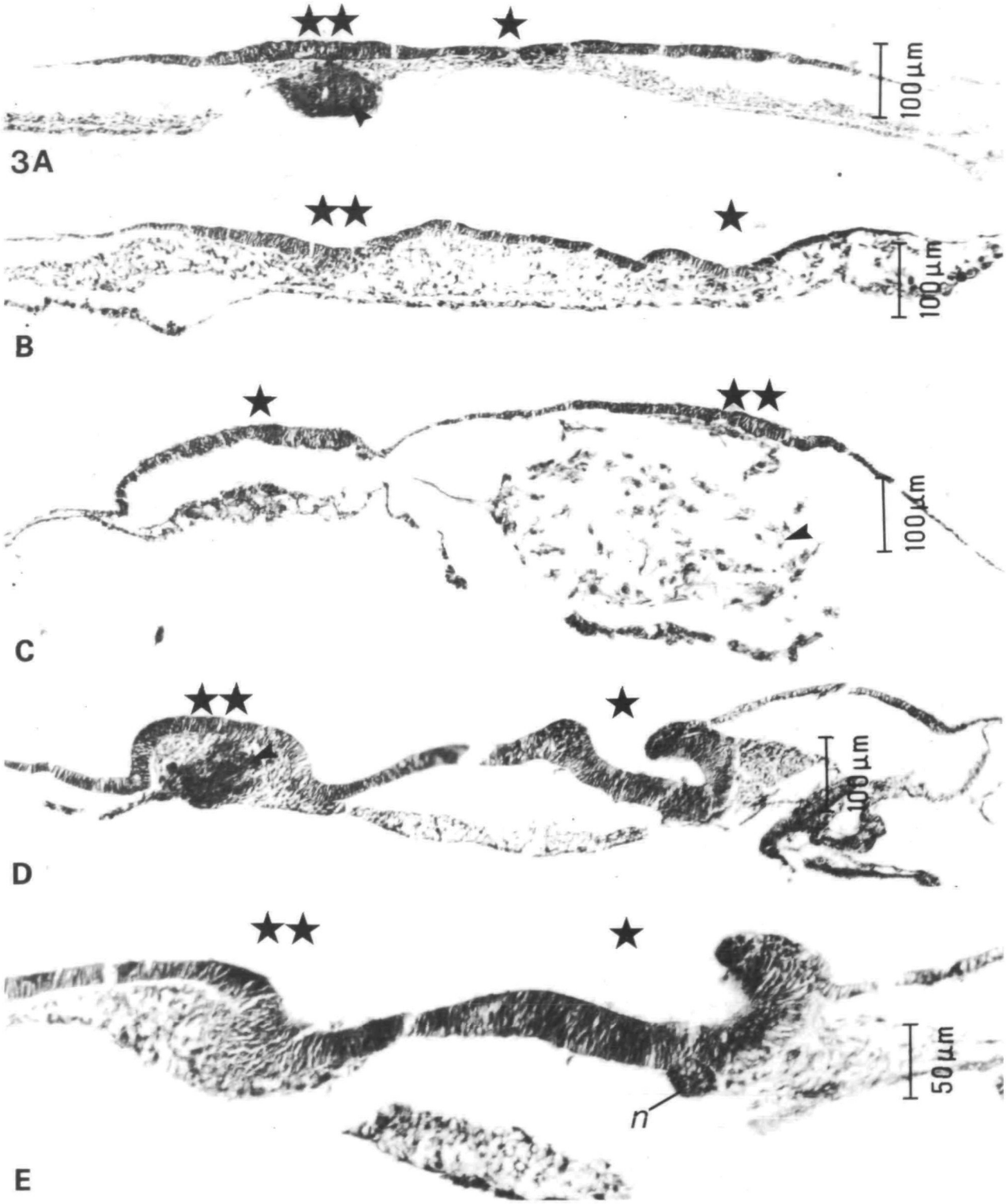

Fig. 3. Transverse sections of embryos grafted with pellets of MRC-5 cells (arrowhead). Grafted cells could be distinguished from host cells in these sections by their size, morphology and affinity for Light Green stain, which was used in processing. The host axis is indicated by *, the induced axis by **. (A) Mesodermal condensation associated with the graft. The epiblast overlying the graft (**) is thickened like a neural plate. (B) A secondary primitive-streak-like structure (**) has formed. (C) A neural-plate-like structure has formed in the epiblast overlying the graft (**); the graft appears as loosely packed cells (arrowhead); note the absence of mesoderm in the region of the graft and the induced neural plate. (D) The graft is surrounded by a mesodermal condensation and the epiblast above this region (**) is thickened like a neural plate. (E) A more cranial transverse section through the same embryo as shown in D. The host (*) and induced (**) neural plates are seen, as is the host notochord (n). The induced neural plate has undergone folding resembling the forming neural tube of the host.

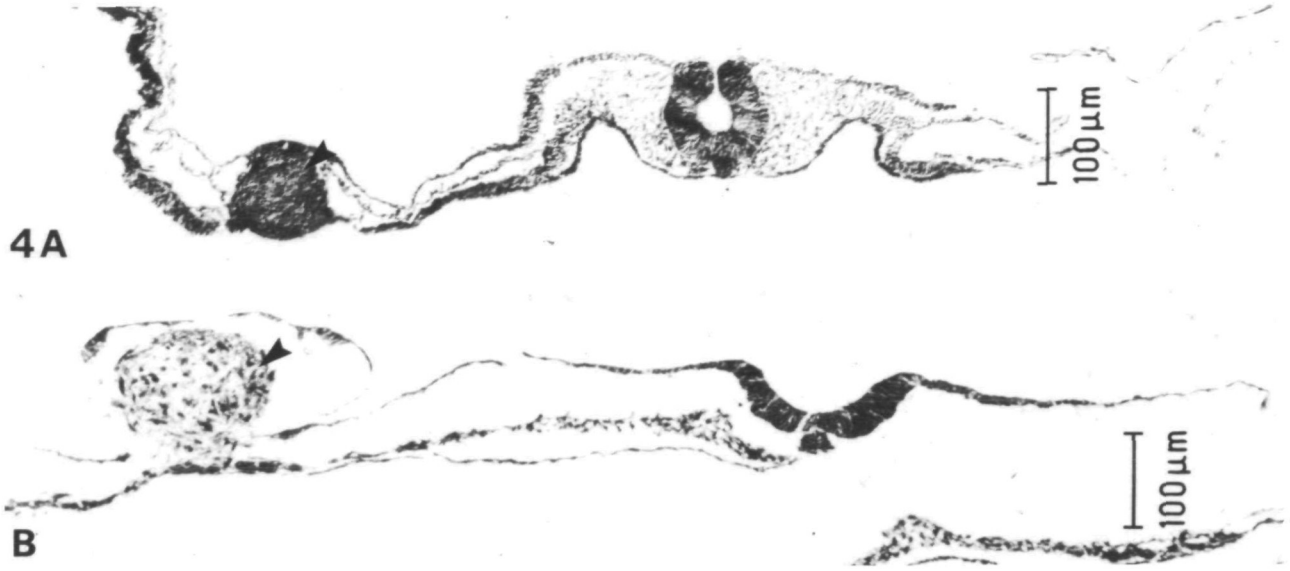

Fig. 4. Transverse sections of embryos grafted with pellets of non-producing cells (arrowhead). (A) Graft of HDF cells, showing normal epiblast overlying the graft. (B) Graft of 3T3 fibroblasts, showing normal epiblast overlying the graft.

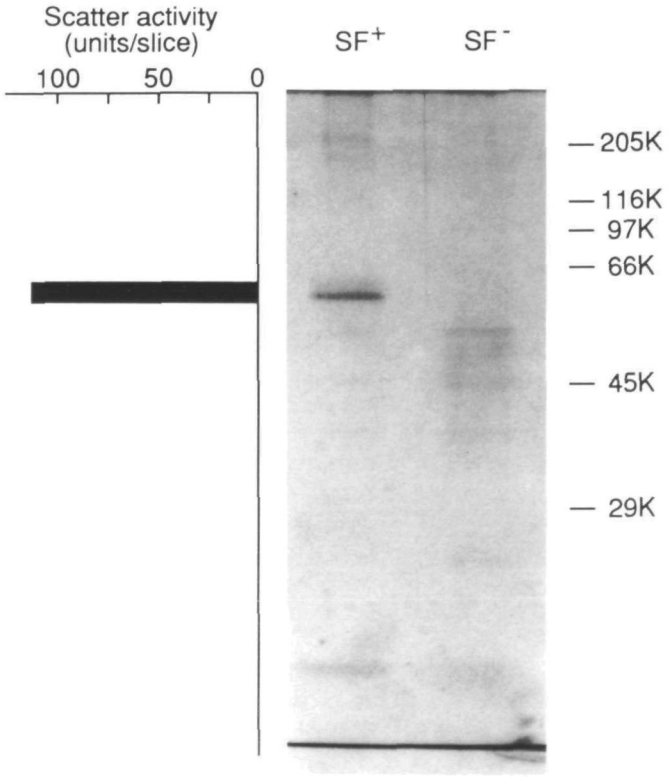

Fig. 5. SDS–PAGE profile of proteins eluted from Mono-S beads. SF+ is derived from clone D4 of *ras*-NIH3T3 cells. SF− is derived from non-producing clone. Molecular mass markers shown on right. A single prominent band is seen in SF+ but not in SF−. Analysis of scatter factor activity eluted from gel slices using the MDCK scattering assay shows the activity confined to the region of this band.

the embryonic axis (c.f. Fig. 1B with Fig. 6B). In histological sections (Fig. 8), some embryos grafted with scatter-factor-containing beads showed primitive-streak-like (Fig. 8A) or neural-plate-like structures, and many had mesodermal condensations (Fig. 8B) around the implanted carrier similar to those seen surrounding grafts of secreting cells. Such partial axial duplications were never seen in control embryos. Embryos grafted with purified scatter factor showed localized effects on the structure of the epiblast overlying the graft (Fig. 8D); in particular, cells had lost their columnar appearance and the cells appeared to be oriented randomly within the tissue as if intercellular junctions had been disrupted. This effect was never seen with grafts of control beads (Fig. 8C,E). The stage-dependence of the abnormalities seen also resembled that seen with grafts of producing cells (c.f. Figs 1C and 6C).

Effects of scatter factor on embryonic chick cells in vitro *(Table 2)*

To determine whether scatter factor acts on chick embryonic cells in a similar way to its effects on MDCK cells, an *in vitro* assay is required. However, no scattering assay similar to the one employing MDCK cells is available for chick cells. As scatter factor is known to cause shape changes observable in single MDCK cells as well as scattering clumps, measurements of cell shape could form the basis for an alternative assay. First, MDCK cells were dissociated and plated on plastic dishes in the presence or absence of *ras*-NIH3T3 conditioned medium or purified scatter factor. They were left overnight and then fixed and stained (as done in the scatter factor assay); the projected outlines of cells were drawn using a MOP

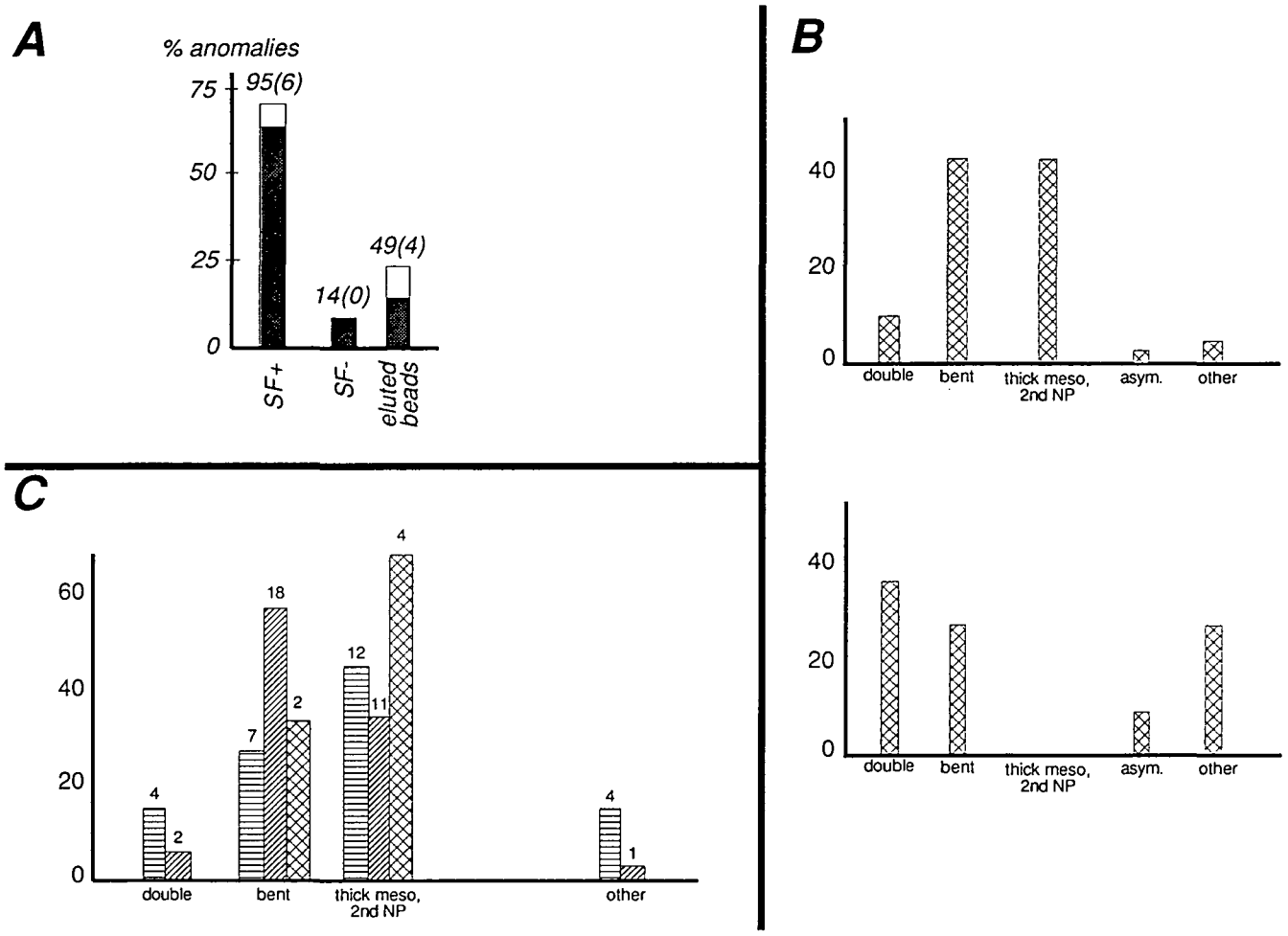

Fig. 6. Summary of results obtained when grafting Mono-S beads containing scatter factor or control beads into embryos. (A) Comparison of the results obtained when grafting beads containing scatter factor (**SF+**) with two types of control beads: beads loaded with medium from non-producing cell line which had been passed over a Mono-S column (**SF−**) and Mono-S beads obtained from a column after elution of the scatter factor peak (**eluted beads**). The shaded part of each bin represents axial bending or duplication and secondary mesodermal or neural structures; the unshaded part of the bin corresponds to other anomalies. Numbers above each bin represent total embryos grafted and, in brackets, embryos that died. (B) Incidence of different types of anomalies, analysed by treatment type. The upper histogram corresponds to embryos grafted with scatter-factor-containing beads, the lower to control beads. The abscissa represents the frequency of each type of abnormality among the embryos; the sum of all the bins in each histogram gives 100 % (all anomalies seen). Abbreviations as in Fig. 1. Supernumerary neural structures and mesodermal thickening are only seen in embryos grafted with scatter-factor-containing Mono-S beads. (C) Incidence of different types of axial anomalies in embryos grafted with scatter-factor-containing Mono-S beads, according to the stage at which the grafting was done. Bins with horizontal shading correspond to embryos grafted with producing cells at stages XII–XIV, those with diagonal shading are embryos grafted at stages 2–3 and the cross-hatched bins correspond to those grafted at stages 3^{+}–4^{+}. The figures above each bin represent the total number of cases of each type of anomaly observed in embryos grafted at the stages indicated. Abbreviations as in B. Bending of the embryonic axis is most frequent in embryos grafted at stage 2–3, while the frequency of double embryos decreases with development.

Videoplan and the shape factor computed (Table 2; Fig. 9). This clearly showed that both *ras*-NIH3T3 conditioned medium and scatter factor purified from it caused a significant decrease in the shape factor computed from the MDCK cells (Table 2). An assay based on individual cell shape could be used on dissociated *area pellucida* cells. Such an assay showed a smaller but still significant decrease in the measured shape factor (Table 2).

Discussion

Effects of scatter factor on early chick embryos

Several abnormalities were seen in embryos that received a graft of scatter factor-secreting cells or purified factor. Condensations of middle layer cells are often seen to surround the graft, even in regions normally devoid of mesoderm. In some cases, a second primitive-streak-like structure is found in the vicinity of

the graft; this structure may represent the source of the middle layer cells that are often found associated with the graft. In epithelial somites near the graft the structure of the somitic epithelium is often disrupted. In some embryos a neural-plate-like thickening of the ectoderm is associated with the graft, while in many cases the embryonic axis displayed one or more dramatic, angular bends.

Table 2. *Effect of conditioned medium and scatter factor on MDCK and chick embryonic cells* in vitro

Treatment	*n*	Area	Perimeter	Shape
(a) MDCK				
Control	75	1502.8 (807.2)	150.2 (36.2)	0.775 (0.160)
*ras*NIH3T3	75	1720.5 (793.0)	209.0 (59.5)	0.519 (0.169)
SF	75	1617.4 (803.8)	213.4 (66.0)	0.471 (0.177)
(b) Chick				
Control	68	2168.4 (1166.1)	233.0 (69.4)	0.520 (0.188)
*ras*NIH3T3	68	1874.3 (1077.5)	246.0 (79.2)	0.413 (0.177)
SF	68	2326.2 (1362.2)	264.4 (87.3)	0.433 (0.172)

Measurements of mean area, perimeter and cell shape of isolated MDCK and chick embryonic *area pellucida* cells spreading on plastic in the presence of *ras*-NIH3T3 conditioned medium, purified scatter factor or control medium. Standard deviations are given in brackets. Comparisons of cell shape between the various treatments (see Methods) revealed statistically significant differences at the 1% level ($P<0.01$) in both chick and MDCK cells for: control *versus* *ras*-NIH3T3 and control *versus* scatter factor. No significant difference ($P>0.05$) was found between *ras*-NIH3T3 and scatter factor for either chick or MDCK cells.

The reaction seen to a graft of scatter-factor-producing cells or to local application of a source of purified factor is reminiscent of the results obtained more than 20 years ago by Mareel and his colleagues (Mareel *et al.* 1968, 1973*a*,*b*), who grafted pellets of HeLa cells into chick embryos. They summarised their results by stating that '*blastoderms showed an elongated condensation following the graft... The host germ was bent towards this structure. In some cases it appeared less developed at the side of the graft... Mesoblast cells were usually crowded against it... the elongated condensation had, deep to it, cells which, together with the ectophyll thickening, were often very suggestive of a process similar to the one observed on sections of normal primitive streak*' (Mareel *et al.* 1968, pp. 250–251). This description could apply accurately to our embryos that received a graft of a source of scatter factor.

Are the effects seen due to scatter factor?

One important question to consider is whether these effects are indeed caused by scatter factor or whether

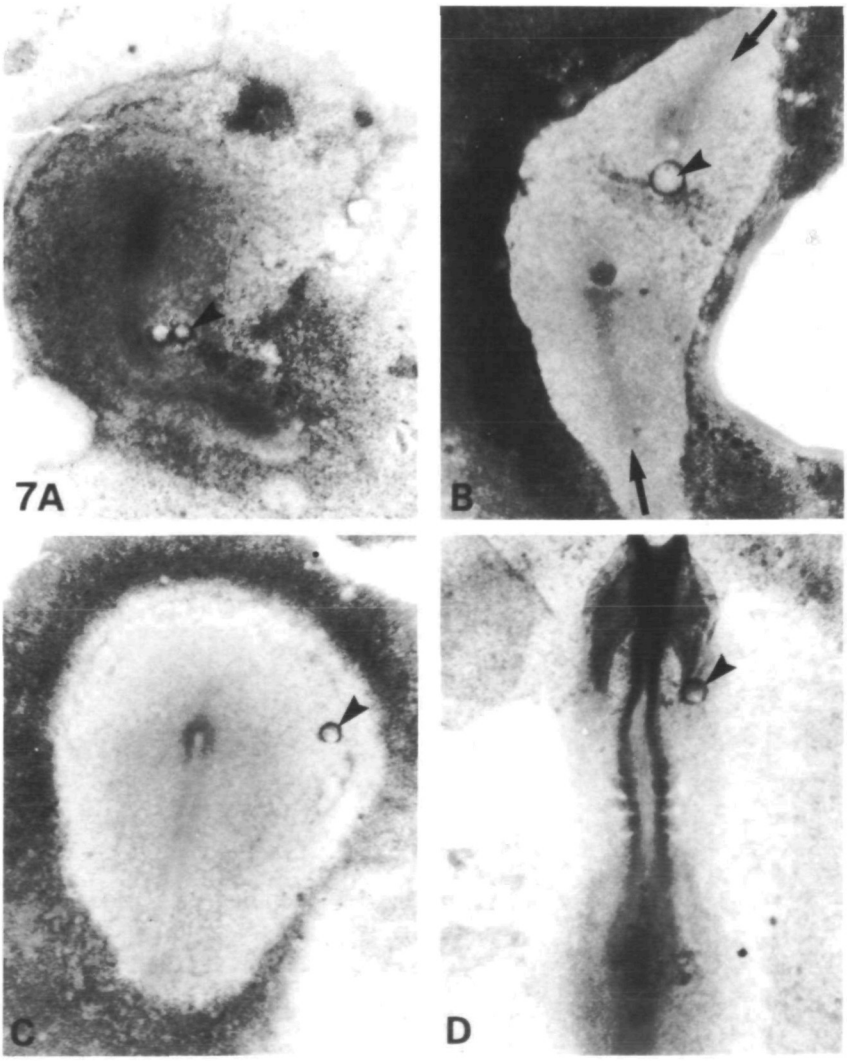

Fig. 7. Fixed and stained embryos grafted with beads (arrowheads) as carriers for purified scatter factor (A, B) and controls (C, D). (A) Two AG1X2 beads carrying Mono-S SF-containing beads: dramatic bending of the embryonic axis. (B) AG1X2 bead with pure scatter factor; double embryo. The caudal end of each of the axes is marked by an arrow. (C) AG1X2 bead as carrier for control (unloaded) Mono-S beads. (D) AG1X2 bead carrying control (SF− type) Mono-S beads; the embryo has been allowed to develop to the 4-somite stage (stage 8).

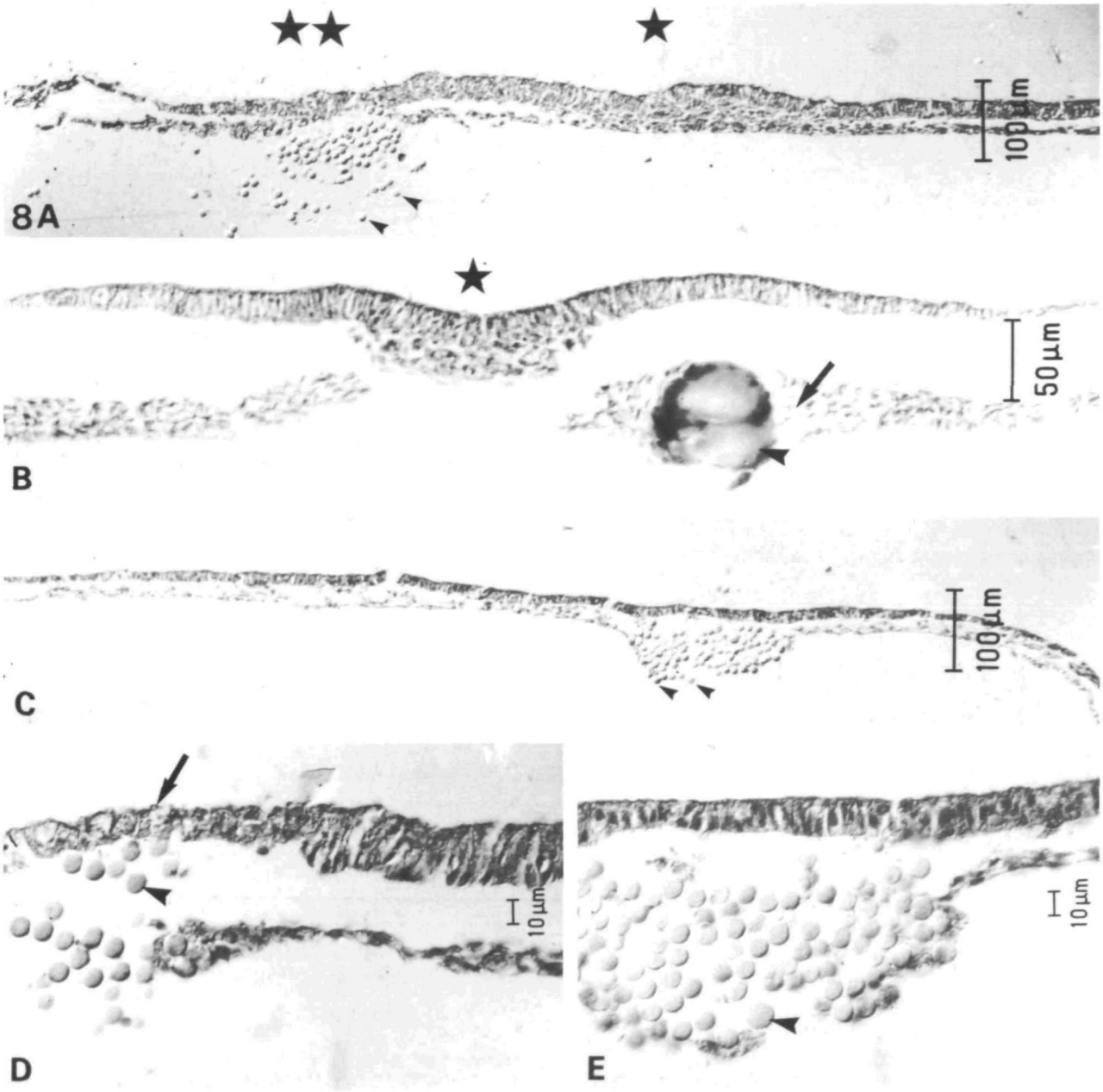

Fig. 8. Transverse sections of embryos grafted with beads carrying pure scatter factor or with control beads. Beads indicated by arrowheads, host axis by * and induced axis by **. (A) Mono-S beads containing SF (AG1X2 carrier used but not contained in this section); the morphology of the germ layers in the region of the graft (**) is altered; (B) AG1X2 bead containing MRC-5 conditioned medium (with scatter factor activity of 1:256) surrounded by a mesoderm condensation (arrow). (C) Mono-S control beads (AG1X2 carrier used but not contained in this section). (D) Mono-S beads containing SF (AG1X2 carrier used but not contained in this section) showing disruption of the epiblast and its basal lamina in the region overlying the graft. (E) High-power view of region of C showing normal epiblast and its basal lamina overlying the control beads.

some other factor(s), secreted by the grafted cells and which co- purify with scatter factor, are responsible. There are three compelling reasons to believe that scatter factor is indeed responsible for the main abnormalities seen. First, there is a strong correlation between the secretion of scatter factor by a particular cell type and its ability to generate the abnormalities listed above. Second, highly purified scatter factor preparations share the effects of secreting cells. Finally, a comparison of Mono-S beads with protein from conditioned media derived from scatter factor producing and non-producing *ras*-NIH3T3 cells revealed that the proteins eluted from the two types of beads look similar by SDS–PAGE except for the presence of a 62×10^3 M_r band in the medium from producing cells (Fig. 5); only beads containing this band produced a

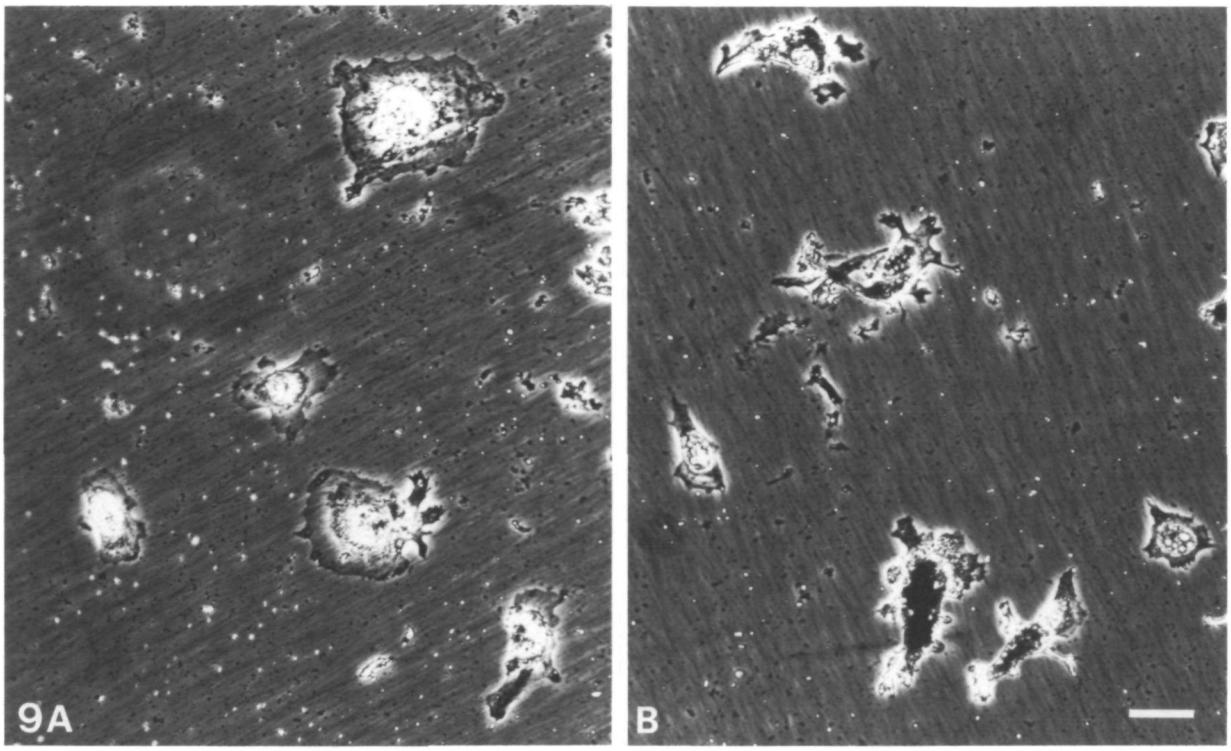

Fig. 9. Fixed and stained dissociated early chick embryonic cells which have been plated in normal medium (A) or *ras*-NIH3T3 conditioned medium (B). Note the difference in the morphology of the cells: those cultured with normal medium have a smoother outline. Scale bar, 35 μm.

significantly greater proportion of extra mesoderm, supernumerary neural plates and bending of the axis when grafted into embryos (Fig. 6). Moreover, when slices of these polyacrylamide gels were tested on MDCK cells, all of the detectable scatter factor activity of purified preparations was contained within a single slice, corresponding to the position of the single major polypeptide seen in such gels (M_r 62×10^3; Fig. 5).

One interesting possibility is that some of the beads used as carriers for purified scatter factor, especially those with a high affinity for the factor (Mono-S beads) may absorb molecules present within the embryo that share some binding properties with scatter factor. This may explain the slightly increased incidence of double embryos and of bending of the embryonic axis in embryos grafted with control Mono-S beads (Fig. 6B) compared to those grafted with non-producing cells (Fig. 1B). It is also possible that the control beads obtained after elution contain residual factor. However, control beads do not cause all the abnormalities seen with scatter-factor-containing beads (Fig. 6B); the incidence of supernumerary neural plates and mesodermal condensations are therefore the best indication of the effects of purified scatter factor.

Does scatter factor act in a similar way on chick embryonic cells and on MDCK cells?

We should also address the question of whether the apparently complex effects seen in embryos are a consequence of a similar action of scatter factor on chick embryonic cells as is seen in cultured MDCK cells, and, if so, which of the embryonic germ layers is affected. In chick embryos, we have found that a 3.5 h exposure to cells secreting the factor is as efficient as a longer exposure in producing axial anomalies (Fig. 1). This finding suggests that the abnormalities seen are a consequence of changes taking place during the early stages of exposure to the factor. What are these early changes? It was observed that the continuity of the epiblast overlying the implanted source of purified scatter factor was disrupted locally, and, in those embryos that were allowed to develop sufficiently, the epithelial morphology of somites near the graft was often similarly disrupted. It seems likely that this 'de-epithelialisation' is analogous to the scattering of MDCK cells seen after exposure to scatter factor.

Perhaps the most unusual effect of the implants is the occurrence of angular bends of the embryonic axis. With the exception of two reports (Mareel *et al.* 1968, after grafting HeLa cells [see above] and Robertson and Gingle, 1977, who applied pulses of cyclic-AMP to chick embryos), this sort of axial bending has never been observed in chick embryos. At present it is difficult to provide an explanation for this axial bending, either after exposure to scatter factor or to pulses of cyclic-AMP.

Is disruption of epithelial continuity the cause of the other abnormalities seen? It is easy to envisage a connection between a localised disruption in the continuity of the epiblast and the presence of mesodermal condensations around the graft: the site at which

epithelial continuity is disrupted could act as an outlet through which the middle layer cells could ingress, as proposed by the model referred to in the Introduction (Stern, 1984). In this sense, scatter factor could mimic some process occurring during normal development during which the primitive streak forms. However, local disruption of epithelial continuity **cannot** account for the appearance of neural-plate-like structures, and it is therefore possible that scatter factor has inducing activity unconnected with de-epithelialisation.

Is a scatter factor-like substance involved in mesodermal or neural induction?

Scatter factor has affinity for heparin (Gherardi *et al.* 1989; Rosen *et al.* 1989), a property reminiscent of another factor which has been shown to have mesoderm-inducing activity in amphibian embryos, basic Fibroblast Growth Factor (bFGF) (Kimelman and Kirschner, 1987; Slack *et al.* 1987; Smith, 1989). However, attempts to establish whether scatter factor is a mesoderm inducer in *Xenopus* animal cap explants have not been successful (J. M. W. Slack and J. C. Smith, unpublished observations). Moreover, it appears that in chick embryos certain cells of the epiblast are predetermined to form mesoderm (Canning and Stern, 1988; Stern and Canning, 1990).

If scatter factor is a primitive-streak-inducing factor in chick embryos, does it induce neural structures directly or as a consequence of an earlier induction of axial mesoderm? In some of the scatter-factor-treated embryos that developed a supernumerary neural-plate-like structure, we have observed that mesoderm was not present close to this structure, suggesting that the induction of neural plate by scatter factor might be caused directly, without axial mesoderm being induced. Moreover, neural-plate-like structures are found even when a source of scatter factor is implanted into older embryos (stage 4–5), by which stage it is difficult to induce further axial mesoderm (see Nieuwkoop *et al.* 1985 for review).

We should also consider the possibility that a substance similar to scatter factor is present in the chick embryo at the time of formation of the embryonic axis. Here we show that early chick embryonic cells *in vitro* can respond to scatter factor from *ras*-NIH3T3 cells. Although we have been unable to demonstrate a similar activity secreted by an embryonic tissue with inducing ability (Hensen's node), chick embryo fibroblasts from later embryos, which can be cultured in greater numbers, do secrete an activity able to scatter MDCK cells (unpublished observations).

Finally, we should consider briefly the reasons why the local application of scatter factor, either by grafting cells or purified factor, is not more effective at producing axial duplications. Such duplications have only rarely been seen after application of **any** factor to higher vertebrate embryos. In those instances in which pure chemicals do cause axial duplications (especially in amphibians, as this effect is unknown in amniotes; Deuchar, 1969; see review by Nieuwkoop *et al.* 1985), this has been ascribed to effects of the substance(s) on the pattern of cell movements rather than to direct inductive effects. Movement of the 'organiser' or source of the inducing signal may be important in determining that the inductive response forms an **axis** rather than a circular patch around the organiser. We might therefore expect that one way to increase the proportion of embryos in which axial duplications are caused might be to graft the scatter factor source on a moving carrier. However, such an experiment is difficult to design.

Conclusions

The results presented in this paper show that scatter factor, applied locally to early chick embryos, produces characteristic axial malformations. Most of these can be obtained when secreting cells or when purified factor is applied to the embryo. Some of the malformations seen could be related to the known effects of scatter factor as an epithelial motility factor. We also propose that in addition to this effect on locomotion, it may have primitive-streak- and/or neural-inducing activity.

This study was supported by grants from the Wellcome Trust (CDS) and the Medical Research Council (GWI, SH). We are particularly grateful to Professor Lewis Wolpert for having initiated the contact between us that led to this study. We would also like to thank Dr Cheryll Tickle for introducing us to the use of AG1-X2 beads.

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(*Accepted 17 September 1990*)