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Mesoderm induction by fibroblast growth factor in early *Xenopus* development

By J. M. W. Slack, B. G. Darlington, L. L. Gillespie, S. F. Godsave, H. V. Isaacs and G. D. Paterno

Imperial Cancer Research Fund, Developmental Biology Unit, Department of Zoology, South Parks Road, Oxford OX1 3PS, U.K.

[Plate 1]

In early amphibian development the mesoderm is formed around the equator of the blastula in response to inductive signals from the endoderm. At the time of its formation the mesoderm consists of a large 'ventral type' zone and a small 'organizer' zone. A screen of candidate substances showed that a small group of heparin binding growth factors (HBGFs) were active as mesoderm inducing agents *in vitro*. The fibroblast growth factors (aFGF and bFGF) and embryonal carcinoma derived growth factor (ECDGF) all show similar potency and can produce ventral inductions at concentrations above about 100 pM. Single blastula ectoderm cells can be induced and will differentiate in a defined medium to form mesodermal tissues and all inner blastula cells are competent to respond to the factors.

Inducing activity can be extracted from *Xenopus* blastulae and can be purified by heparin affinity chromatography. Antibody neutralization and Western blotting experiments identify this activity as bFGF. The amounts present are small but would be sufficient to evoke ventral inductions *in vivo*. It is not yet known whether the bFGF is localized to the endoderm, although it is known that inducing activity secreted by endodermal cells can be neutralized by heparin.

The competence of ectoderm to respond to FGF rises from about the 128-cell-stage and falls again by the onset of gastrulation. This change is paralleled by a rise and fall of binding of ^{125}I-labelled aFGF. Chemical cross-linking reveals that this binding is attributable to a receptor of molecular mass about 130 kilodaltons (kDa). The receptor is present both in the marginal zone, which responds to the signal *in vivo*, and in the animal pole region, which is not induced *in vivo* but which will respond to HBGFs *in vitro*.

In intact embryos we believe that the ventral type mesoderm forms the somites, kidney and other intermediate structures as well as the blood islands of the ventral midline. These intermediate structures are induced as a function of distance from the organizer in a process called 'dorsalization'. Lithium salts have a dorsalizing effect on whole embryos and also on explants from the ventral marginal zone, causing them to form large blocks of muscle. Lithium will also cause large muscle blocks to form when applied to ectoderm explants together with FGF.

It is difficult to extend these results directly to mammalian embryos, but we have shown that the products of the murine *int-2* gene and of the human *k-fgf* genes are active as mesoderm inducing factors.

Introduction

For most types of animal embryo the specification of different regions to follow the appropriate developmental pathways depends on a series of inductive interactions (Slack 1983). The explanation of these processes depends on the identification of inducing factors, understanding how they are produced and transported and the nature of the responses that competent cells

make to them. Inducing factors from heterologous sources that can affect amphibian embryos have been known for many years (Needham 1942), but progress was halted by a limited understanding of the normal biology of the embryo, by inappropriate methods of assay, and by low specificity of the responses. The modern era of research can perhaps be dated from 1969, when Nieuwkoop first defined the process of mesoderm induction as something that occurred during the blastula stage of development and which was clearly distinct from neural induction. The past five years have seen an explosion of new work on mesoderm induction (Smith 1989) with the result that it is probably now the best understood inductive interaction in vertebrate development.

The process of mesoderm induction

The existence of inductive interactions in the early amphibian embryo can be predicted by comparing the fate map with the specification map for an early developmental stage (figure 1). The fate map shows what happens in the course of normal undisturbed development, and is established by injecting individual blastomeres with fluorescent tracer molecules and finding which parts of the body these end up in at later stages (Dale & Slack 1987*a*). The specification map is constructed by explanting small pieces of tissue and allowing them to develop in isolation in a neutral medium, thus revealing the intrinsic developmental commitment of the piece at the time of explantation (Dale & Slack 1987*b*). It will be evident from figure 1 that the two maps differ in a number of important respects. First, the fate map of the 32-cell stage shows a substantial contribution to the mesoderm from the second tier of cells whereas the specification map shows that the second tier, like the first tier, forms only epidermis. This implies that some signal from the vegetal region is necessary for mesodermal development of these cells. Secondly, in the fate map the majority of the somitic muscle comes from the ventral half of the embryo, but in the specification map ventral halves form ventral type mesoderm (blood islands and mesenchyme) but little muscle. This shows that some signal from the dorsal region is necessary to promote part of the ventral mesoderm to a somitic level of development. Finally, in the fate map the dorsal part of the ectoderm forms the neural plate, whereas in the specification map it forms epidermis. This reveals the familiar process of neural induction that occurs during gastrulation and will not be considered in this paper.

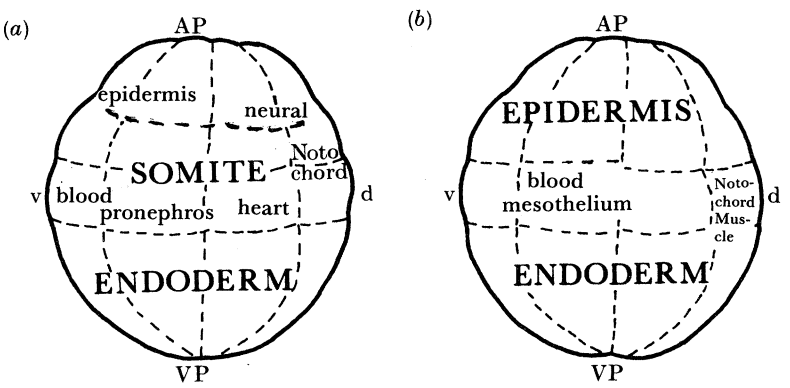

Figure 1. (*a*) Fate map: (*b*) specification map for the 32-cell stage *Xenopus* embryo. The fate map was compiled by injection of a fluorescent lineage label into each blastomere, the specification map was deduced from the self-differentiation of small explants in neutral medium. Abbreviations: AP, animal pole; VP, vegetal pole; v, ventral; d, dorsal.

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The inferences drawn from the maps are reinforced by direct tissue recombination experiments. If tissue from the animal pole region is cultured in combination with vegetal tissue then a substantial proportion forms mesodermal tissues (Nieuwkoop 1969; Dale *et al.* 1985; Gurdon *et al.* 1985). Careful quantitative studies have shown that this is a genuine instructive process and not an effect of selecting out a precommitted subpopulation of cells. The competence of ectoderm to respond to the signal(s) extends from the early blastula into the early gastrula stages and then declines sharply (Jones & Woodland 1987). The regional character of the induction depends on the region from which the inducing tissue is taken. In experiments in which individual blastomeres from the 32 cell stage were used as the inducer (figure 2) it was shown that only the most dorsal blastomere pair from the vegetal octet would induce notochord mesoderm and the remainder would induce ventral or intermediate types of mesoderm (Dale & Slack 1987*b*). The immediate product of mesoderm induction thus seems to consist of a ring of cells around the equator of the embryo of which the larger part, about the ventralmost 300° of circumference, consists of ventral mesoderm, and the dorsalmost 60° is the region we call the 'organizer'.

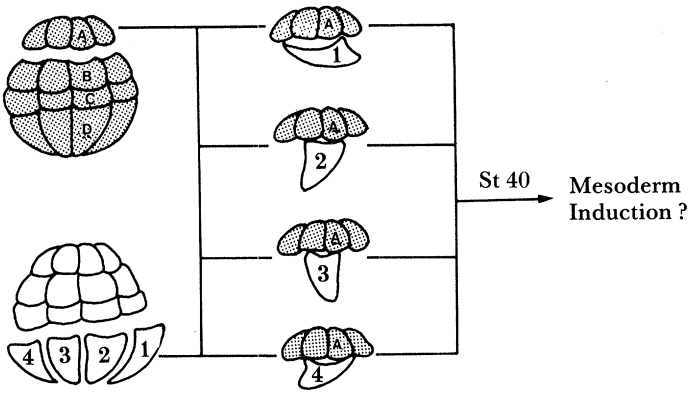

FIGURE 2. Regional specificity of mesoderm induction. In the experiment shown the inducer was a single cell from the vegetal octet of the 32-cell stage whereas the responding tissue was the first cell tier from another, labelled embryo. Only the most dorsal of the vegetal blastomeres will induce notochord.

By the end of neurulation the mesoderm ends up as a cylinder with a number of tissue types arranged in dorsal–ventral sequence: notochord, somite, kidney, lateral plate and blood islands. The intermediate types (somite and kidney) appear to be formed from the original ventral mesoderm in response to a signal from the organizer. This can be shown by making tissue combinations between organizer and ventral mesoderm (Slack & Forman 1980; Smith & Slack 1983; Dale & Slack 1987*b*). We call this process 'dorsalization' and believe that it is a distinct process from mesoderm induction, probably occurring during gastrulation.

The overall picture we have of the early stages is shown in figure 3 and enables us to predict the existence of three kinds of inducing factor: one that will induce ventral mesoderm from ectoderm; another that will induce organizer type mesoderm from ectoderm; and a third that will dorsalize already formed ventral mesoderm. There are now pure factors that will do each of these three jobs.

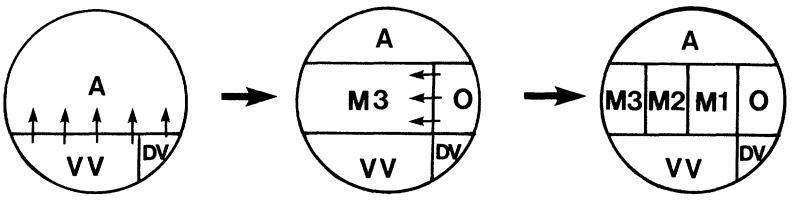

FIGURE 3. The 'three signal model' advanced to explain the data from grafting, explantation and combination experiments. In the first phase a large ventral mesodermal region and a small organizer region are induced by signals from the vegetal hemisphere, now thought to be bFGF and XTC-MIF, respectively. In the second phase the ventral mesoderm becomes regionalized into differently committed zones depending on distance from the organizer. This step is mimicked by treatment with lithium salts.

Effect of FGF on ectoderm explants

In 1986 we tested several pure substances in an attempt to identify mesoderm inducing factors, using the serial dilution assay described by Godsave *et al.* (1988). Only three substances were active and they were basic fibroblast growth factor (bFGF), embryonal carcinoma derived growth factor (ECDGF) and acidic fibroblast growth factor (aFGF), all of which belong to the small group of heparin binding growth factors (Slack *et al.* 1987). As will be shown, all these growth factors behave as inducers of ventral mesoderm. The activity of bFGF was also noticed at this time by Kimelman & Kirschner (1987).

Work in other laboratories has also shown that two factors belonging to the TGF family are active. These are TGF-β-2 (Rosa *et al.* 1988) and the mesoderm inducing factor (XTC-MIF) purified from the culture medium of a *Xenopus* cell line (Smith 1987; Smith *et al.* 1988). The XTC-MIF at least behaves like an inducer of the organizer. In this paper the behaviour of the FGFs will be considered in some detail and attention will be drawn to the respects in which XTC-MIF is different. In fact, the properties of aFGF and bFGF in their capacity as MIFs are very similar indeed, so in what follows 'FGF' will be used to refer to either form indifferently.

Untreated explants from around the animal pole of *Xenopus* blastulae develop into solid masses of epidermal cells (figure 4*a*, plate 1). It can be shown by using antibodies to epidermal markers that 100% of cells become epidermal. Mesoderm inductions can be invoked by FGF concentrations in excess of 1–2 ng ml^{-1} (about 100 pM). After explants are exposed to FGF nothing much appears to happen for the first few hours, the explants round up with their blastocoelic surface inside and the cells continue to cleave just like untreated explants. However, it is the first 90 min or so of exposure that are critical. After this time the FGF can be withdrawn without affecting the course of the subsequent events. Then, while control embryos are undergoing gastrulation, the explants elongate with the original closure point at

DESCRIPTION OF PLATE 1

FIGURE 4. (*a–c*) Ectoderm explants from *Xenopus* blastulae cultured for three days. (*a*) Untreated, forms epidermis only, (*b*) Treated with 4 units ml^{-1} of *Xenopus* bFGF to give ventral induction. (*c*) Treated with 32 units ml^{-1} to give muscle-containing induction. (*d–e*) Single, internal, blastula ectoderm cells cultured *in vitro*. (*d*) Untreated; stained with an epidermis-specific antibody to the cytokeratin XK70. (*e*) Treated with bovine bFGF; stained with the muscle-specific antibody 12/101. (*f–h*) Effects of lithium. (*f*) Whole embryo hyperdorsalized by treatment with lithium at the early blastula stage. (*g*) Isolated ventral marginal explant, untreated, showing ventral mesodermal differentiation. (*h*) Ventral marginal explant treated with lithium, showing large muscle mass.

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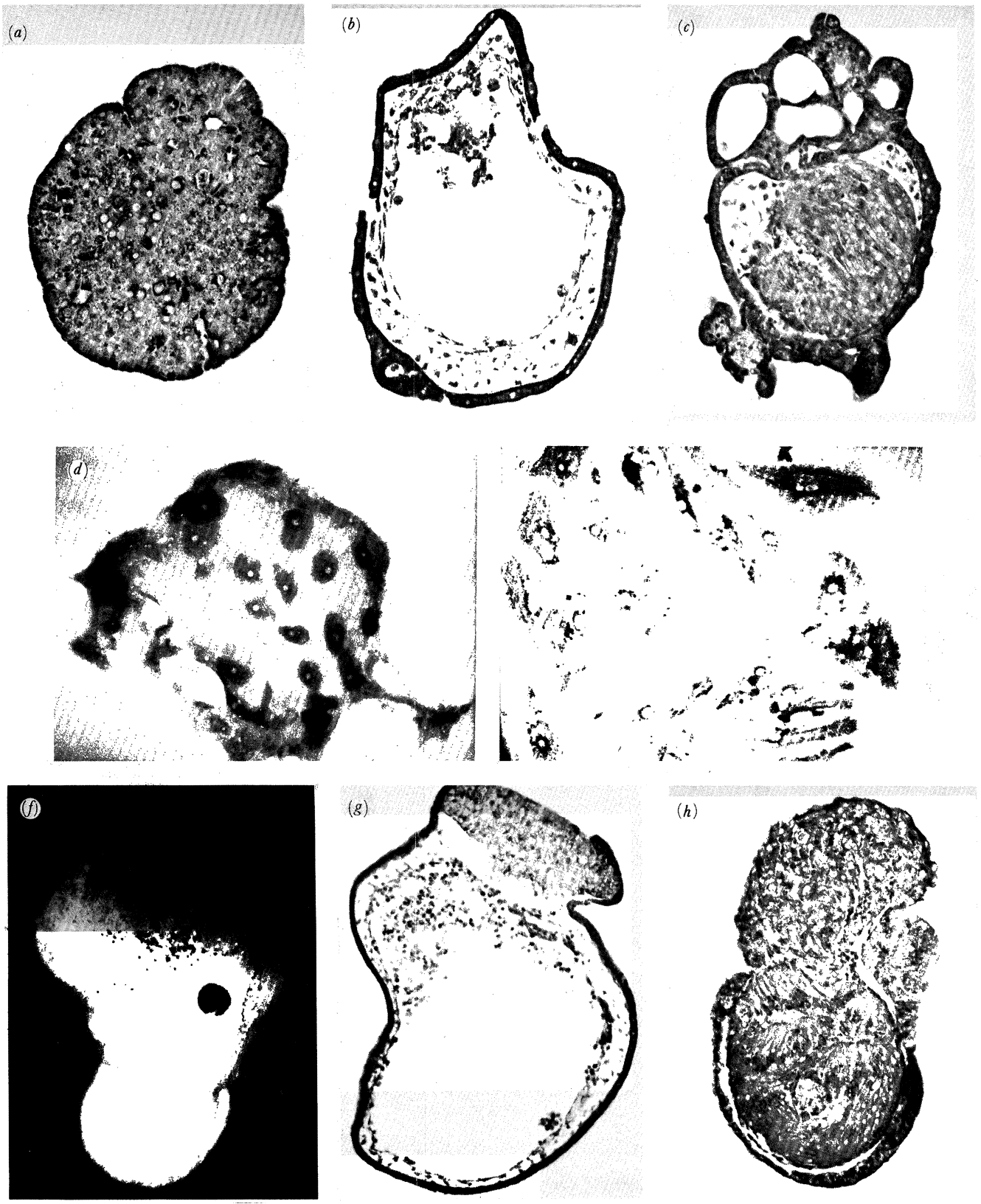

FIGURE 4. For description see opposite.

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one end and the original animal pole at the other. Within a batch the degree of elongation depends on the applied dose, but between batches there is considerable variation. After 24–36 h of culture the induced explants starts to swell and soon become transparent. These vesicles invariably contain mesodermal tissues although the quantity and type depends on the applied dose (Godsave *et al.* 1988; Slack *et al.* 1988). At low doses inductions consist of small amounts of mesenchyme and mesothelium with the occasional wisp of muscle (figure 4*b*), whereas at higher doses we see increasing amounts of mesenchyme and increasing amounts of muscle (figure 4*c*). This dose-response curve is significantly different from that obtained with XTC-MIF, which will induce notochord reliably at a low multiple of the minimum inducing concentration (Smith *et al.* 1988) and also causes the induced tissue to acquire organizer capability (Cooke & Smith 1989). XTC-MIF also seems to work faster, taking perhaps 30 min to achieve a maximum effect.

It should be emphasized that neither group of MIFs has any mitogenic effect on *Xenopus* blastula ectoderm cells that are already cleaving every 30 min in the absence of growth factors and are probably incapable of further stimulation.

We have examined the location of ^{125}I-labelled FGF in explants and find that it binds mainly to those plasma membranes that are exposed at the blastocoelic surface. There is little binding to the plasma membrane of the external surface (oocyte-derived or O-membrane) and little penetration into the cell mass (Darlington 1989). The maximal response to the high doses consists of about 20% muscle by cell composition with an additional 10–20% of mesenchyme and this probably represents all the cells that were exposed on the blastocoelic surface of the explant at the time of treatment. The fact that many cells in induced explants are still epidermal may be entirely due to the limited penetration of the FGF as our studies of single cells lead us to believe that all cells without O-membranes are potentially inducible.

bFGF IN THE *XENOPUS* EMBRYO

Obviously, the minimum requirement for identification of an endogenous inducing factor is that the substance should be present in the embryo at the developmental stage when the relevant events are happening, and in amounts that are capable of exhibiting the observed degree of biological activity. Our results show that it is possible to purify a MIF from *Xenopus* blastulae using heparin affinity chromatography and that it consists of two proteins of M_r 19000 and 14000 which react with antibodies against bFGF (Slack & Isaacs 1989) (figure 5*a*, *b* herein). The quantity in blastulae is about 10 ng ml^{-1}, which is sufficient to account for the ventral induction. It is also found in unfertilized eggs and in whole ovary, suggesting that it is synthesized and perhaps prelocalized before fertilization. The biological properties and specific activity of the *Xenopus* bFGF seem similar to the bovine bFGF, which has been used for most of our experiments on the responses of animal cells. All the MIF activity in a crude embryo or ovary extract can be inhibited by a neutralizing antibody to bFGF, but not by antibodies to aFGF or TGF-β-2. Parallel work by Kimelman *et al.* (1988) has also shown the presence of bFGF mRNA and protein in *Xenopus* blastulae.

We would obviously predict that the bFGF would be secreted by the cells of the vegetal hemisphere. So far, immunolocalization on embryo sections has not proved successful, probably because of the small quantities present. We have shown that the MIF released by vegetal cells in transfilter experiments can be neutralized by heparin, as can both *Xenopus* and

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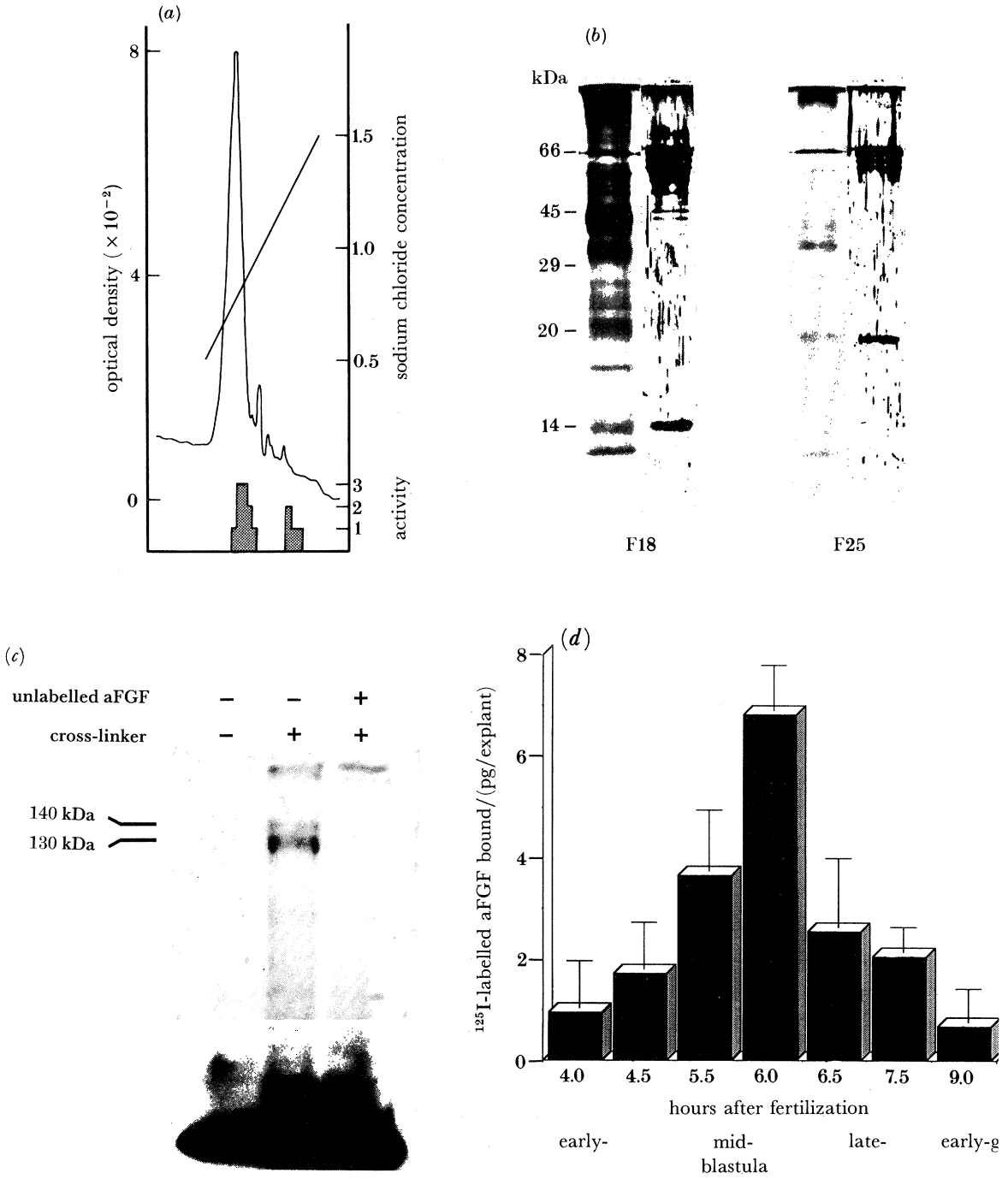

FIGURE 5. (*a*) Purification of mesoderm inducing activity from *Xenopus* ovary by heparin affinity HPLC. (*b*) SDS gels of peak activity fractions. For each, the left track is silver stained and the right is a Western blot stained with an antibody against bovine bFGF. (*c*) Identification of the FGF receptor on *Xenopus* blastula ectoderm by cross-linking of ^{125} I-labelled aFGF. (*d*) Rise and fall of ^{125}I-labelled aFGF binding activity with embryonic stage.

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bovine bFGF, but not by anti-bFGF antibodies. This may mean that the bFGF is secreted as part of some complex not recognized by our neutralizing antibody, but further work is necessary to prove beyond doubt that the vegetal cells really secrete bFGF. One problem in this regard is the well known fact tht bFGF lacks a classical signal sequence for secretion (Abraham *et al.* 1986), and so there remains some uncertainty about its mechanism of release from cells.

Response of the ectoderm

We have studied the onset of competence to FGF in the ectoderm by exposing for a period of 90 min explants taken from different stages and this shows that competence begins at about stage 7. We have studied the loss of competence by permanent exposure of ectoderm explants taken from different stages and this shows that competence is lost between stages 9 and 10 (Slack *et al.* 1988). The competence for FGF seems to rise at about the same time as competence for the natural MIF(s) but falls rather earlier, as there are about 3 h between stages 9 and 10.5 at 22–24 °C. Competence to respond to XTC-MIF also seems to persist into gastrulation, until stage 10.5–11, according to our measurements (Darlington 1989). We have found that with appropriate medium supplements it is possible to induce single, isolated cells and to obtain mesodermal differentiation of the resulting clone (Godsave & Slack 1989) (figure 4*d*, *e* herein). All cells from the inner surface of the animal hemisphere appear to be competent to respond.

As inductions arise in response to low FGF concentrations, we expected that an essential molecular component required for competence would be a specific receptor. We have probed for a receptor on explanted tissues using ^{125}I-labelled aFGF and the cross-linking agent BS_3. This has shown that a receptor is present and appears as two gel bands of M_r about 130 and 140, similar to the mammalian FGF receptor (Gillespie *et al.* 1989); (figure 5*c* herein). Binding studies show that about 70–80 % of bound ^{125}I-labelled FGF can be competed out by an excess of unlabelled FGF. Assuming that this represents binding to the specific receptor then the density is about 3×10^8 molecules per square millimetre of cell surface is within the range of values measured for mammalian cells. The binding curve shows a half-maximal value of about 3–4 nM and a plateau at about 10 nM, which is very similar to the dose response curve for muscle formation. This suggests that the receptor binding is a limiting step in the response. If it were not then a maximal response, in this case a maximal percentage of cells induced, would be obtained at an FGF concentration below that required to saturate the receptors.

The receptor density has been studied by binding of ^{125}I-labelled aFGF to ectoderm explants taken from different embryonic stages. The competable binding rises by a factor of 10 between the early and middle blastula, and falls again to the starting level by the onset of gastrulation. This closely parallels the rise and fall of competence to respond to FGF and suggests that competence is indeed controlled by receptor density.

Competition experiments have shown that both aFGF and bFGF bind to the same receptor but TGF-β-2 does not. This again resembles the situation in mammalian cells and makes it probable that the extended period of competence that ectoderm explants show when treated with XTC-MIF is due to the presence of separate TGF-β receptors.

We have measured the regional distribution of FGF receptors in stage 8 blastulae by binding studies on explants (Gillespie *et al.* 1989). This shows, as predicted, that FGF receptors are present both in the marginal zone region, which normally responds to the signal *in vivo*, and in the animal pole region which can respond in experimental situations but would not normally

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do so *in vivo*. There is a slight excess of receptor density in the marginal zone but this is only 50% more than the animal pole value, so it would seem that the normal extent of mesoderm induction is determined by the extent of the signal and not by the presence of a more highly competent tissue in the marginal zone. There is no difference in receptor density between dorsal and ventral regions of the animal hemisphere, so this cannot account for the difference between dorsal and ventral inductions.

Lithium mimics the dorsalizing signal

Lithium treatment of intact early *Xenopus* embryos produces embryos with grossly enlarged heads and dorsal parts and minute posteriorventral parts (Kao *et al.* 1986; Cooke & Smith 1988) (figure 4*f*). We can deduce from the fate map that a larger than normal part of the blastula mesoderm has been dorsalized and might predict that lithium elevates some critical metabolic intermediate that is normally elevated by the dorsalizing signal from the organizer. It has been shown that lithium will dorsalize isolated ventral marginal explants to the level of large muscle masses (Kao & Elinson 1988; Slack *et al.* 1988) (figure 4*g*, *h* herein). The distinction between this effect and the original mesoderm induction is clear. When blastula ectoderm is exposed to lithium nothing happens, but when ectoderm is exposed to FGF and lithium, it behaves like the ventral mesoderm treated with lithium and produces large muscle masses. Lithium is known to affect signal transduction pathways by the inhibition of the enzyme inositol monophosphatase (Hallcher & Sherman 1980) and of certain G proteins (Avissar *et al.* 1988), but these pathways are not yet sufficiently understood in *Xenopus* for us confidently to predict the biochemical consequences of lithium treatment.

Oncogenes and mammalian development

In an attempt to extend our findings into the realm of mammalian development we have examined some of the FGF-like oncogenes that have recently been discovered. This has been done by *in vitro* transcription of complementary deoxyribonucleic acids (cDNAs) from plasmids containing SP6/T7 bacteriophage promoters, followed by translation in a rabbit reticulocyte lysate. The lysate is assayed directly by treating ectoderm explants with series of dilutions, and the specific activity determined by measurement of the concentration of the translated protein (Paterno *et al.* 1989). So far, we have examined kFGF, which is the product of the human *ks* and *hst* oncogenes (Delli-Bovi *et al.* 1987; Taira *et al.* 1987) and INT-2, the product of the murine *int-2* oncogene (R. Smith *et al.* 1988). Both are active as mesoderm inducing factors. The specific activity of the kFGF is similar to that of the aFGF and bFGF, whereas the specific activity of INT-2 is very much lower. Considering the factors as a group, there is a good correlation between their mesoderm inducing activity and their mitogenic activity when tested on mammalian fibroblasts and this suggests that similar signal transduction machinery is being used for the two processes.

The INT-2 protein is of considerable interest to students of mammalian development because the gene is expressed in several discrete locations in the mouse embryo (Wilkinson *et al.* 1988, 1989). These include the pimitive streak, where the mesoderm is being formed and where a mesoderm inducing factor might be expected to be found. They also include three sites where the pathways of differentiation are controlled by epithelial–mesenchymal interactions:

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the endoderm of the first three pharyngeal pouches, the hindbrain adjacent to the developing otocyst, and the mesenchyme of developing teeth, so it is possible that the same inducing factor is used several times for different purposes. Like *int-2*, the *k-fgf* gene is not thought to be expressed in the adult and so is presumed to have an embryonic function. At the time of writing there is no information about its expression pattern in the embryo, but it is known to be secreted by the undifferentiated cells of certain embryonal carcinoma cell lines (Paterno *et al.* 1989), which suggests that it too may be acting as a mitogenic factor or an inducing factor in early development.

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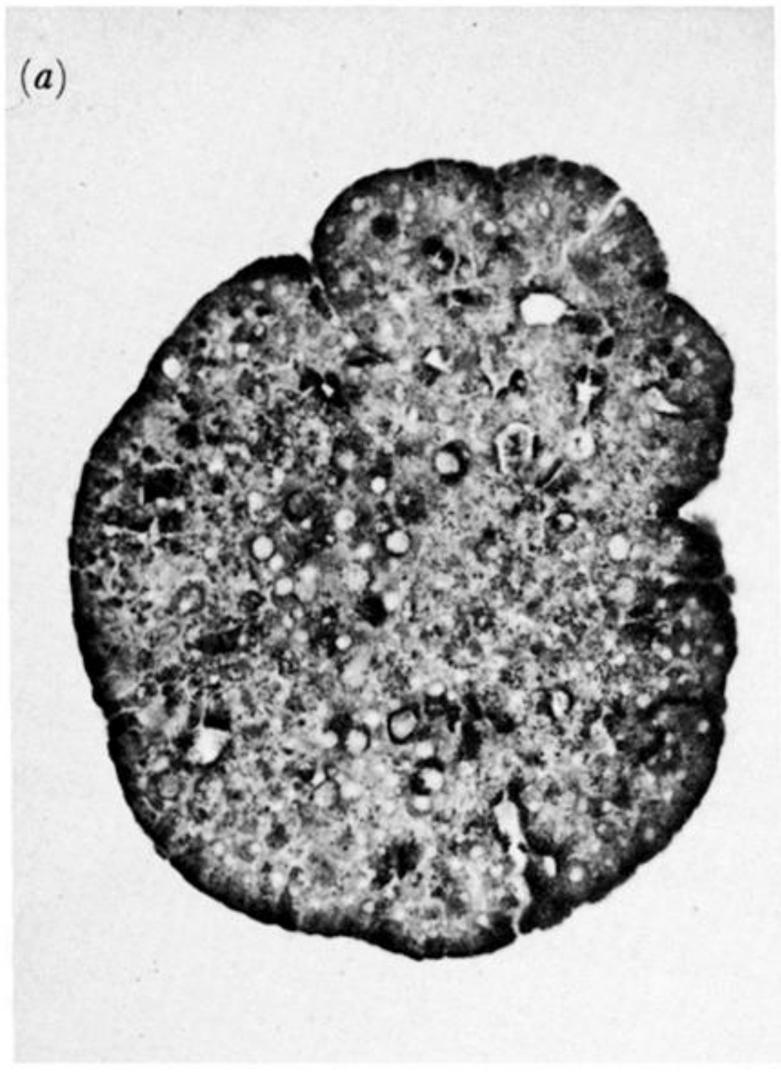
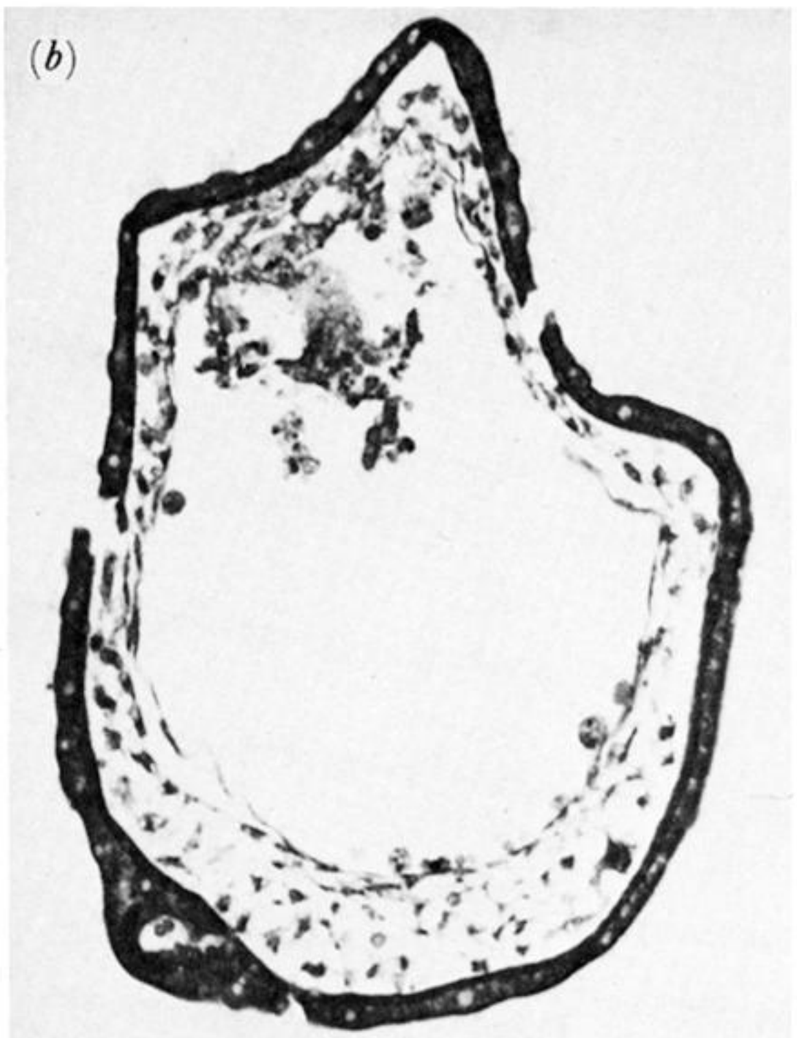
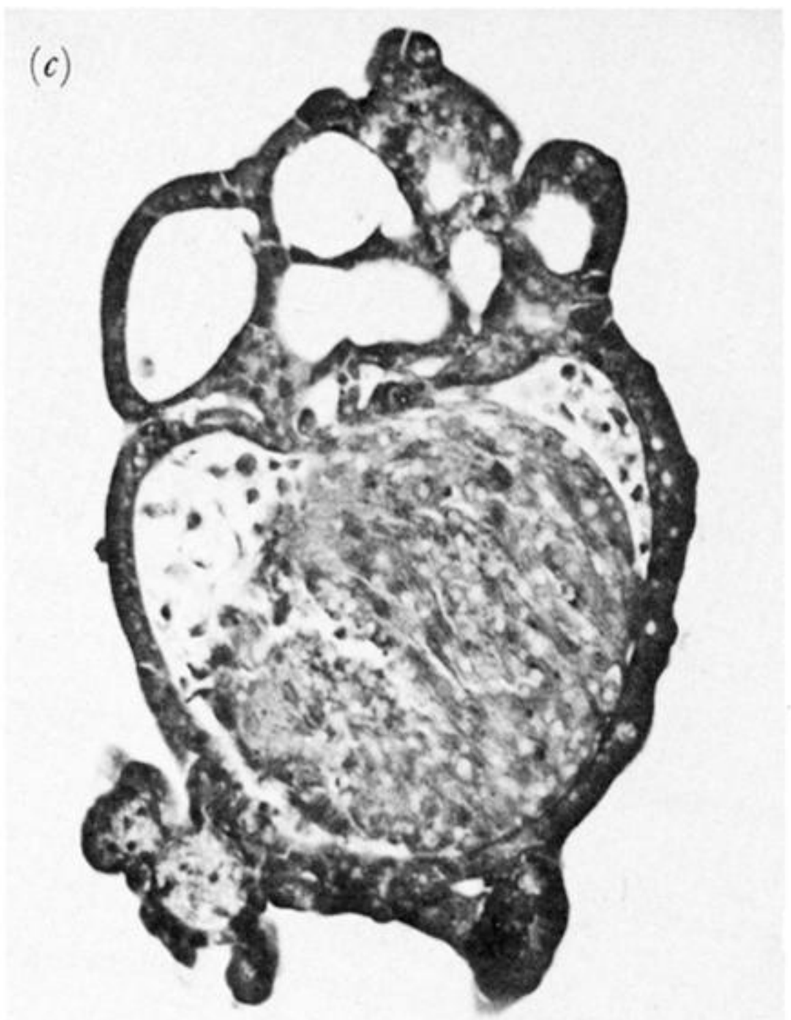
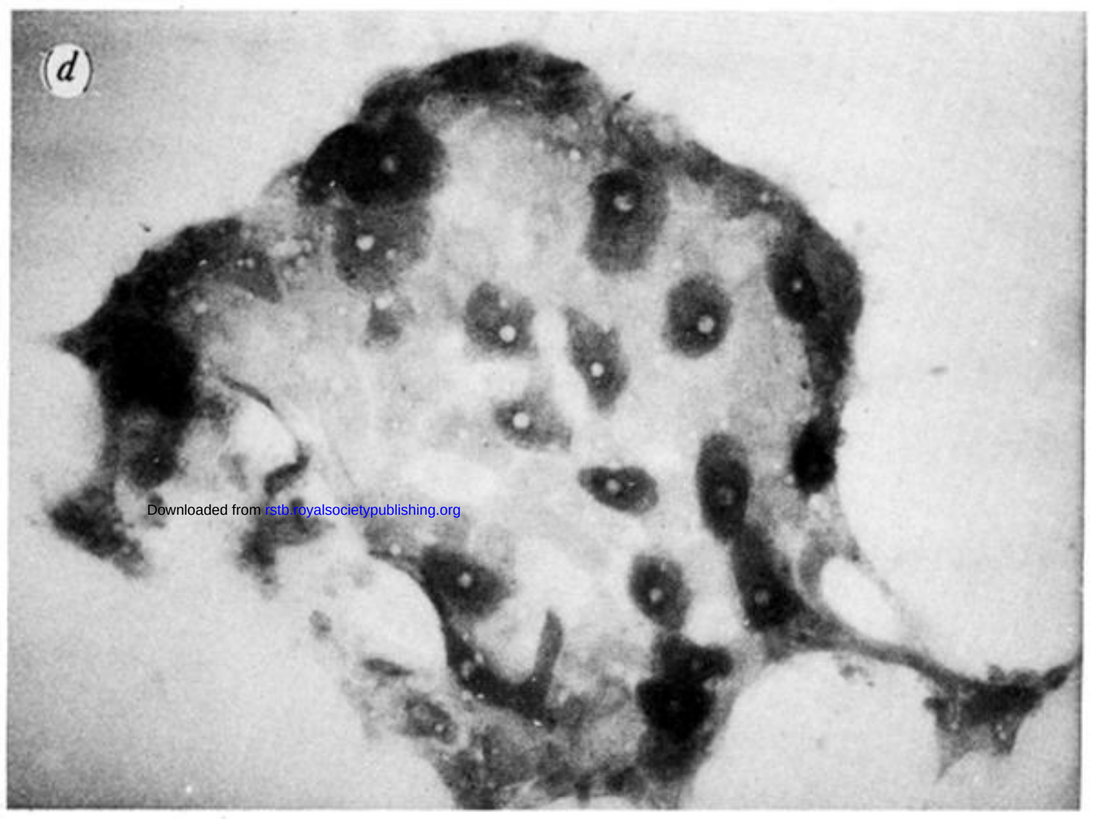
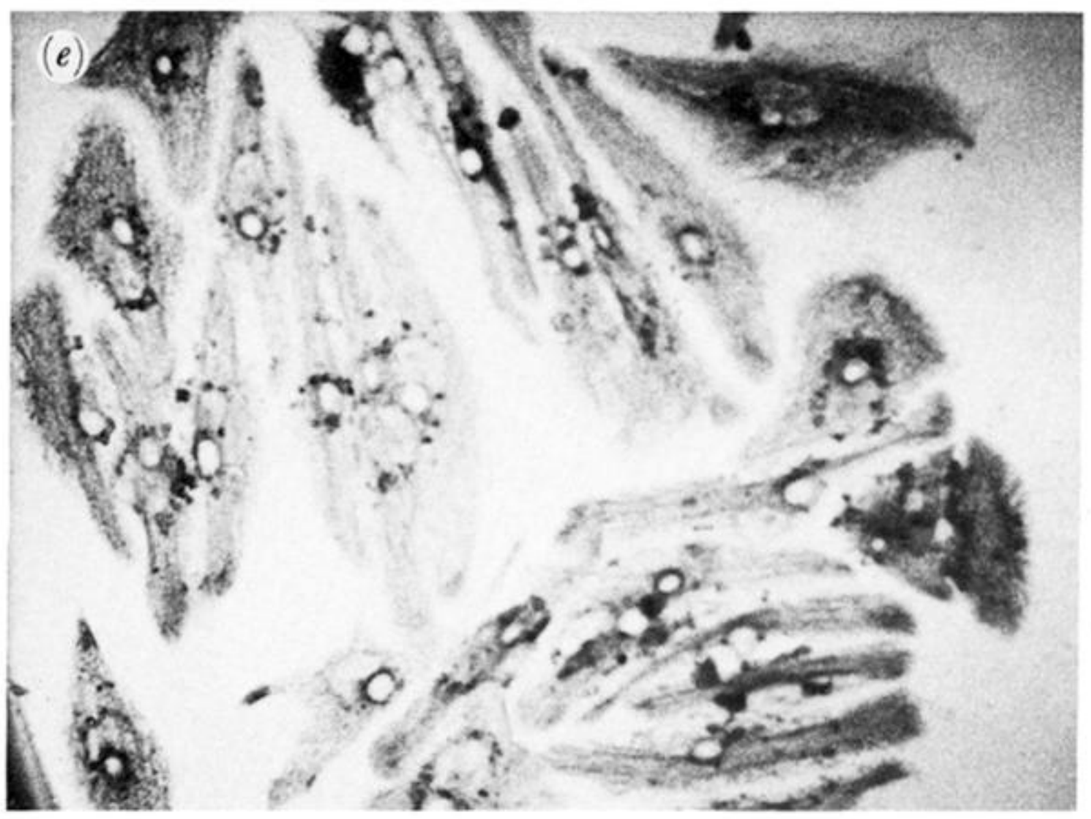
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

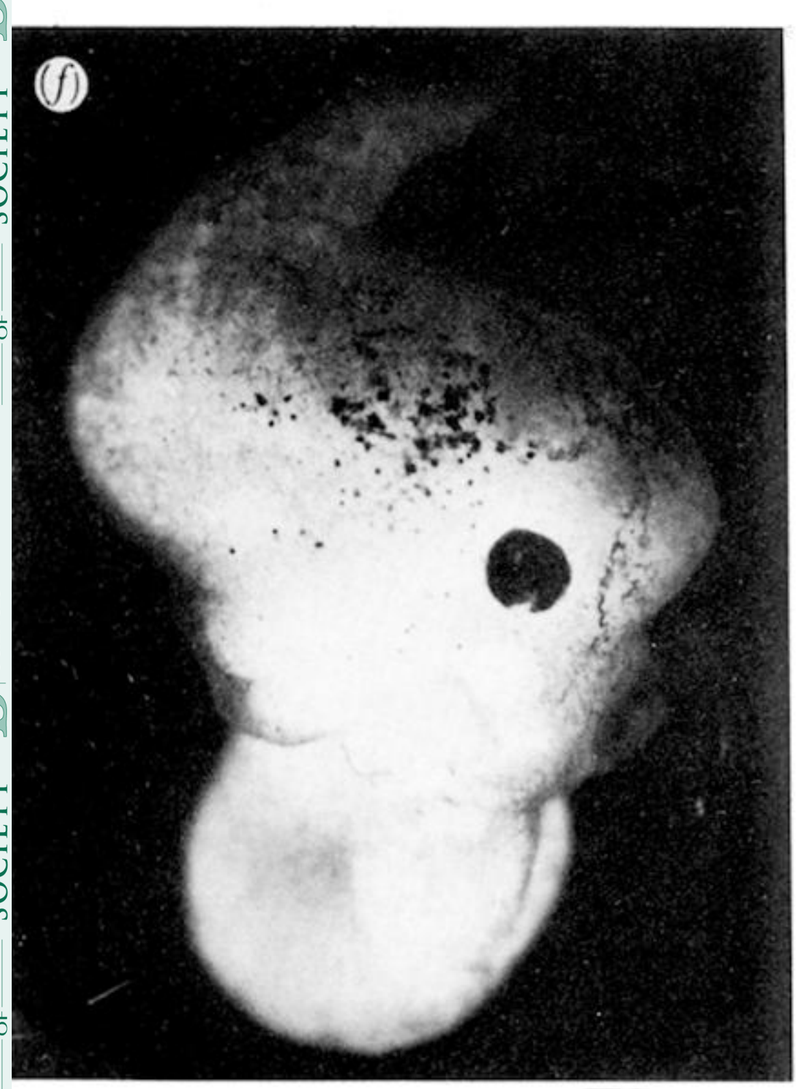


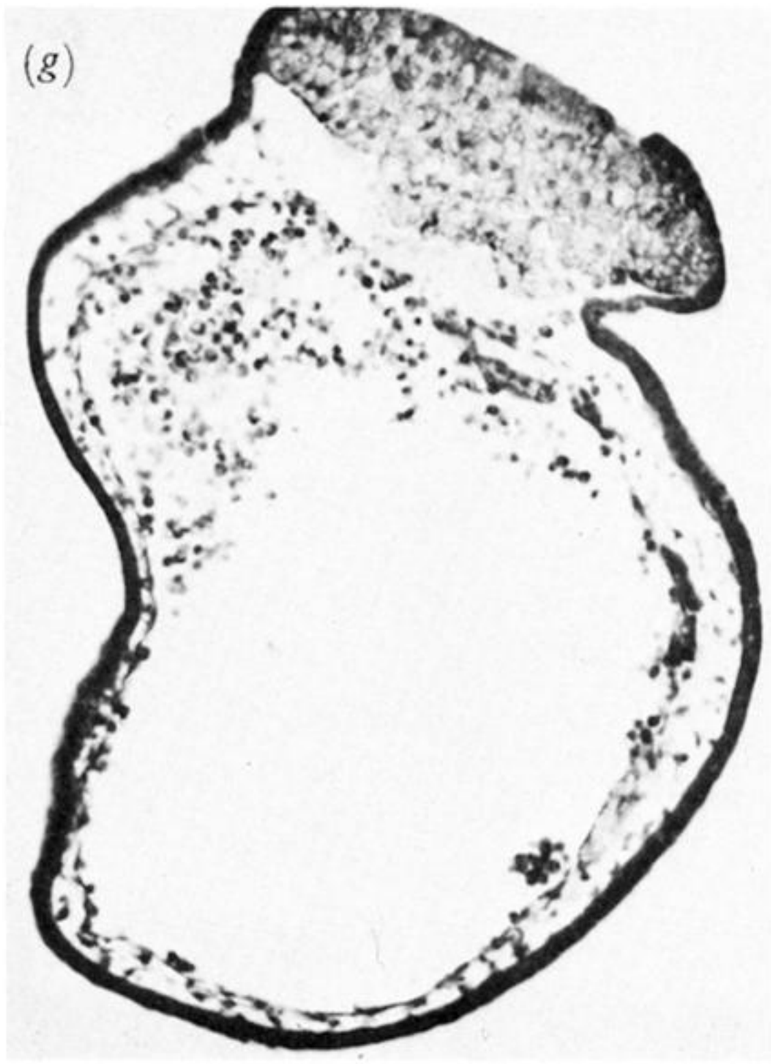












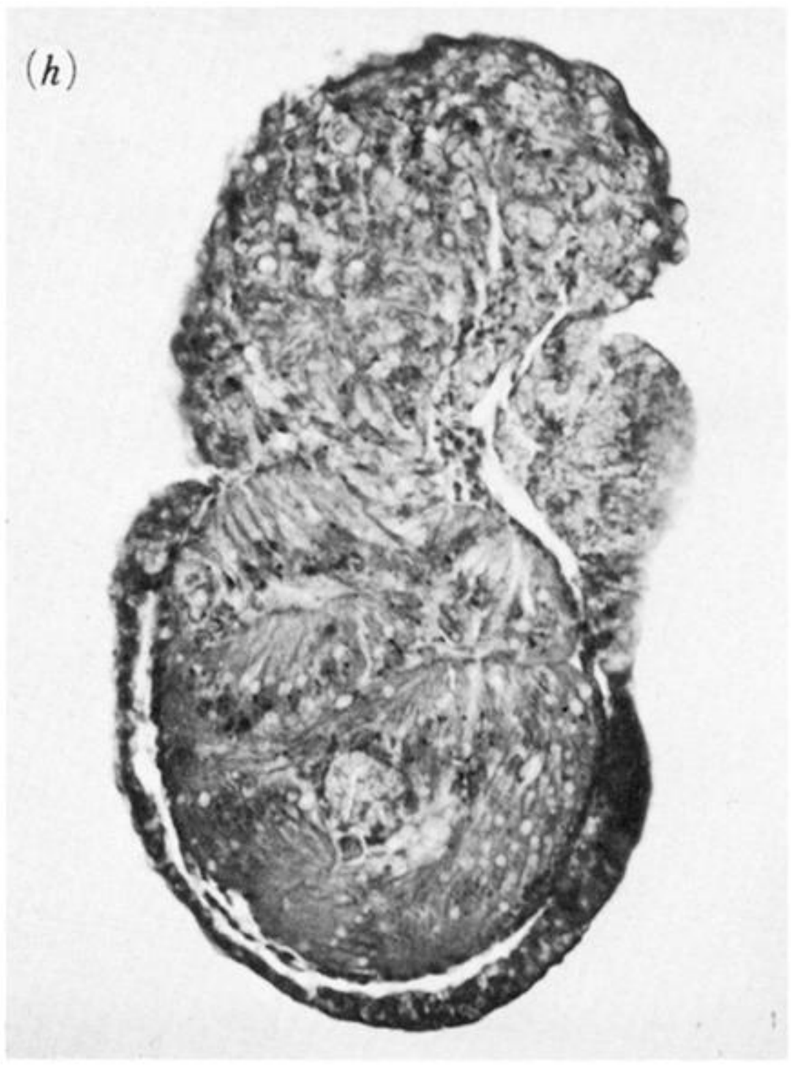
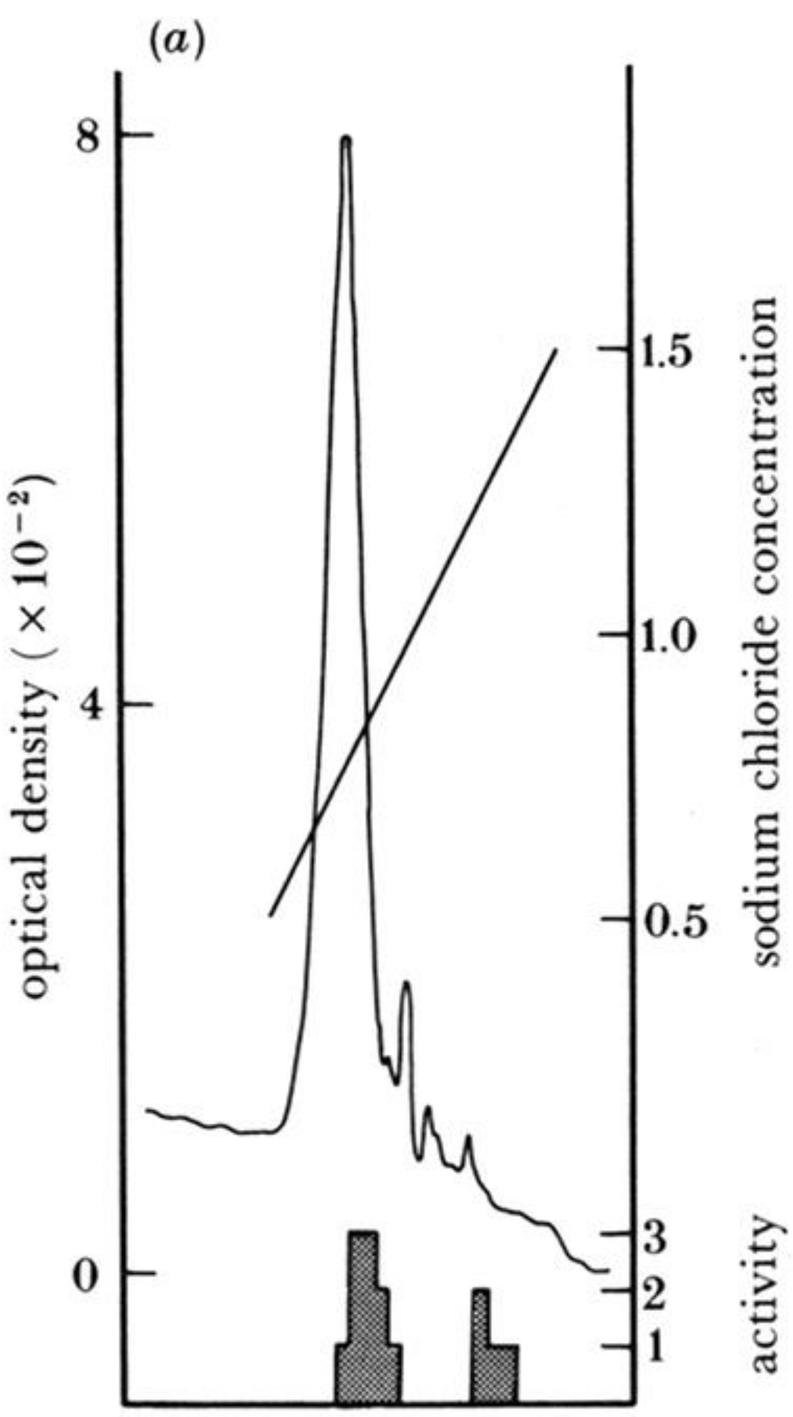
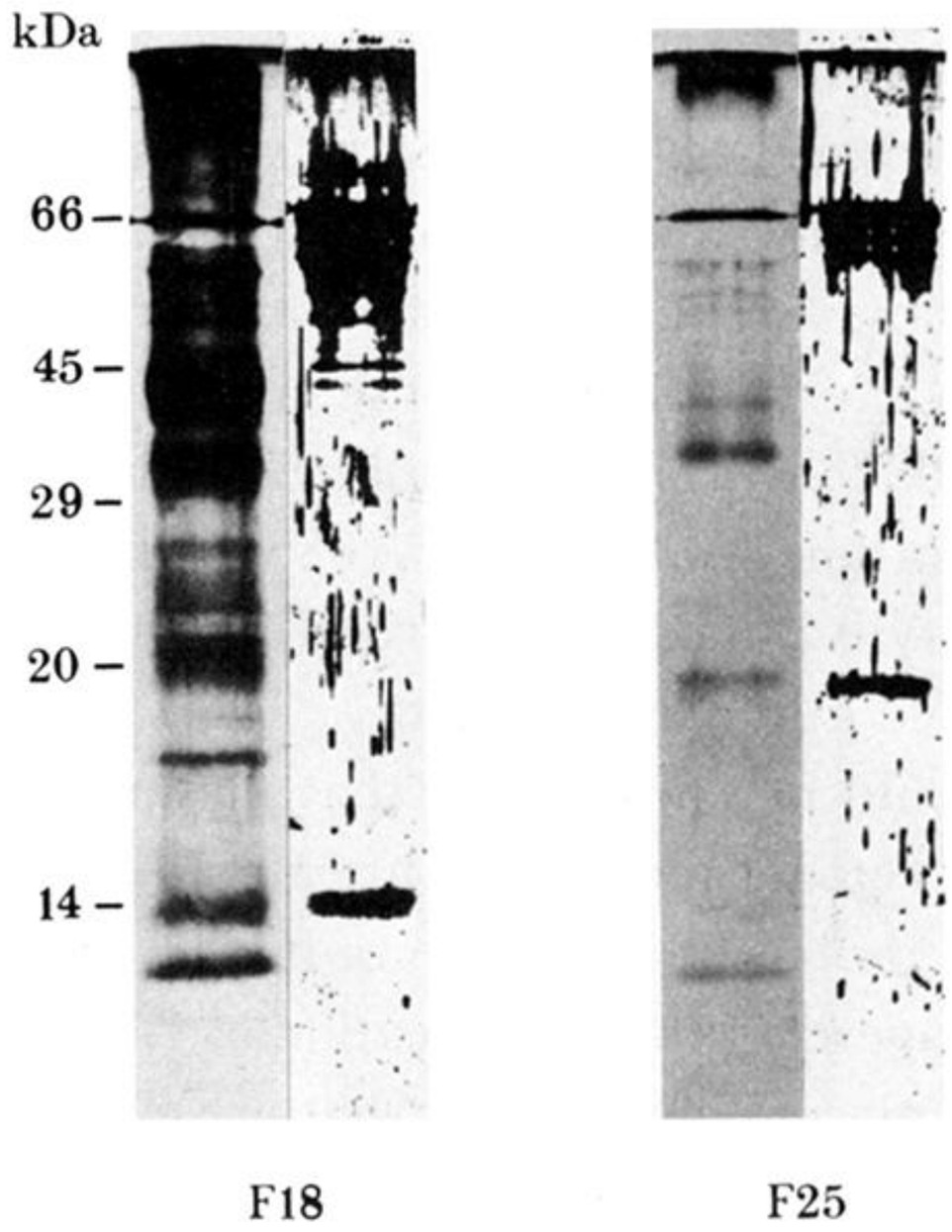
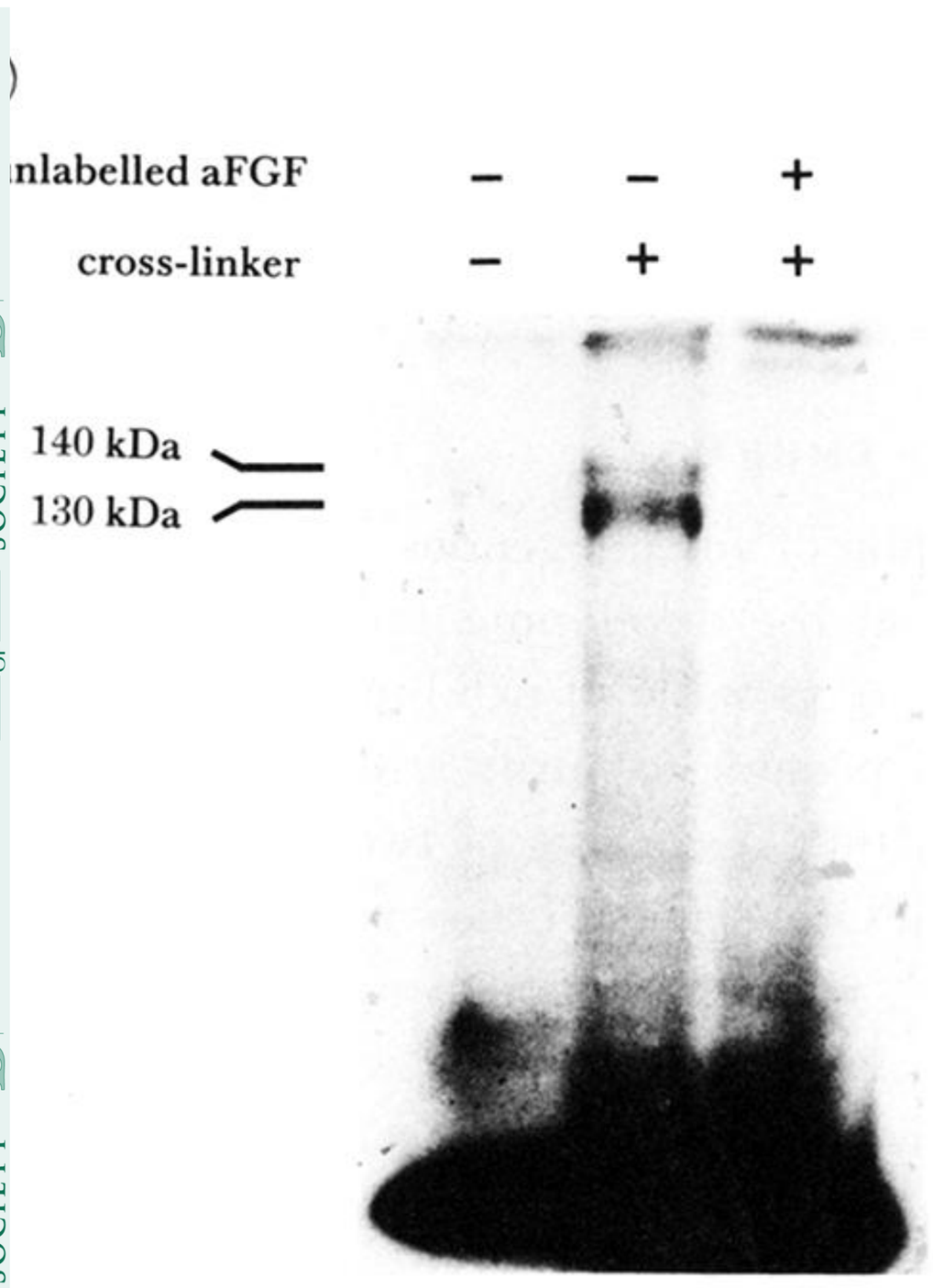


FIGURE 4. For description see opposite.

(*Facing p.* 78)







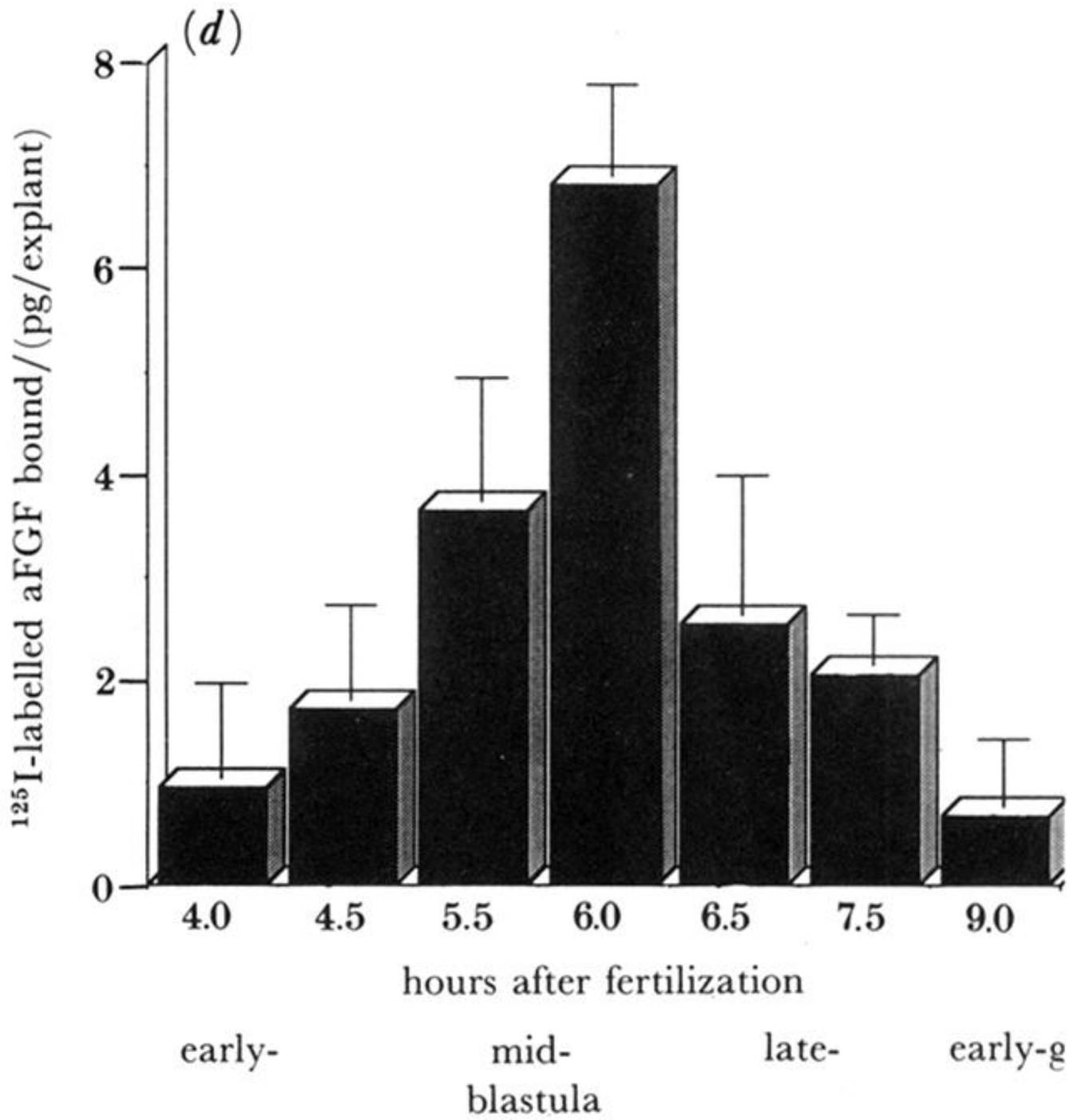


FIGURE 5. (*a*) Purification of mesoderm inducing activity from *Xenopus* ovary by heparin affinity HPLC. (*b*) SDS gels of peak activity fractions. For each, the left track is silver stained and the right is a Western blot stained with an antibody against bovine bFGF. (*c*) Identification of the FGF receptor on *Xenopus* blastula ectoderm by cross-linking of ^{125} I-labelled aFGF. (*d*) Rise and fall of ^{125}I-labelled aFGF binding activity with embryonic stage.