Cortical Grafting in *Xenopus laevis*

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WITH ONE PLATE

INTRODUCTION

The amphibian egg bears a cortex as the periphery of its cytoplasm. The interpretation by Dalcq & Pasteels (1937) of experiments performed by Pasteels (1938, 1940, 1941), in which fertile eggs of *Rana fusca* were centrifuged or inverted, led to the suggestion that the cortex possesses properties which control the course of development to some degree. It appeared that the properties of the cortex of a fertile egg were such that a particular portion of it, that lying in the grey crescent region, was involved in the determination of the site of the dorsal lip when gastrulation occurred and thus of the general axiality of the embryo. In these experiments the relation between the cortex and the underlying cytoplasm was disturbed and it was possible to alter the natural relations of any given piece of the cortex with the cytoplasm beneath. But it was not possible to alter the relationship of one part of the cortex to another. In consequence it was impossible to perform any experiment which might reveal how the properties of the cortex are organized within itself. Moreover, since it was impossible to move a piece of cortex from one embryo to another, it could not be denied that the structure of morphogenetic importance which was resistant to centrifugation or inversion might be other than the cortex. Lehmann (1945) suggested the subcortical marginal plasm, though the experiments of Pasteels (1946) make this suggestion improbable. Grafting the cortex from one embryo to another allows a test of this point as well as permitting investigation into the organization of any morphogenetic properties which the cortex may have: such an organization was suggested by Dalcq (1938).

MATERIALS AND METHODS

The eggs, fertile or unfertilized, were stripped of their membranes by hand and stored in a normal Holtfreter solution buffered to pH 6·2 with a 0·001 M 2-amino-2-hydroxymethyl-1,3-propanediol-sodium dihydrogen phosphate buffer.

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Hereafter this buffer system will be called a tris-phosphate buffer. Donor embryos and those receiving grafts were either uncleaved eggs or eggs in the midst of their first cleavage. Four or five minutes before grafting the donor embryo was moved to a 0-002 M solution of the chelating agent, ethylene-diamine tetra-acetate in calcium-free Holtfreter solution, buffered to pH 8-1 with 0-002 M 2-amino-2-hydroxymethyl-1,3-propanediol and hydrochloric acid. A minute before grafting the recipient embryo was placed in this solution next to the donor. After 4–5 minutes it was possible to lift off portions of the cortical structure using fine tungsten needles, leaving an undisturbed cytoplasm beneath. The grafts were usually square or oblong in shape, measuring about 150 μ each way. This size of graft represents about 5 per cent of the whole cortical area. Using this method it has so far been impossible to remove pieces of cortex from regions near the vegetal pole. A fresh pair of needles was used for each graft. Six pieces of the cortex were fixed for electron microscopy using osmic fixation and embedded in Araldite by Mr. M. Kidd, who sectioned and examined them and to whom I am very grateful. The cortex removed in this manner varies from 0·5 to 3·0 μ in thickness; it contains a true cell surface at the periphery, a thin hyaline layer, and mitochondria, pigment granules, &c., within. Dollander (1951) described a similar structure for the cortex of various urodele eggs. It was, of course, impossible to decide whether these pieces corresponded exactly with that part of the cortex which has been shown to be resistant to centrifugation and inversion. But the stability and strength it possessed in comparison to the underlying cytoplasm make this very probable.

In order to insert the graft in the receiving embryo, a slight cut was made at the site for grafting; this cut slowly opened out. The graft was led into place with the needles and gently laid on the cut. Care was taken to ensure that it lay with its cell surface outermost and that no portion overlapped the surrounding cortex. To cause the graft to heal in, it was necessary to do no more than add sufficient calcium ion to the medium and to drop the pH enough for wound healing to take place. This was done by adding equal volumes of 0·03 M calcium chloride solution and normal Holtfreter solution buffered with tris-phosphate to pH 6·2. About 5 ml. of each were added to the Petri dish, which contained about 5 ml. of the solution of chelating agent. The medium was then slowly replaced with normal Holtfreter at pH 6·2 by repeated additions and removals. Healing was very rapid and if the graft was properly placed it appeared to merge in with the surrounding cortex after a few minutes. Grafts put in upside down were thrown out during the healing process. If the graft was noticeably either more or less pigmented than the surrounding cortex it was possible to locate it for the first day or so of subsequent development.

Control embryos were of two sorts: first, unoperated embryos which had been treated in the same solutions as the grafted embryos, and secondly sham-operated embryos in the midst of the first cleavage in which a cut in the cortex was made but which received no graft. Experimental embryos and controls were cultivated
in glass dishes. A few cleavage stages after the operation the medium was replaced by a 0.5 to 0.6 strength Holtfreter solution buffered at pH 6.2 with a 0.0005 M tris-phosphate buffer. This medium also contained 0.5 ml. per litre of a supplementary medium, the addition of which appeared to help towards the healthy survival of the embryos, though this point has not been tested properly. The reason for the use of this solution is that the chelating agent will have removed other metallic ions as well as calcium from the embryo. To prepare this solution the following chemicals were dissolved in one litre of glass-distilled water and autoclaved:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity (g)</th>
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</thead>
<tbody>
<tr>
<td>Magnesium sulphate, heptahydrate</td>
<td>6.00</td>
</tr>
<tr>
<td>Ferric sulphate</td>
<td>0.60</td>
</tr>
<tr>
<td>Manganese sulphate</td>
<td>0.02</td>
</tr>
<tr>
<td>Zinc chloride</td>
<td>0.01</td>
</tr>
<tr>
<td>Copper sulphate, pentahydrate</td>
<td>0.005</td>
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</tbody>
</table>

Control embryos were transferred to this medium as well. This appeared to be the most satisfactory culture medium until the embryos had reached Nieuwkoop's stage 26. Most embryos were fixed at this stage but some were cultured for 5 days: they were transferred to 0.2 strength Holtfreter solution after stage 26. For these, as well as for those at stage 26, Smith's formol-bichromate reagent was used for fixation. They were embedded in celloidin-paraffin and sectioned at 8-μ thickness.

Grafting operations were carried out using material from various parts of the cortex, thus:

Animal pole placed at animal pole
  "  " grey crescent
  "  " vegetal pole
Grey crescent placed at grey crescent
  "  " mid-ventral margin
Mid-ventral margin placed at grey crescent.

Some grafts were taken from unfertilized eggs; these grafts were placed in embryos in the midst of their first cleavage. Grafts from fertile embryos were taken either from uncleaved eggs or from embryos in the midst of their first cleavage and were placed in embryos in the midst of their first cleavage.

RESULTS

Control embryos

The unoperated embryos (23) all developed normally to stage 26. Embryos which were sham-operated received wounds in one of four sites: at the animal pole, the grey crescent, the presumptive ventral margin, or the vegetal pole. Since
the first cleavage was in progress these wounds were made, when necessary, not in the furrow but to the left or the right of it. Those with animal pole wounds (10 embryos) showed displacement of their first few cleavage planes, but thereafter developed normally save that the brain cavities were rather constricted and their walls thicker than usual. This displacement of the early cleavage planes was also noted in the embryos with wounds at other sites. The 9 embryos wounded at the vegetal pole and the 11 wounded at the ventral margin otherwise developed normally. Of the 13 embryos with grey crescent wounds, 11 developed normally, but 2 developed into the 'ring' type of embryo described by Schechtman (1942), in which invagination fails to occur properly. Since his 'ring' type embryos were produced by isolating the presumptive dorsal lip from the lateral marginal regions in the very early gastrula, it is possible that, in some manner, the wounding described here can leave the dorsal lip isolated.

Grafts using unfertile material

Two series of experiments, each with 8 embryos, were done. In the first, material slightly to the left of the animal pole was grafted to the same site; in the second it was grafted to the vegetal pole. In all cases the first cleavage, which was in progress in the receiving embryo, ceased and regressed. Grafts placed beside the animal pole allowed the appearance of furrows in the unpigmented part of the egg. A number of these, arranged irregularly, appeared, but it is uncertain whether they were cleavage furrows or not. In such embryos the pigmented part of the egg appeared to lie like a coherent cap on the remainder of the cytoplasm and never entered into this furrow formation. After some hours these processes stopped and the embryos became very fragile. Grafts placed at the vegetal pole also resulted in the cessation and regression of the cleavage furrow. In these embryos the pigmentation altered so that a broad equatorial band of pigment with lighter caps formed. The embryos gradually shaped themselves into fragile flattened balls.

Grafts using donor material of same age as receiving embryo

Each of the six types of graft mentioned earlier was taken from embryos in which the first cleavage was in progress. Since the first cleavage was in progress it was necessary both to take and to insert the grafts not medially but slightly to the right or to the left side of the embryo. After the grafts had healed in, the next few cleavage planes were often slightly abnormal in position. This abnormality was most pronounced on the side of the embryo to which the graft was made. Otherwise development appeared to be normal until the beginning of gastrulation.

When animal pole grafts were transplanted to the same site in a second embryo, gastrulation and neurulation took place normally in all the ten embryos used. However, subsequently, slight disturbances to the brain structure developed in four embryos, which appeared to be very similar to those in the control series.
Grafts of the animal pole material to the grey crescent region (9 in number) produced a typical situation at gastrulation: a central portion of the dorsal lip failed to form and invagination proceeded at two dorsal lips on either side of a slight protuberance. As a result two normal and nearly parallel axes were formed (Text-fig. 1 A, B; Plate, fig. D). The graft appears to form a slight amount of neural tissue composed of rather folded layers of cells.

Animal pole material grafted at the vegetal pole was easily distinguished by its pigmentation. In embryos with these grafts the early stages of gastrulation proceeded normally. But after a while a very unusual form of exogastrulation took place. Instead of the exogastrulate being only a slumped heap of cells it was a coherent ball of cells which had a slightly pigmented surface. So far as could
be seen it appeared that this pigmented surface contained the graft. A few loose
cells lay around this ball. The balls appeared to contain about fifty cells apiece.
Once this ball had left the embryo no further exogastrulation occurred. These
balls could be separated from the embryos and cultured for several days, over
which period they show no differentiation. Nine such embryos received this type
of graft and behaved in this manner, and in all cases this exogastrulation re-
moved so little material from the embryo that subsequent development was
normal. Since exogastrulation occurred only twice in the other types of embryos
and only to a very slight degree, it seems likely that it is a direct consequence of
the insertion of the graft.

Grey crescent material grafted to its own site led to entirely normal develop-
ment in 8 out of 11 embryos. The three that did not develop normally produced
ring embryos similar to those found in the control group. When the grey crescent
was transplanted to the ventral marginal region two dorsal blastopore lips were
formed. One was in the normal position and the other at or near the site of the
graft. These embryos (11) all produced these double dorsal lips which Pasteels
(1941) was able to produce by suitable inversion of the egg. Invagination
occurred at both lips, for a pair of axes were found in all these embryos (Plate,
fig. A). These axes were arranged during early neurula stages so that their
anterior ends pointed away from each other. During later stages they tended to
become more parallel, as can be seen in Text-fig. 1 c, d, e, which show external
views of such an embryo. Both axes are substantially complete, and two cross-
sections of one of these embryos are shown in the Plate, figs. B, C.

Ten ventral margin grafts transplanted to the grey crescent region behaved in
a manner rather similar to animal pole material at the same site. Gastrulation
occurred on either side of the graft, leading to two entirely separate axes
developed from the dorsal side of the blastopore. These axes were parallel in five
cases, and at nearly parallel positions in the other five. The axes are similar in
direction.

**Grafts using donor material younger than receiving embryo**

In order to test whether differences in age between donor and recipient in-
fluenced the experimental result, two sets of experiments were done. In the first,
5 embryos were given grafts at the vegetal pole, the material coming from the
animal pole region of uncleaved fertile eggs. These embryos received their grafts
while they were in the midst of their first cleavage. In the second, grey crescent
material was placed in the ventral margin; the respective ages of graft and
receiving embryo were the same as in the first. All these embryos showed a lag
in cleavage in that half of the embryo which had the graft. This lag was from half
to rather more than one cleavage stage behind the ungrafted half of the embryo.
These effects are shown in Text-fig. 2. The discordance in cleavage age between
the two sides of the embryo disappeared by about the sixth cleavage. Apart from
these differences the embryos showed normal development until gastrulation began. Thereafter they behaved like similar grafts taken from embryos in the midst of their first cleavage, as described above.

DISCUSSION

No profound effect on morphogenesis was produced by wounding the embryo, when this alone was done, as in the control series. There were, however, a small number of 'ring' embryos formed when the grey crescent was wounded. A few such embryos turned up in the experimental series when the operation was done at the same site, which suggests that they have a common cause. These 'ring' embryos are discussed below. The normal development of most of the control embryos suggests that slight wounding is not normally of noticeable morphogenetic effect. Likewise, those experiments in which a graft was made to the same type of site as that from which it was derived gave embryos which developed nearly normally. These may be regarded as forming part of the control experiments. At this point it should be remarked that the grafts do not replace portions
of the cortex of the embryos in which they are placed; instead, they are additions
to a complete cortex from which nothing is removed. These grafts do not repre-
sent more than about 5 per cent. of the surface area. In those embryos in which
a 'control' operation was done it might have been expected that the added
cortical material would result in the development of additional amounts of those
organs in whose construction it was involved. But these grafts do not have such
an effect, or at least any noticeable one. This argues against an extremely rigid
control of morphogenesis in which each point of the cortex necessarily represents
a certain future structure. This conclusion was clearly indicated by the work of
Dalcq & Pasteels (1937), provided that it could be accepted that the cortex was
the structure of morphogenetic importance which resisted centrifugation.

In designing the experiment to test the morphogenetic importance of the
cortex it was decided to make those grafts which most closely resembled the
experiments which led Dalcq & Pasteels (1937) and Pasteels, (1940, 1941) to
propose the existence of a cortical field. By suitable centrifugation or inversion
these authors were able to bring the junction of the light and heavy yolks close to
and equidistant from the grey crescent in two places. Dorsal lips formed at both
these sites, though neither lay at the site of the grey crescent. On this type of
experiment in particular they based the idea that the dorsal lip of the blastopore
and the subsequent formation of a nervous system, &c., was situated at a place
determined by the interaction of two morphogenetic factors. One, the yolk
gradient, was localized in the yolk, the other was present in the cortex. The
ventral margin region is close to the junction between the two types of yolk. In
consequence the grafting of grey crescent material to such a region should induce
the formation of a dorsal lip there if the cortex does possess these properties. The
grafting experiments show that this type of grafting has exactly this effect, and
provides evidence for the definite existence of morphogenetic properties in the
cortex.

Grafting experiments allow investigation of the manner in which one part of
the cortex interacts with another. Since the cortical structure remained intact
under centrifugation or inversion it was impossible to do this heretofore. The
results of grafting between various sites which have been described lead to
limited conclusions, for only a few of the very many possible grafts have as yet
been done. Moreover, it should be remembered that the results described apply
to Xenopus alone—it is possible that different degrees of cortical interaction
occur in other species.

Nevertheless, it is possible to draw some conclusions about the nature of the
cortical field. The results suggest that no one portion of the cortex controls the
remainder completely, for no graft is completely altered by, or completely alters,
the surrounding cortex. Each graft retains, in respect of its ability to invaginate
at gastrulation or not, the properties it would have shown in its normal position.
Moreover, it is of interest to note that Schechtman (1942) found that grafts of
cellular portions of the very early gastrula made in the same way behaved in a
very similar manner in gastrulation. These autonomous characters shown by portions of the cortex in gastrulation may not extend to their other morphogenetic properties. At present there is no information on such matters as the regulation of size in organs formed from the graft or nearby tissues. Nor is it known what is the precise course of differentiation in the cells derived from the graft and around it. Dollander (1950) showed that a reorganization of the cortical field might occur after ligature of early stages of Triturus helveticus, which indicates a fair degree of interaction between parts of the cortex.

Attention should be drawn to several other matters. In these experiments no close check was kept on the size of graft: what would be the effect of larger or smaller grafts? In addition, the graft was inserted without regard to its orientation—presumably in the number of experiments done a very wide range of orientations were used. That the results of each type of grafting were very consistent suggests that orientation, at least in grafts of the size used, does not affect the display of the cortical properties. Some of the embryos wounded in the grey crescent region produced 'ring' embryos, as did some of those receiving grafts in this region. Schechtman (1942) was able to show in Hyla regilla that separation of the lateral marginal regions from the dorsal lip shortly before gastrulation, by the interposition of inert materials, produced such embryos. It is possible that the 'ring' embryos described here are due to such an effect, if it be supposed that grafting can sometimes damage the cortex in such a way that barriers to the interaction of dorsal lip and lateral marginal zones are formed.

The significance of the results of grafting infertile material to fertile embryos is at present obscure. They do, however, show that the cortex of fertile and infertile eggs is different and that this difference can be transferred at least from infertile to fertile eggs. That the cortex is deeply involved in fertilization and cleavage is of course well known (see Waddington, 1956). The experiments of Runnström (1958) with sea-urchin eggs may be a close parallel to these results. He was able to prevent the cortical reaction from spreading to the whole egg on fertilization: in consequence cleavage was inhibited. It would be very interesting to know what happens to mitotic processes in such embryos. Closely related are the grafts between embryos of differing age which again show a transfer of properties with the graft and whose results relate to mechanisms of cleavage control.

**SUMMARY**

1. A technique for the grafting of cortical material from one embryo to another at early cleavage stages is described. This method may also be used for the grafting of cortical material from infertile eggs to early cleavage stages.

2. Grafts transplanted to embryos during first cleavage to the same site as that from which they were derived, resulted in normal development. Grafts obtained from grey crescent material and transplanted to the ventral margin induced the appearance of a secondary dorsal lip and subsequent secondary axis. Those of
animal pole or ventral margin material placed in the grey crescent resulted in the failure of invagination during gastrulation at the graft site, and this causes the splitting of the dorsal lip into two and the appearance of double axes. Animal pole material placed at the vegetal pole produces a peculiar form of exogastrulation.

3. Cortical material from infertile eggs if grafted into embryos during their first cleavage inhibits further development. Cortical material from fertile uncleaved eggs, if placed in embryos in the midst of their first cleavage, appears to cause a lag in cleavage in that half of the embryo which received the graft.

4. These results show that the cortical material definitely possesses morphogenetic properties which may be transferred with it. These are concerned with gastrulation, and the experiments are discussed in relation to the ‘cortical field’ theory of Dalcq & Pasteels. This technique of grafting allows investigation of the organization of the cortical field.

RÉSUMÉ

Transplantations du cortex chez Xenopus laevis

1. Une technique décrite ici permet de transplanter du matériel cortical d’un œuf à un autre aux stades initiaux de la segmentation. Cette méthode peut aussi être employée pour la transplantation de matériel cortical des œufs non fécondés aux œufs en début de clivage.

2. Les greffes reportées pendant la première segmentation au même endroit que celui dont elles provenaient ont été suivies d’un développement normal. Les transplantations de matériel du croissant gris à la région marginale ventrale ont causé l’apparition d’une lèvre blastoporale secondaire et subséquemment d’un système secondaire d’organes axiaux. Lorsque du matériel provenant du pôle animal ou de la zone marginale ventrale est placé dans le croissant gris, l’in-vagination gastruléenne fait défaut à cet endroit, d’où une séparation en deux de la lèvre dorsale avec formation de deux systèmes axiaux.

3. Les greffes à des œufs en première segmentation de matériel cortical provenant d’œufs vierges inhibent leur développement ultérieur. Lorsque du matériel cortical provenant d’œufs fécondés indivis est implanté dans des œufs en voie de leur première division, il produit un retard de la segmentation dans la moitié de l’œuf qui a reçu le greffon.

ACKNOWLEDGEMENTS

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REFERENCES


EXPLANATION OF PLATE

Fig. A. Whole embryo of Xenopus laevis at neural plate stage. Secondary neural plate and dorsal lip induced by graft from grey crescent to ventral margin visible on upper end of embryo. ×15.

Fig. B. Transverse section through whole embryo at stage 26, which received same type of graft as illustrated in fig. A. Two neural tubes visible, one with notochord, the second anterior to notochord. Celestin blue and eosin.

Fig. C. Transverse section through same embryo as in fig. B, but more posterior. Two neural tubes and notochords visible.

Fig. D. Transverse section through embryo at stage 26 which received graft of animal pole material in grey crescent. Two closely contiguous neural tubes and notochords are to be seen. Celestin blue and eosin.

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