A mesoderm-inducing factor is produced by a *Xenopus* cell line

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Summary

Inductive interactions play a major role in the diversification of cell types during vertebrate development. These interactions have been extensively studied in amphibian embryos (usually *Xenopus laevis*) where the earliest is mesoderm induction, in which an equatorial mesodermal rudiment is induced from the animal hemisphere under the influence of a signal from the vegetal hemisphere. The molecular basis of mesoderm induction is unknown, although Tiedemann has isolated a protein from 9- to 13-day chick embryos that has the properties one would expect of a mesoderm-inducing factor. However, the relevance of this molecule to the events of early amphibian development is unclear, and it is a matter of some importance to discover a *Xenopus* mesoderm-inducing factor.

In this paper I show that the *Xenopus* XTC cell line secretes mesoderm-inducing activity into the culture medium. Isolated animal pole regions cultured in XTC-conditioned medium differentiate into muscle and notochord, while controls form 'atypical epidermis'. Three different cell lines – XL, XL177 and KR – secrete no such activity. Preliminary characterization of the XTC mesoderm-inducing activity indicates that the active principle is heat stable, trypsin sensitive, nondialysable, and has an apparent relative molecular mass of about 16000. Work is in progress to characterize the activity further and to discover whether the mesoderm-inducing factor is also present in normal embryos.

Key words: *Xenopus*, mesoderm induction, morphogen, amphibian embryo.

Introduction

The basic body plan of the amphibian embryo arises through a sequence of inductive interactions (reviewed by Smith, Dale & Slack, 1985). The first is mesoderm induction, in which an equatorial mesodermal rudiment is induced from the animal hemisphere under the influence of a signal from the vegetal hemisphere (Nieuwkoop, 1969, 1973; Dale, Smith & Slack, 1985; Gurdon, Fairman, Mohun & Brennan, 1985). In the absence of this signal the animal hemisphere would become ectoderm.

The molecular basis of mesoderm induction is unknown, but Tiedemann and his colleagues have isolated a substance that has the characteristics we would expect of a 'vegetalizing' or 'mesoderm-inducing' factor (Tiedemann & Tiedemann, 1959; Born, Geithe, Tiedemann, Tiedemann & Kocher-Becker, 1972; Geithe, Asashima, Asahi, Born, Tiedemann & Tiedemann, 1981; Schwartz, Tiedemann & Tiedemann, 1981). This factor is a protein of relative molecular mass 28–30000 isolated from 9- to

13-day chick embryos. When it is applied, in the form of an insoluble pellet, to amphibian blastula ectoderm it causes the formation of a range of mesodermal cell types, including notochord, muscle, kidney and blood (Asashima & Grunz, 1983; Grunz, 1983). The relevance of this chick-derived factor to the events of early amphibian development is not clear, but there is evidence that a molecule with similar activity is also present in early *Xenopus* embryos (Faulhaber, 1972; Faulhaber & Lyra, 1974). However, this factor has not yet been purified: *Xenopus* embryos are small, the substance is likely to be present only at low concentrations and the high concentration of yolk proteins interferes with fractionation. An alternative source of the *Xenopus* factor is urgently required.

The work described in this paper stemmed from the observation that cell surface molecules characteristic of dorsal or ventral compartments in *Drosophila* wing imaginal discs (Wilcox, Brower & Smith, 1981; Brower, Wilcox, Piovant, Smith & Reger, 1984) are present on several *Drosophila* cell lines (Wilcox, Brown, Piovant, Smith & White, 1984). This

suggested that developmentally significant molecules may be expressed by cell lines from other species, and accordingly I tested two *Xenopus* cell lines for meso-derm-inducing activity. Pellets of the XTC cell line (Pudney, Varma & Leake, 1973), placed in close contact with isolated animal pole regions, proved to have powerful mesoderm-inducing activity whereas pellets of XL cells (Anizet, Huwe, Pays & Picard, 1981) had none. It was further found that XTC cells release an active factor into the culture medium while conditioned medium from XL cells, and XL177 and KR cells (Ellison, Mathisen & Miller, 1985), had no mesoderm-inducing activity. The XTC cell line thus provides a convenient and unlimited source of a *Xenopus* mesoderm-inducing factor which may be identical to the natural molecule. Preliminary characterization of the active principle shows that it is heat stable, trypsin sensitive, nondialysable and has an apparent relative molecular mass of 16000. Purification of the factor is in progress.

Materials and methods

Embryos

Embryos of *Xenopus laevis* were obtained by artificial fertilization as described by Smith & Slack (1983). They were chemically dejellied using 2% cysteine hydrochloride (pH 7·8–8·1), washed and transferred to Petri dishes coated with 1% Noble Agar and containing 5% or 10% normal amphibian medium (NAM: Slack, 1984). The embryos were staged according to Nieuwkoop & Faber (1967).

Cells

Four cell lines were used. XTC (Pudney *et al.* 1973) and XL (Anizet *et al.* 1981) cells were supplied by Dr E. A. Jones (University of Warwick). XL177 and KR cells (Ellison *et al.* 1985) were supplied by Dr L. Miller (University of Illinois, Chicago). The cells were maintained at 25°C in Leibovitz L-15 medium diluted to 61% and supplemented with foetal calf serum to 10%. They were usually subcultured once a week at a 1:5 or 1:10 split ratio and fed once a week.

Pellets of cells to be cultured in contact with explanted *Xenopus* animal pole regions were prepared by seeding 1–2×10⁵ cells into the wells of Nunc microwell plates. The wells had previously received 50 μl of molten 1% Noble Agar, which sets to form a shallow cup. The cells roll down the walls of the cups to aggregate into compact masses (F. M. Watt, personal communication).

Conditioned media

Conditioned medium was prepared from confluent cultures of XTC, XL, XL177 or KR cells. The cells were rinsed three times with serum-free L15 medium diluted to 65% and then incubated in the same medium. A volume of 4 ml was used in an 80 cm² flask or 10 ml in a 175 cm² flask. After 24 to 48 h the conditioned medium was removed, centrifuged to remove dead cells and stored at 4°C.

Operations

Manipulations involving the cell lines were carried out in 61% L15 medium supplemented with 10% foetal calf serum. Operations involving only embryo explants were performed in half-strength NAM. Use of half-strength NAM prevented loss of the inner cells of animal pole pieces (see Asashima & Grunz, 1983). In all experiments the piece of animal pole 'test tissue' was a disc of tissue from the centre of the pigmented hemisphere of the stage-7·5 to -8 embryo subtending a solid angle of about 60° (Dale *et al.* 1985). This was dissected out using electrolytically sharpened tungsten needles or mounted eyebrow hairs (a gift of Dr E. A. Jones, Warwick) and hand-ground forceps. In some experiments this piece of tissue was allowed to develop in isolation, in half-strength NAM, in diluted L15 medium or in conditioned medium. In other experiments it was pressed against a pellet of XTC or XL cells and then allowed to develop. In control experiments the test tissue was combined with the vegetal pole region of an embryo at the same stage (Dale *et al.* 1985). Combinations and explants were cultured at room temperature (18–22°C) either for 48 h (controls reach stage 35–38) or for 66 h (controls reach stage 39–41) before analysis.

Histological analysis

Specimens required for histological analysis were fixed for 48 h in a solution of 10% formalin, 2% glacial acetic acid, 50% alcohol and 38% NAM, followed by 48 h in 10% formol saline. They were then block stained in Grenacher's borax carmine before embedding in paraffin wax and sectioning at 10 μm. The sections were counterstained with 0·1% naphthalene black in saturated picric acid (see Slack & Forman, 1980) before mounting in DPX.

Immunofluorescence analysis

Specimens required for immunofluorescence staining were fixed in 2% trichloroacetic acid at 4°C for one hour to overnight. They were dehydrated in ethanol and embedded in polyethylene glycol 400 distearate (Koch) plus 1% cetyl alcohol (Koch-Light) at 40°C (Dreyer, Wang, Wedlich & Hausen, 1983). Sections were cut at 10 μm and brought to PBS-A through an acetone series. The sections were analysed by indirect immunofluorescence exactly as described by Dale *et al.* (1985).

SDS–polyacrylamide gel electrophoresis and immunoblotting

Specimens to be analysed by 'Western' blotting were dissolved directly in gel sample buffer (Laemmli, 1970). They were boiled for 3 min, microfuged and loaded onto gels immediately or stored at −70°C. The samples were run on linear 5–15% polyacrylamide gradient gels using the buffer system of Laemmli (1970). After electrophoresis the separated proteins were transferred to nitrocellulose as described by Towbin, Staehelin & Gordon (1979). The transfer buffer contained 0·1% SDS to facilitate passage of the myosin heavy chain (Nielsen, Manchester, Towbin, Gordon & Thomas, 1982).

After blotting the nitrocellulose membrane was 'blocked' overnight at room temperature in 10% normal goat serum and 4% bovine serum albumin (BSA), in PBS-A. It was

then incubated for 1 h in the appropriate first antibody(ies) diluted in 4 % BSA in PBS-A. After washing in five changes of PBS-A over 1 h, the membrane was incubated in a 1/500 dilution of peroxidase-conjugated affinity-purified goat anti-rabbit IgG (Miles) for 1 h and then washed again. The membrane was probed for peroxidase activity as described by Adams (1981).

Antibodies

Three antibodies were used in this study.

(1) MHC2 is a rabbit antiserum raised against adult *Xenopus laevis* myosin heavy chain and characterized by Western blotting and immunofluorescence (see Dale *et al.* 1985). This antibody stains muscle from stage 35 onwards and does not react with any tissue at earlier stages. An antibody with similar characteristics was raised against a total adult *Xenopus laevis* myosin preparation and this was also used in the present experiments.

(2) 12/101 is a monoclonal antibody raised against newt muscle (Kintner & Brockes, 1984). Using the immunofluorescence procedure described above this antibody recognizes *Xenopus* somite muscle from stage 23 onwards.

(3) A rabbit antiserum was prepared against adult *Xenopus* keratin-like protein II (Reeves, 1975). On sections this antibody interacts with larval and adult epidermis and on Western blots it recognizes at least four bands between $M_r = 50\,000$ and $M_r = 60\,000$.

Gel filtration

Samples of serum-free conditioned medium were concentrated to 1/10 of their original volume by ultrafiltration with Amicon YM2, YM5 or YM10 membranes. Ammonium sulphate was added to 90 % of saturation and the suspensions were stirred for 30 min. After centrifugation at 10 000 g for 10–20 min pellets were dissolved in 2–5 ml of column buffer (see Results), microfuged and loaded onto a 40×2·6 cm column of Ultrogel AcA 54 (LKB). The column was run at 24 ml h^{-1} and 4 ml fractions were collected. The

column was calibrated with bovine serum albumin ($M_r = 66\,000$), ovalbumin ($M_r = 45\,000$), soybean trypsin inhibitor ($M_r = 20\,100$) and cytochrome C ($M_r = 12\,300$).

Protein determinations were made according to Bradford (1976).

Results

Pellets of XTC, but not XL, cells induce mesoderm from animal pole explants

When the animal pole region of a midblastula (stage 7·5–8) *Xenopus* embryo is dissected out and cultured alone in a simple salts solution it forms epidermis; the vegetal pole region under similar conditions fails to differentiate while the equatorial region forms most of the structures present in a normal embryo (Dale *et al.* 1985). The phenomenon of mesoderm induction can be demonstrated by placing a piece of animal pole tissue in contact with a vegetal pole piece (Fig. 1A); significant quantities of mesodermal cell types, including notochord, muscle, kidney and blood, are formed from the animal pole component (Nakamura, Takasaki & Ishihara, 1970; Sudarwati & Nieuwkoop, 1971; Dale *et al.* 1985; Gurdon *et al.* 1985). In preliminary experiments to test *Xenopus* cell lines for mesoderm-inducing activity, pellets of XTC and XL cells were prepared as described in the Materials and Methods section. Pieces of these pellets, roughly the size of a vegetal pole isolate, were placed in contact with stage 7·5–8 animal pole regions (Fig. 1B) and the combinations were allowed to develop for about 65 h before being fixed, sectioned and analysed by indirect immunofluorescence using antibodies specific for muscle and epidermis. Like others (Gurdon *et al.* 1985) I used muscle development as the
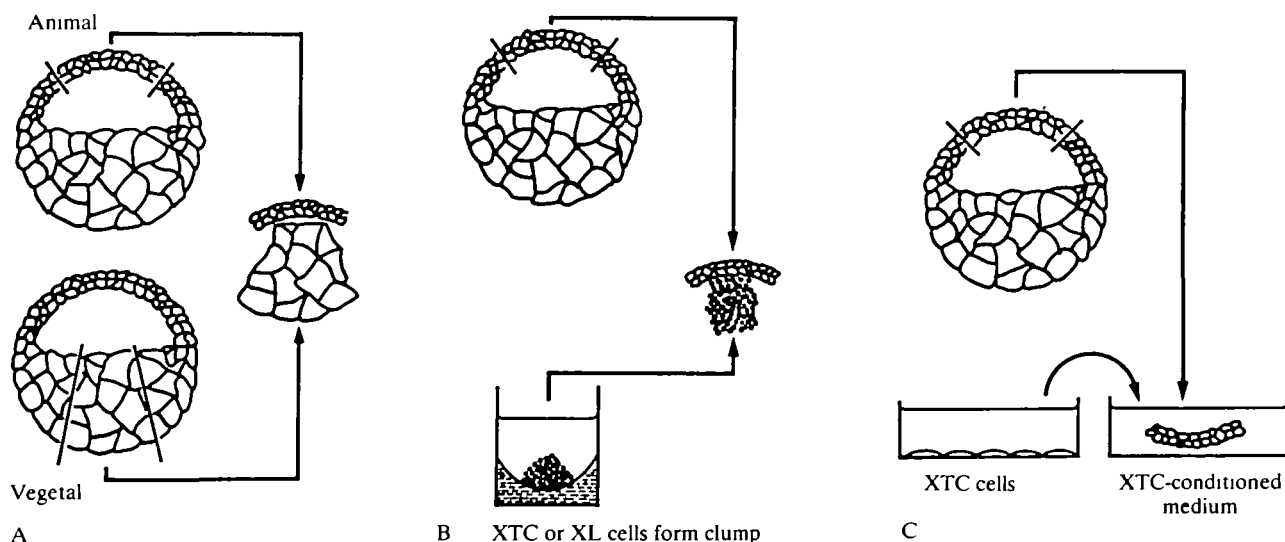

Fig. 1. The experimental design. (A) Mesoderm induction is demonstrated by combining animal pole tissue with vegetal pole tissue. (B) A pellet of XTC or XL cells is pressed against the blastocoel surface of an isolated animal pole region. (C) Conditioned medium from XTC cells is used as the culture medium for isolated animal pole regions.

criterion for mesoderm induction because muscle is both the most abundant mesoderm-derived tissue (Cooke, 1983) and the most abundant cell type to arise from animal–vegetal combinations (Dale *et al.* 1985).

24 combinations of animal pole regions with XL cells were made and in no case was muscle development observed (Fig. 2A,B); most cells from the animal pole component developed as epidermis, the cells staining with an antibody to keratin. In contrast, muscle development was observed in the animal pole component of 24 out of 25 combinations involving XTC cells (Fig. 2C,D). The muscle cells were usually positioned near the centre of the animal pole region, sometimes, but not always, adjacent to the XTC cells. The external surface of the animal pole region invariably developed as epidermis. In control experiments, also carried out at stage 7·5–8, muscle

development occurred in all of 13 animal–vegetal combinations but in none of 8 animal pole explants allowed to develop in isolation.

XTC cells secrete a mesoderm-inducing factor

The above results suggested that XTC, but not XL, cells produce a mesoderm-inducing factor. To investigate this further, serum-free conditioned medium from both cell lines (prepared as described in the Materials and Methods) was tested for inducing activity by using it as the culture medium for newly dissected midblastula animal pole pieces (Fig. 1C). After about 66h of culture the explants were fixed, sectioned and analysed by indirect immunofluorescence. In preliminary trials, explants cultured in XTC-conditioned medium frequently formed large
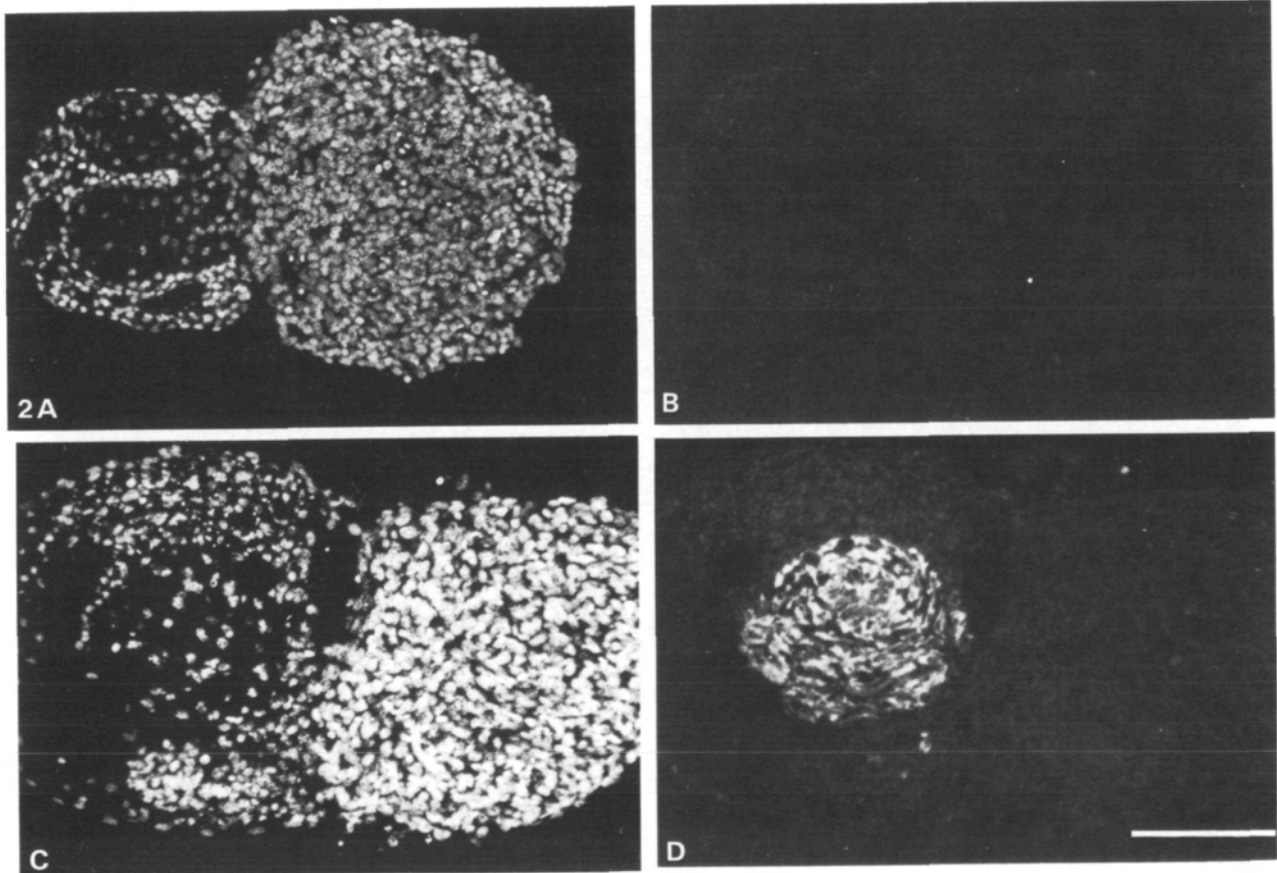
Fig. 2. Mesoderm induction by a pellet of XTC cells but not by a pellet of XL cells. Isolated *Xenopus* blastula animal pole regions were pressed against a pellet of XTC or XL cells and allowed to develop for 65 h at 18–22°C in modified L15 medium containing 10 % foetal calf serum. After fixation and sectioning, samples were analysed by indirect immunofluorescence using an antibody raised against *Xenopus* myosin heavy chain. (A,B) A combination between XL cells and *Xenopus* animal pole tissue stained both with 4′,6-diamidino-2-phenylindole-dihydrochloride (DAPI), to show nuclei, and with anti-myosin heavy chain. (A) DAPI staining. The animal pole component, with fewer cells, is to the left. (B) The section is negative for myosin heavy chain. (C,D) A combination between XTC cells and animal pole tissue stained with DAPI and anti-myosin heavy chain. (C) DAPI staining. The animal pole component is to the left. (D) The animal pole cells have formed muscle.
   Scale bar in (D) is 200 μm and also applies to (A), (B) and (C).
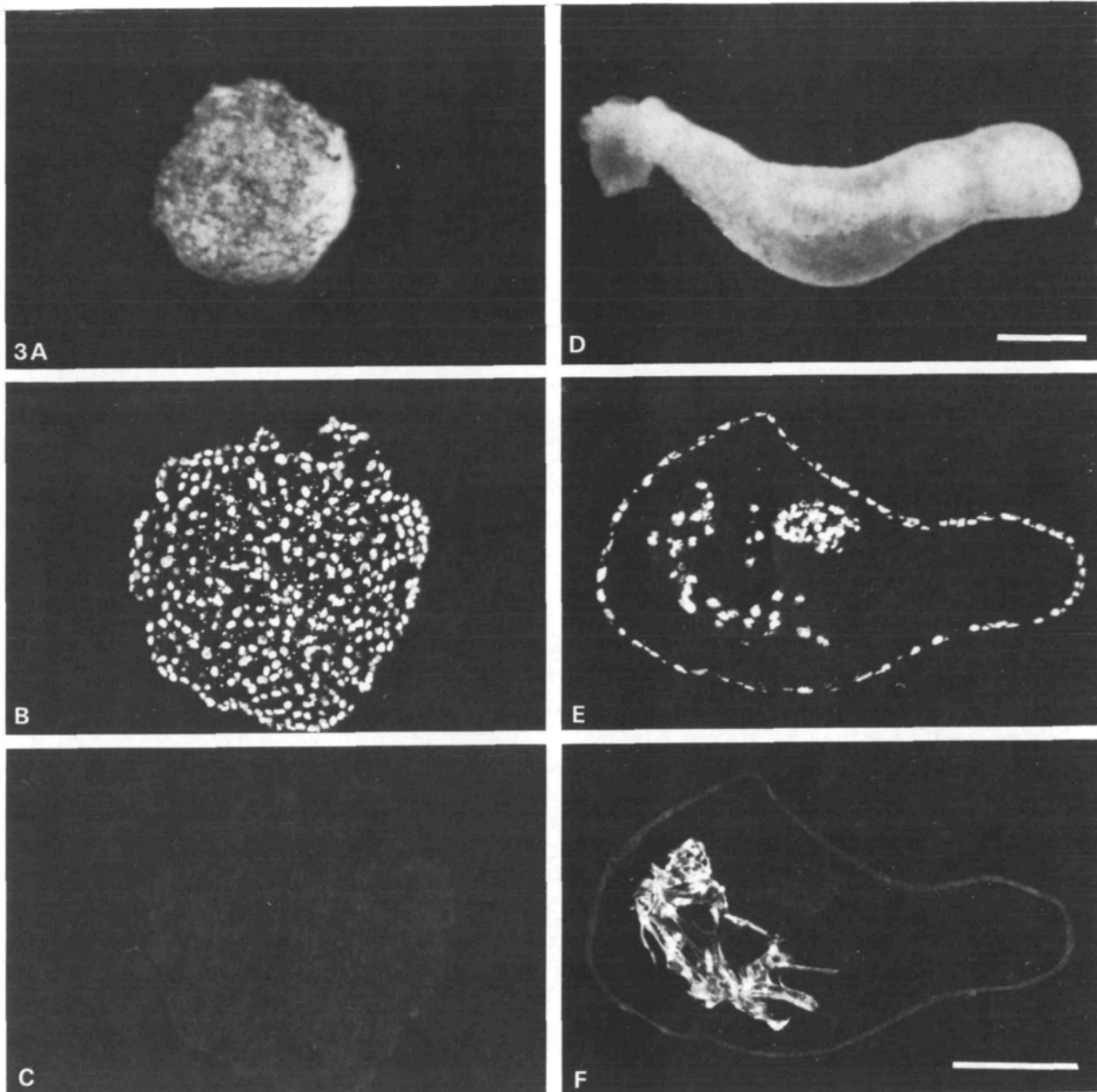
Fig. 3. Mesoderm induction by XTC-, but not XL-, conditioned medium. Midblastula-stage animal pole regions were cultured in heated conditioned media for about 16 h before being transferred to half-strength NAM for about 48 h. They were then fixed and analysed by indirect immunofluorescence. (A–C) Explants cultured in XL-conditioned medium. (A) This explant has formed a wrinkled ciliated sphere. (B) A section (of a different explant) stained with DAPI to show the nuclei. (C) The same section stained with 12/101; no muscle is formed. (D–F) Explants cultured in XTC-conditioned medium. (D) This explant has become elongated and acquired some internal structure. (E) A section (of a different explant) stained with DAPI. (F) The same section stained with 12/101; large amounts of muscle have been formed.

Scale bar in (D) is 500 μm and also applies to (A). Scale bar in (F) is 200 μm and also applies to (B), (C) and (E).

amounts of muscle, while results with XL-conditioned medium were negative. During these experiments it was found (see below) that heating XTC-conditioned medium to 95 °C for 5 min enhanced mesoderm-inducing activity tenfold. Since this observation, 16 series of experiments with different batches of heated and unheated XTC-conditioned medium have been analysed by immunofluorescence, and 6 with XL-conditioned medium, involving a total of 736 explants.

Explants cultured in XL-conditioned medium, whether heated or not, usually formed spheres of wrinkled, ciliated epidermis (Fig. 3A) which contained no cells reacting with the anti-muscle antibodies (Fig. 3B,C). One batch of XL-conditioned medium did, however, have weak inductive activity, with a single explant forming wisps of muscle. By contrast, explants cultured in XTC-conditioned medium formed elongated structures (Fig. 3D), presaged by a period of dramatic cell movement at the time when donor embryos would have entered gastrulation (Symes & Smith, in preparation). Sections of such explants fixed after 65 h of culture revealed large masses of muscle inside a covering of epidermis (Fig. 3E,F). Different batches of XTC-conditioned medium differed in their specific activities, but before heating they were usually active at dilutions of 1:2 to 1:4 (about 8–16 μg protein ml^{-1}) while after heating activity was normally detectable at dilutions of 1:16 to 1:32 (1–2 μg protein ml^{-1}).

XTC-conditioned medium induces a variety of mesodermal cell types

Although muscle development is used here as the criterion for mesoderm induction, XTC-conditioned medium induces other mesodermal cell types, including notochord, mesenchyme and mesothelium (Fig. 4A). Structures resembling kidney tubules are occasionally formed (Fig. 4A) but red blood cells have not yet been unequivocally identified; this is currently under investigation using an antibody to *Xenopus* globins.

Ectodermal differentiation is also affected by XTC-conditioned medium. In uninduced animal pole explants the cells form 'atypical epidermis', in which all the cells stain with antibodies to keratin (Dale *et al.* 1985; Smith *et al.* 1985) but which lacks the normal histological features of *Xenopus* larval epidermis (Fig. 4C). In induced explants, however, the epidermis surrounding the induced mesodermal cell types more closely resembles normal skin (Fig. 4A,B). Some of the animal pole ectoderm, furthermore, forms neural cell types, including neuroepithelium and melanocytes (Fig. 4B). It is probable that the nervous tissue arises from interaction between newly induced mesoderm and uninduced ectoderm (Suzuki, Yoshimura & Yano, 1986), but it is not possible to exclude the possibility that XTC-conditioned medium also contains neural induction activity.

The effects of the concentration of conditioned medium and of the stage of the responding tissue on the pattern of cell differentiation are under investigation.

Mesoderm-inducing activity is heat stable, nondialysable and trypsin sensitive

Histological analysis is necessary to study the spatial pattern of cellular differentiation in induced explants but it is rather slow and inconvenient for preparing dose–response curves of inducing activity or for
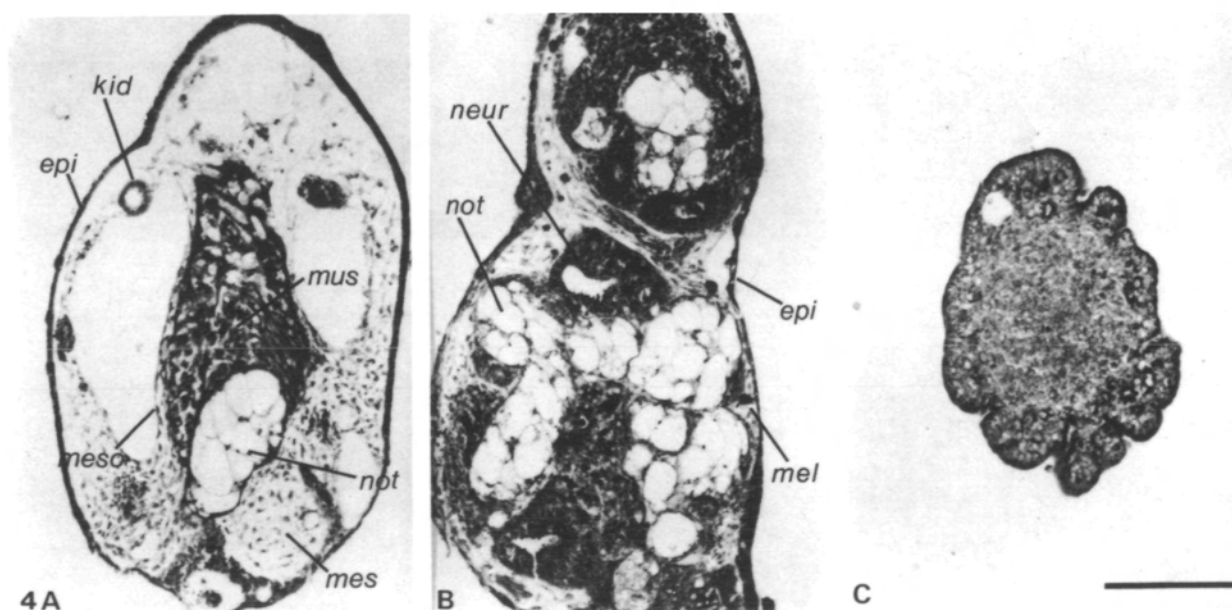

Fig. 4. Histological cell types formed in response to heated XTC-conditioned medium. (A) An explant showing notochord (*not*), muscle (*mus*), kidney (*kid*), mesenchyme (*mes*) and mesothelium (*meso*). The epidermis (*epi*) shows good histological differentiation. (B) An explant demonstrating large amounts of notochord (*not*) with neuroepithelium (*neur*) and melanocytes (*mel*). The epidermis (*epi*) is well differentiated. (C) An explant cultured in half-strength NAM forms 'atypical epidermis'. Scale bar in (C) is 200 μm and also applies to (A) and (B).

testing column fractions during purification. Further-more, histological analysis does not permit a rapid visual comparison of different treatments. Fig. 5 therefore shows the result of an experiment in which groups of three animal pole isolates were incubated in different concentrations of heated or unheated XTC- or XL-conditioned medium, solubilized in gel sample buffer, and run on a 5–15 % polyacrylamide gradient gel. The separated proteins were blotted onto nitro-cellulose and probed simultaneously with the anti-body to *Xenopus* myosin heavy chain, to detect muscle and the antibody to *Xenopus* keratin, specific for epidermis, to confirm that the explants were viable. The presence of myosin heavy chain in these experiments is taken as indicating that mesoderm induction has occurred. The experiment shows that this batch of unheated XL-conditioned medium lacks mesoderm-inducing activity completely and that ac-tivity is only just detectable after heating to 95 °C for 5 min. Other batches of heated XL-conditioned medium tested in the same way were completely inactive. By contrast, unheated XTC-conditioned medium has strong mesoderm-inducing activity at a 1:3 dilution (protein concentration of $6.7 \mu g \, ml^{-1}$) and after heating to 95 °C for 5 min it is active at a 1:30

dilution ($0.7 \mu g \, ml^{-1}$). Further experiments demon-strated that mesoderm-inducing activity is retained even after heating to 95 °C for 1 h, although activity is somewhat reduced, returning to the unheated level (data not shown).

A similar approach was adopted to demonstrate that XTC mesoderm-inducing activity is nondia-lysable and excluded by Sephadex G-25. Two mol-ecular-weight cut-off sizes were used for dialysis: $M_r = 6-8000$ and $M_r = 12-14000$ (membranes ob-tained from BRL). Mesoderm-inducing activity was retained by both membranes although there were slight losses which could be prevented by the in-clusion of 0.02 % Tween 20 in the conditioned me-dium (data not shown).

The trypsin sensitivity of XTC mesoderm-inducing activity was established by incubating heated serum-free conditioned medium with $500 \mu g \, ml^{-1}$, $100 \mu g \, ml^{-1}$ or $20 \mu g \, ml^{-1}$ trypsin (Type IX, Sigma, 15 000 BAEE units per mg protein) at 37 °C for 1 h. Soybean trypsin inhibitor was then added to $1250 \mu g$ ml^{-1}, $250 \mu g \, ml^{-1}$ or $50 \mu g \, ml^{-1}$ and serial dilutions were assayed for mesoderm-inducing activity. Two experiments were carried out and in both the XTC-
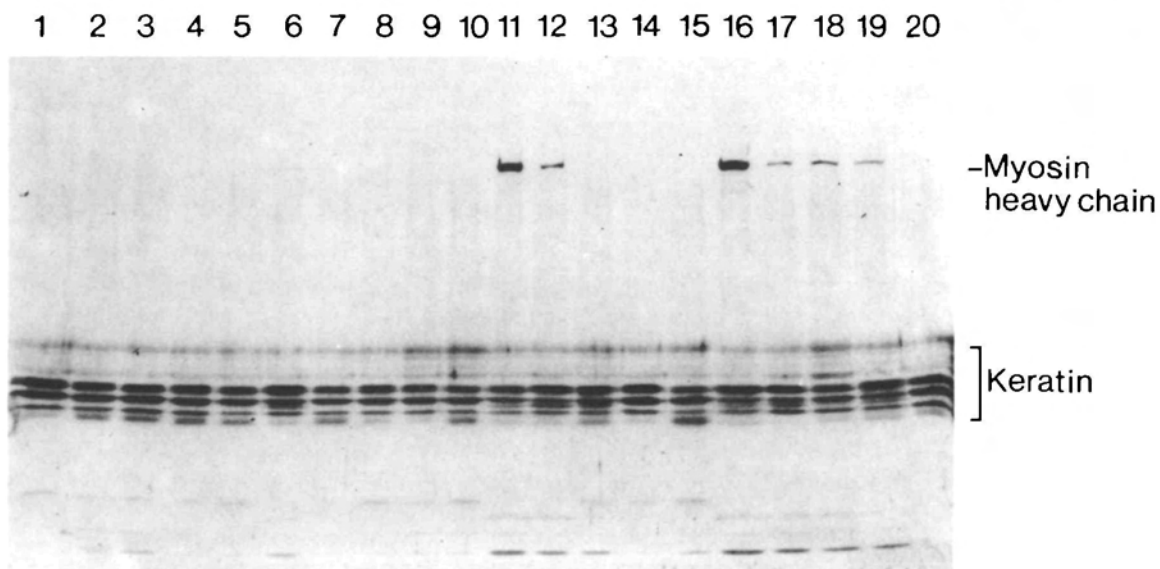
Fig. 5. Titration of XTC- and XL-conditioned media by Western blotting. Groups of three animal pole explants were exposed for 16 h to serial dilutions of XL-conditioned medium, heated or unheated, or XTC-conditioned medium, heated or unheated. They were then transferred to half-strength NAM and cultured for 48 h until controls reached stage 40. Samples were solubilized in gel sample buffer, boiled and run on a linear 5–15 % polyacrylamide gradient gel. The separated proteins were transferred electrophoretically to nitrocellulose and probed simultaneously with an antibody recognizing *Xenopus* myosin heavy chain ($M_r = 205000$) and an antibody against *Xenopus* keratin-like protein II (which recognizes at least four bands between $M_r = 50000$ and $M_r = 60000$). Tracks 1–5: XL-conditioned medium (unheated) at protein concentrations of 41.4, 13.8, 4.1, 1.4 and $0.4 \mu g \, ml^{-1}$ respectively. Tracks 6–10: XL-conditioned medium (heated to 95 °C for 5 min) at 34.2, 11.4, 3.4, 1.1 and $0.3 \mu g \, ml^{-1}$ respectively. Tracks 11–15: XTC-conditioned medium (unheated) at 20, 6.7, 2.0, 0.7 and $0.2 \mu g \, ml^{-1}$ respectively. Tracks 16–20: XTC-conditioned medium (heated to 95 °C for 5 min) at 20, 6.7, 2.0, 0.7 and $0.2 \mu g \, ml^{-1}$ respectively. Note that XL-conditioned medium is only slightly active even after heating, and that the mesoderm-inducing activity of XTC-conditioned medium is enhanced by a factor of 10 as a result of heating.

Table 1. *Mesoderm-inducing activity in XTC-conditioned medium is trypsin sensitive*

Treatment	TCA precipitable counts remaining (%)	Conditioned medium concentration (%)			
		100	33	10	3·3
No trypsin	100	+	+	+	+
20 µg ml⁻¹ trypsin	31	+	+	+	−
100 µg ml⁻¹ trypsin	24	+	±	−	−
500 µg ml⁻¹ trypsin	24	−	−	−	−
500 µg ml⁻¹ trypsin + trypsin inhibitor	89	+	+	+	+

Conditioned medium was prepared from twelve confluent 100 mm plates of XTC cells. Eight plates received 3·5 ml of modified serum-free L15 medium while four received the same volume of medium with the methionine concentration reduced to 10 % of the normal level and with 100 µCi of [³⁵S]methionine (Amersham). Incubation in serum-free medium was for 24 h and trypsin treatment of heated conditioned medium was for 1 h at 37°C. The proteolytic effect of trypsin treatment was assessed on the radioactive samples. One aliquot of each sample was TCA precipitated and counted in a scintillation counter while another was acetone precipitated, dissolved in gel sample buffer and run on a 5–15 % polyacrylamide gradient gel. The gel was prepared for fluorography (Bonner & Laskey, 1974) and exposed to preflashed X-ray film; the results are described in the text. Mesoderm-inducing activity of the trypsin-treated conditioned media was assayed by 'Western' blotting, as described in the text. A '+' sign indicates a strong myosin heavy chain band. '±' indicates a weak signal and '−' indicates no visible signal.

conditioned medium was made radioactive by including [³⁵S]methionine in the serum-free culture. This facilitated analysis of the effectiveness of the trypsin.

The results of one experiment are shown in Table 1; the other gave similar results. 500 µg ml⁻¹ trypsin completely abolished mesoderm-inducing activity, while 100 µg ml⁻¹ removed at least 90 % of the activity and 20 µg ml⁻¹ removed at least 67 %. Poly-acrylamide gel electrophoresis of the trypsin-treated samples followed by fluorography showed that all three concentrations of trypsin removed high molecular weight ($M_r > 30000$) components but that some lower molecular weight proteins were resistant to 20 µg ml⁻¹ and 100 µg ml⁻¹ trypsin. Virtually no bands were visible after 500 µg ml⁻¹ trypsin. In control experiments simultaneous addition of 500 µg ml⁻¹ trypsin and 1250 µg ml⁻¹ trypsin inhibitor to heated XTC-conditioned medium did not abolish mesoderm-inducing activity (Table 1) and addition of these components to modified L15 medium did not introduce mesoderm-inducing activity (data not shown).

Characterization of mesoderm-inducing activity using gel filtration

A preliminary characterization of the mesoderm-inducing activity of XTC-conditioned medium was carried out using gel filtration. 100 ml batches of heated conditioned medium were concentrated by ultrafiltration and ammonium sulphate precipitation, and the resulting pellet was dissolved in column buffer (2–5 ml). Initial experiments, in which the column was run in 0·1 M- to 0·5 M-NaCl, buffered with 10 mM-sodium phosphate at pH 7·4, gave variable results. Out of eight experiments, three gave an apparent relative molecular mass of 10–13000 for mesoderm-inducing activity. Four of the remaining five experiments again showed activity at $M_r =$ 10–13000 but with an additional, larger, peak around $M_r = 60$–66000 and one experiment showed activity exclusively at $M_r = 66000$.

It seemed possible that this variability was due to interaction of mesoderm-inducing activity with bovine serum albumin, the major protein component of XTC-conditioned medium, and which is derived from the foetal calf serum used in the cell growth medium. In an attempt to abolish such an interaction, columns were run in the presence of low concentrations of detergent, either 0·1 % sodium deoxycholate or 0·1 % Brij 58, neither of which show significant u.v. absorbance. Five experiments (three with sodium deoxycholate and two with Brij) gave similar results, with apparent M_r's for mesoderm-inducing activity of 13–18000, and an average of 16000. Fig. 6 shows a typical result, using 0·1 % sodium deoxycholate and conditioned medium prepared in the presence of [³⁵S]methionine.

XTC and XL cells

Why should XTC cells secrete a mesoderm-inducing factor while XL cells, and XL177 and KR cells (data not shown) do not? One trivial possibility is that the XTC cells simply grow faster, but this is not the case: in this laboratory the XTC and XL lines both grow with a doubling time of 39 h (data not shown). A more interesting possibility is that the XTC cells are of endodermal origin and have 'remembered' their early embryological function, while the other lines were derived from ectodermal, or perhaps meso-dermal, cell types. Unfortunately, in the absence of germ-layer-specific markers, it is not possible to answer this question directly and the alternative approach of studying the derivation of the cell lines, gives only limited information. Thus, the XTC cell line was derived from a metamorphosing tadpole that had had its skin, eyes, intestine and tail removed (Pudney *et al.* 1973); the XL line was derived from whole swimming larvae (Anizet *et al.* 1981); the XL177 line was derived from tadpoles that had had their epidermis removed (Miller & Daniel, 1977); and the KR line is a clone of the adult kidney line A6 (Rafferty, 1969; see Ellison *et al.* 1985).

Differences in the polyacrylamide gel electrophoresis pattern of proteins secreted by the cell lines are, however, consistent with the suggestion that they are of different origins. Fig. 7 shows a one-dimensional polyacrylamide gel of the proteins synthesized by XTC and XL cells. The cell-associated proteins of the two cell lines are similar, but the secreted protein patterns differ significantly. The patterns are not altered by brief heat treatment. It is probable that a low M_r protein peculiar to the XTC line is responsible

for mesoderm induction and this is now under investigation.

Discussion

The results described in this paper show that the *Xenopus* XTC cell line secretes mesoderm-inducing activity capable of converting animal pole ectoderm to mesodermal cell types, including notochord, muscle, mesenchyme and mesothelium. Preliminary
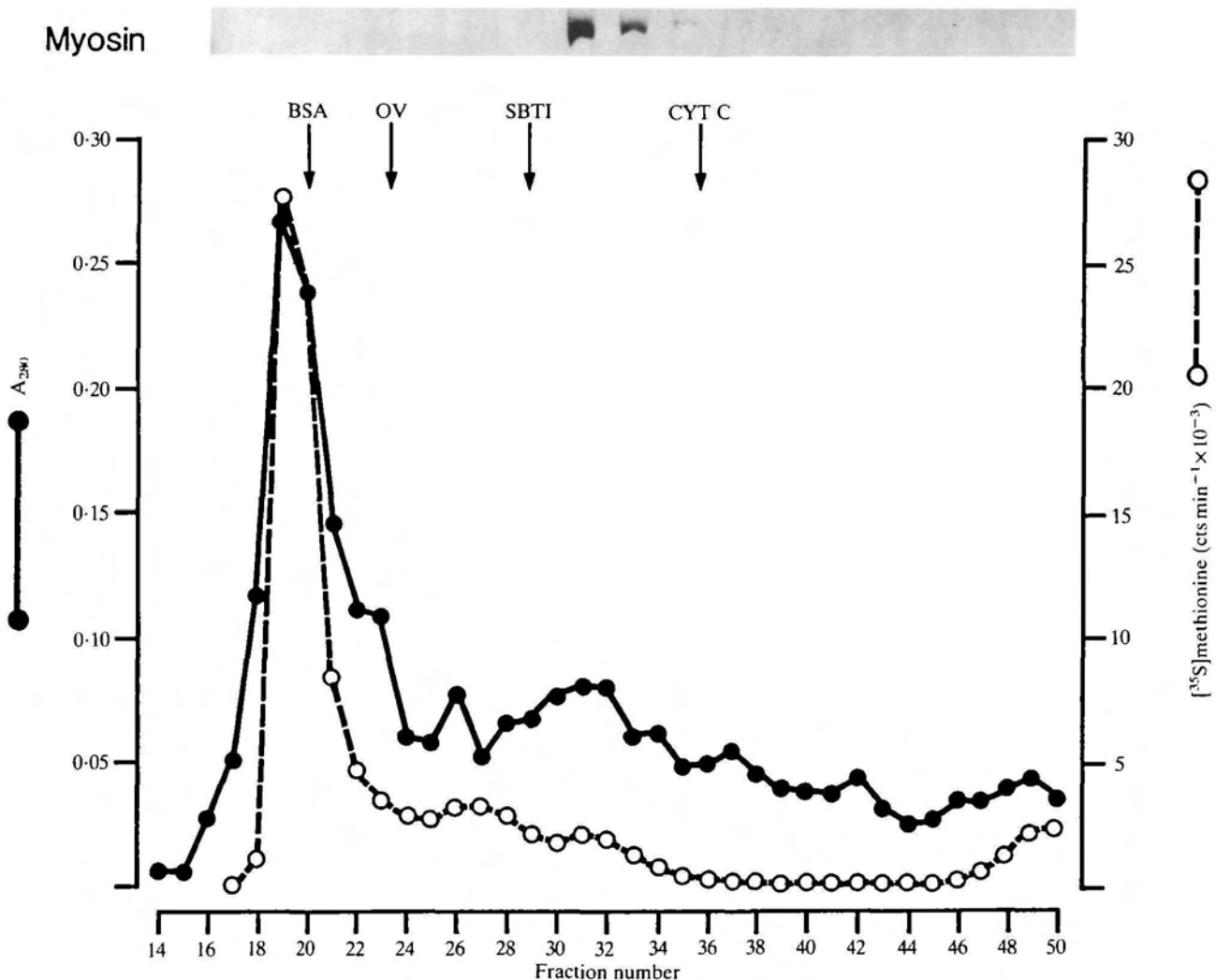
Fig. 6. The mesoderm-inducing activity of XTC-conditioned medium has an apparent relative molecular mass of about 16 000. 120 ml of serum-free XTC-conditioned medium was prepared from ten confluent 150 mm tissue culture plates. One plate contained serum-free L15 with 10 % of the normal methionine concentration and 20 μCi of [^{35}S]methionine. The conditioned medium was centrifuged to remove cell debris, heated to 95°C for 5 min, and centrifuged at 10 000 g for 20 min. This medium was concentrated with an Amicon YM2 membrane, ammonium sulphate precipitated and dissolved in 5 ml of 0·1 M-NaCl, 1 mM-EDTA, 20 mM-tris pH 8·0, 0·1 % sodium deoxycholate. 4 ml of this sample was applied to an AcA 54 gel filtration column equilibrated in the same buffer, and 4 ml fractions were collected. The fractions were assayed at a 1/20 dilution. The figure shows protein absorbance at 280 nm (●) and ^{35}S-radioactivity (○). Mesoderm-inducing activity was assayed by Western blotting and the region of the blot containing the myosin heavy chain is shown above the graph. Only the odd-numbered fractions are presented here. Fractions 31 and 33 induced substantial quantities of myosin heavy chain while a very weak band is visible at fraction 35. Arrows indicate relative molecular mass markers: BSA ($M_r = 66\,000$), ovalbumin (OV; $M_r = 45\,000$), soybean trypsin inhibitor (SBTI; $M_r = 20\,100$) and cytochrome C (CYT C; $M_r = 12\,300$).
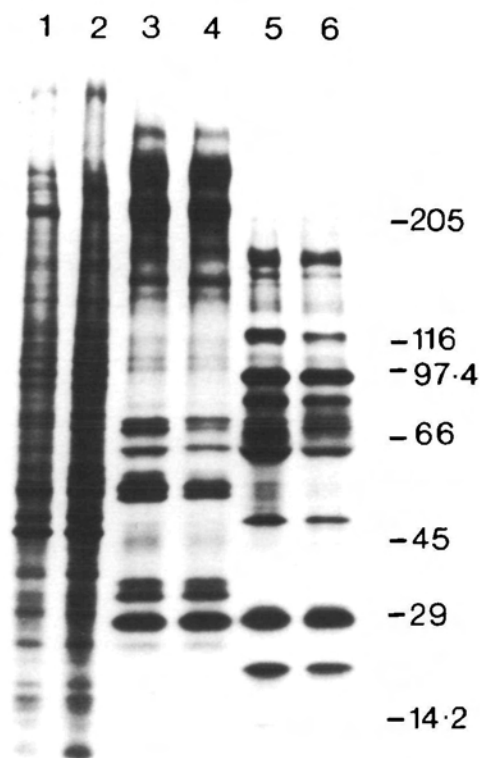
Fig. 7. Protein synthesis by XTC and XL cells. Confluent cultures of XTC or XL cells were rinsed three times with serum-free modified L15 medium and then incubated for 24 h in the same medium but with the methionine concentration at 10 % of the normal level, and with 25 μCi ml^{-1} [^{35}S]methionine. Samples of labelled conditioned medium, either heated or unheated, were prepared for gel electrophoresis by acetone precipitation and solubilization in gel sample buffer. The labelled XTC and XL cells were rinsed in 70 % PBS-A and solubilized directly into gel sample buffer. After boiling, equal counts were run on a linear 5–15 % polyacrylamide gradient gel, which was fluorographed and exposed to X-ray film. Lane 1, proteins associated with XTC cells; lane 2, proteins associated with XL cells. Lane 3, XTC-conditioned medium; lane 4, heated XTC-conditioned medium; lane 5, XL-conditioned medium; lane 6, heated XL-conditioned medium. Relative molecular mass markers are shown ($\times 10^{-3}$).

characterization of this factor shows that it is heat stable, nondialysable, trypsin sensitive, and has an apparent relative molecular mass of about 16 000.

The relationship of the *Xenopus* cell line meso-derm-inducing activity to other mesoderm-inducing factors is unknown. The best characterized of these is Tiedemann's 'vegetalizing factor', which is isolated from 9- to 13-day chicken embryos, and has an apparent relative molecular mass of 28–30 000, although this separates into smaller chains of $M_r =$ 13–15 000 in formic acid (Geithe *et al.* 1981). Other sources of mesoderm-inducing factors include guinea pig bone marrow (Toivonen, 1953), HeLa cells (Saxen & Toivonen, 1958), carp swimbladder (Kawakami, 1976) and even *Xenopus* blastulae and gastrulae (Faulhaber, 1972; Faulhaber & Lyra, 1974), although the limited amount of material available from the latter source rules it out as a useful starting material for purification.

In the absence, therefore, of an alternative source of a *Xenopus* inducing factor it is tempting to speculate that the XTC factor is identical to a natural inducer molecule. One way to test this will be to raise antibodies against the factor and use them to localize the antigen in normal embryos and to interfere with its action (Woodland & Jones, 1985). Similar experiments may be possible by cloning the gene for the factor and microinjecting anti-sense RNA into vegetal pole blastomeres of early embryos (Melton, 1985; Weintraub, Izant & Harland, 1985); it is possible that the maternal mRNA for a mesoderm-inducing factor is localized in the vegetal hemisphere (Rebagliati, Weeks, Harvey & Melton, 1985).

Even if the XTC mesoderm-inducing factor is not identical to a natural molecule the results described in this paper offer an opportunity to analyse the response of cells in the animal hemisphere to mesoderm induction. One distinct advantage of the XTC factor in this regard is that, unlike the 'vegetalizing factor', it is active in soluble form; this allows much more accurate quantitation of mesoderm-inducing activity (see Yamada & Takata, 1961). Thus it will be important to assess the effects of different concentrations of inducing factor and of the developmental stage of the responding animal pole on which mesodermal cell types are induced. Attempts to study this question with the vegetalizing factor (Grunz, 1983) suggest that brief treatment or low concentrations of inducer cause the formation of blood cells and heart structures while longer treatments, or higher concentrations, tend to induce more dorsal structures like somite and notochord. This would seem to support the suggestion that the complex pattern of cell types in the mesoderm can arise from the diffusion of a single inductive factor (Nieuwkoop, 1973; Weyer, Nieuwkoop & Lindenmayer, 1977), although with Dale and Slack I have argued that at least two signals are required, one specifying dorsal and one specifying ventral structures (Dale *et al.* 1985; Smith *et al.* 1985). The apparent absence of red blood cells in animal pole explants treated with XTC-conditioned medium might suggest that if two signals *are* required, the XTC factor is the dorsal one, with the ventral factor as yet undiscovered. However, an alternative explanation for the absence of red blood cells would be that erythrocyte differentiation is dependent on the presence of endoderm (Capuron & Maufroid, 1981; Deparis & Jaylet, 1984).

Another question that can now be approached is that of the immediate biochemical response to mesoderm induction; preliminary results indicate that 15 min exposure to high concentrations of XTC-conditioned medium is sufficient to 'mesodermalize' animal pole cells (Cooke & Smith, unpublished observations) and this suggests that the cellular response to induction is quite rapid. In contrast, at least 2 h exposure to vegetal pole tissue is required for subsequent muscle-specific actin gene expression by animal pole cells (Gurdon *et al.* 1985). This may be due to lower levels of morphogenetic activity in the natural inducer.

Finally, it is of interest that heat treatment of XTC-conditioned medium enhances its activity by a factor of ten (Fig. 5). A similar observation has been made with a mesoderm-inducing extract of carp swimbladder, where one suggestion was that heating destroyed an inhibitor of mesoderm induction (Kawakami, Noda, Kurihara & Okuma, 1977), and indeed an inhibitor of the chick vegetalizing factor has been isolated by Born, Tiedemann & Tiedemann (1972). Such an inhibitor, if more diffusible than the 'activator', could explain why the mesoderm only forms in an equatorial band around the embryo and not throughout the entire animal hemisphere (Meinhardt, 1982).

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