

Cytokeratin filament assembly in the preimplantation mouse embryo

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Summary

The timing, spatial distribution and control of cyto-keratin assembly during mouse early development has been studied using a monoclonal antibody, TROMA-1, which recognizes a 55 000 M_r trophecto-dermal cyto-keratin (ENDO A). This protein was first detected in immunoblots at the 4-cell stage, and became more abundant at the 16-cell stage and later. Immunofluorescence analysis revealed assembled cyto-keratin filaments in some 8-cell blastomeres, but not at earlier stages. At the 16-cell stage, filaments were found in both polarized (presumptive trophecto-derm; TE) and apolar (presumptive inner cell mass; ICM) cells in similar proportions, although polarized cells possessed more filaments than apolar cells. By the late 32-cell, early blastocyst, stage, all polarized (TE) cells contained extensive filament networks whereas cells positioned inside the embryo tended to have lost

their filaments. The presence of filaments in inside cells at the 16-cell stage and in ICM cells was confirmed by immunoelectron microscopy. Lineage tracing techniques demonstrated that those cells in the ICM of early blastocysts which did possess filaments were almost exclusively the progeny of polar 16-cell blastomeres, suggesting that these filaments were directly inherited from outside cells at the 16- to 32-cell transition. Inhibitor studies revealed that proximate protein synthesis but not mRNA synthesis is required for filament assembly at the 8-cell stage. These results demonstrate that there are quantitative rather than qualitative differences in the expression of cyto-keratin filaments in the inner cell mass and trophecto-derm cells of the mouse embryo.

Key words: cyto-keratin, intermediate filament, mouse embryo.

Introduction

The formation of the expanded blastocyst marks the earliest establishment of two distinct and committed cell populations in the mammalian embryo. The two constituent tissues of the blastocyst, the trophecto-derm and the inner cell mass (ICM), differ in their properties, developmental potentials and fates. We now have a clear and quantitative account of the lineage relationships leading to blastocyst formation, and an awareness of how these relationships may be modulated *in situ* (Graham & Deussen, 1978; Graham & Lehtonen, 1979; Surani & Barton, 1984; Pedersen, Wu & Balakier, 1986; Fleming, 1987; Dyce, George, Goodall & Fleming, 1987; Garbutt, Johnson & George, 1987; reviewed Johnson, Chisholm, Fleming & Houlston, 1986). There has also been much progress towards understanding the cellular processes underlying these events (Ziomek &

Johnson, 1980, 1981, 1982; Johnson & Ziomek, 1981, 1983; Kimber, Surani & Barton, 1982; Johnson & Maro, 1985; reviewed Johnson & Maro, 1986). However, details of the associated molecular changes remain obscure.

That the two cell subpopulations differ in their protein biosynthetic activities has been known for some years (Van Blerkom, Barton & Johnson, 1976; Handyside & Johnson, 1978; Howe & Solter, 1979; Dewey, Filler & Mintz, 1978), and there is evidence that the two tissues have correspondingly different messenger RNA populations (Johnson, 1979; Duprey *et al.* 1985). A subgroup of the proteins characteristic of the biosynthetic pattern of trophecto-derm has been identified as a set of cyto-keratins (Brûlet, Babinet, Kemler & Jacob, 1980; Jackson *et al.* 1980), and the presence of certain cyto-keratin mRNAs and proteins, as well as the assembly of cyto-keratin filaments, has been detected in trophecto-derm but not in ICM cells

(Brûlet *et al.* 1980; Jackson *et al.* 1980; Paulin, Babinet, Weber & Osborn, 1980; Lehtonen *et al.* 1983; Duprey *et al.* 1985). However, the presence and synthesis of cytokeratin proteins and mRNAs can be detected prior to the blastocyst stage (Handyside & Johnson, 1978; Lehtonen *et al.* 1983; Oshima *et al.* 1983; Duprey *et al.* 1985), and both proteins and message also appear in a subpopulation of ICM cells later in development as the primary endoderm is formed (Brûlet *et al.* 1980; Paulin *et al.* 1980; Jackson *et al.* 1981; Duprey *et al.* 1985). Thus, the cytokeratins constitute a family of proteins whose expression is modulated during blastocyst formation and development (data from different papers compared and summarized in Table 1).

One feature that emerges clearly from the data in Table 1 is that cytokeratin proteins are present and/or synthesized in advance of the unequivocal detection of assembled intermediate filaments and well in advance of the establishment of a definitive trophoctodermal cell population at around the 32-cell stage. In this paper, we have used the TROMA-1 monoclonal antibody (Kemler *et al.* 1981) directed

against cytokeratin ENDO A (Brûlet *et al.* 1980; Oshima *et al.* 1983) to undertake a thorough immunocytochemical analysis of filament assembly during cleavage, compaction and blastocyst formation. We find that trophoctoderm and ICM cells and their precursors are distinguished quantitatively rather than qualitatively in their possession of assembled cytokeratin filaments, and suggest that the expression and assembly of filaments may be modulated by cell interaction.

Materials and methods

Recovery of embryos

3- to 4-week-old female MF1 mice (Central Animal Services, Cambridge, UK) were superovulated by intraperitoneal injection of 5–10 i.u. of pregnant mare's serum gonadotrophin (PMS, Intervet) and human chorionic gonadotrophin (hCG, Intervet) 44–48 h apart. Unfertilized eggs were recovered at 14–20 h post-hCG and freed of their cumulus cells by brief exposure to 0.1 M-hyaluronidase (Sigma). In order to obtain embryos, the females were paired overnight with HC-CFLP males (Interfauna) and

Table 1. Summary of published work on cytokeratins in early mouse embryos

Feature	(reference)	Embryo stage					blastocyst	
		oocyte	2-cell	4- to 8-cell	'morula'	TE	ICM	
Presence of mRNA	(1)		–	+		++	–	
Presence of protein (immunoblotting)	(2)	+	+	+	+	++	?	
			57K (doublet) and 54K		additional 50K protein			
Protein synthesis (immunoprecipitation)	(3)	–	–	+	+	++	?	
					50K, 55K (ENDO A (=y), ENDO B (=x))			
Protein synthesis (extraction of labelled protein)	(4)					+	?	
						46K, 54K, 61K (x,y,z)		
	(5)					+	?	
						3 proteins 45–60K		
Presence of filaments (by transmission electron microscopy)	(2)	(+)	(+)	(+)	(+)	+	–	
		associated with	'paracrystalline arrays'		?	filament arrays		
	(4)	–	–	–	+	++	–	
Presence of assembled protein (immunofluorescence)	(4)	–	–	–	+	++	–	
	(6)					++	–	
	(7)	+	+	+	+	++	–	
		granular	granular	granular	granular	filaments		

–, absent; (+), trace; +, present.

References: 1, Duprey *et al.* 1985; 2, Lehtonen *et al.* 1983; 3, Oshima *et al.* 1983; 4, Jackson *et al.* 1980; 5, Brûlet *et al.* 1980; 6, Paulin *et al.* 1980; 7, Lehtonen, 1985.

ENDO A (y) and ENDO B (x) are respectively probably equivalent to trophoctodermal marker proteins T2/T3 and T16 (Handyside & Johnson, 1978). It appears that the ICM does not synthesize cytokeratin protein (Van Blerkom *et al.* 1975; Handyside & Johnson, 1978) although specific probes have not been used to test this.

inspected for vaginal plugs the next day. Embryos at different stages were recovered by flushing from the oviduct at the appropriate time post-hCG with Medium 2 + 4 mg ml⁻¹ bovine serum albumin (M2 + BSA; Fulton & Whittingham, 1978) and cultured in Medium 16 containing 4 mg ml⁻¹ BSA (M16 + BSA; Whittingham & Wales, 1969) under oil at 37°C in 5% CO₂ in air in Falcon plastic dishes.

Embryo manipulations

Zonae pellucidae were removed by brief incubation in acid Tyrode's solution (Nicholson, Yanagimachi & Yanagimachi, 1975). Subsequent decompaction was achieved by 10–15 min incubation in Ca²⁺-free M2 containing 6 mg ml⁻¹ BSA. For disaggregation, decompact embryos were gently blown apart using flame-polished micropipettes. Mid 16-cell-stage and later stage embryos were disaggregated after 5–30 min incubation at 37°C in 25 mg ml⁻¹ trypsin and 10 mg ml⁻¹ EDTA in Ca²⁺-free M2 + BSA. Expanded blastocysts were incubated for 30 min in cytochalasin D (CCD; Sigma), diluted to 0.5 µM in M2 + BSA from a 1 mM stock in DMSO before disaggregation in Ca²⁺-free M2 + BSA. Zona-free embryos and isolated cells were cultured in Sterilin tissue culture dishes.

To obtain natural 2/8 or 2/16 cell pairs, cultures of single late 1/4 or 1/8 cells were inspected hourly for evidence of division, and newly formed pairs were collected and designated 0 h old. Timing of whole 8-cell embryos was also achieved by hourly examination of late 4-cell embryos, and embryos that took less than 2 h to complete the 4- to 8-cell transition selected. The 8- to 16-cell transition was followed similarly by observation of the decompaction, division and subsequent recompaction of compact 8-cell embryos, 16-cell embryos being designated 0 h at recompaction (Fleming, 1987). Blastocysts were designated 0 h old upon the first appearance of a blastocoel during hourly inspections (Chisholm *et al.* 1985).

Immunosurgery of blastocysts to recover ICMs was achieved by incubation of zona-free embryos for 5–10 min in 15 µl heat-inactivated rabbit anti-mouse antiserum (1/10 in M2 + BSA) at 37°C, thorough washing, and a further 5 min incubation in agarose-adsorbed rat serum containing complement activity. Embryos were transferred to M2 + BSA at 37°C for 20–30 min, and the lysed outer cells removed using a flame-polished micropipette. Isolated cells were cultured in Sterilin culture dishes in Dulbecco's Modified Eagle's Medium (DMEM; Flow) supplemented with 60 µg ml⁻¹ penicillin and 50 µg ml⁻¹ streptomycin and containing 10% v/v heat-inactivated fetal calf serum (FCS; Flow).

Vital labelling techniques

Polarized and apolar cells were distinguished on the basis of their surface organization by incubating them in Concanavalin A–Rhodamine (Rh–Con A; Polysciences) or tetramethylrhodamine isothiocyanate conjugated peanut agglutinin (Rh–PNA; Sigma) at 0.5 mg ml⁻¹ in M2 + BSA + 0.02% sodium azide (BDH) for 5–10 min. For selective labelling of outer cells in intact embryos, recompact 16-cell embryos or blastocysts were incubated for 30 s in 1 mg ml⁻¹ Rh–PNA or 2/16 pairs in 0.5 mg ml⁻¹ Rh–PNA for 20 s (Fleming, 1987).

For lineage analysis, a monodisperse suspension of carboxylated red fluorescent latex (R-Latex) particles (Fluoresbrite, Polysciences; 0.2 µm particles, 2–5% solids) diluted 1/25 in M2 + BSA was used (Fleming & George, 1987). Embryos were incubated in R-latex for 30 s, washed in M2 + BSA and cultured further. The latex adheres to, and is internalized by, the outer cells only and is consistently distributed to all their progeny.

Drugs

α-amanitin (Boehringer Mannheim) was used at 11 µg ml⁻¹, which almost completely inhibits RNA polymerase II in mouse embryos (Levey & Brinster, 1978; Braude, 1979). Puromycin (Sigma; a translational inhibitor) was used at 20 mM and found to reduce protein synthesis in our embryos to 3% of control values within 1 h.

Cell fixation and immunocytological staining

Zona-free embryos were placed in specially designed chambers as described in Maro, Johnson, Pickering & Flach (1984) except that the chambers were routinely coated with either 0.1 mg ml⁻¹ Con A in phosphate-buffered saline (PBS) or with 1/10 phytohaemagglutinin (PHA, Gibco) in PBS when Rh–Con A had been used to label cells. Chambers containing samples were centrifuged at 450 g for 10 min at 20–30°C. Three fixation regimes were tested, the first being used routinely. (i) Embryos were fixed for 30 min at 20°C in 1.8% formaldehyde in PBS, washed for 10 min in PBS containing 50 mM-NH₄Cl, extracted for 10 min in PBS containing 0.25% Triton X-100 and finally washed in PBS. (ii) Embryos were washed quickly in PHEM buffer (10 mM-EGTA, 2 mM-MgCl₂, 60 mM-Pipes, 25 mM-Hepes, pH 6.9; derived from Schliwa *et al.* 1981), extracted for 10 min at 20°C in PHEM buffer containing 0.25% Triton X-100 containing 0.1 mM-phenyl methyl sulphonyl fluoride (Sigma), washed in PHEM buffer and fixed for 30 min in 1.8% formaldehyde in PHEM buffer. They were washed and neutralized as above. (iii) Some ICMs were fixed in 2% paraformaldehyde in PBS for 30 min then washed in PBS and neutralized in NH₄Cl.

Immunocytological staining was performed as described in Maro *et al.* (1984) using the TROMA-1 monoclonal antibody (Kemler *et al.* 1981) followed by FITC-conjugated goat anti-rat serum (Miles) at a dilution of 1/50 in PBS. In order to stain chromosomes, Hoechst dye 33258 (5 µg ml⁻¹; Sigma) was included with the second antibody. Control embryos exposed to the second layer only gave no evidence of specific staining.

Photomicroscopy

The coverslips were removed from the chambers and samples were mounted in 'Citifluor' (City University, London) and viewed on a Leitz Ortholux II microscope with filter sets L2 for FITC-labelled reagents, N2 for rhodamine and A for Hoechst dye. Photographs were taken on Kodak Tri-X using a Leitz Vario-Orthomat photographic system.

Immunoelectron microscopy

For electron microscopy, the method of Houlston, Pickering & Maro (1987) was adapted. Cells were washed quickly

in PHEM buffer, extracted for 5 min in PHEM buffer containing 0.5% Brij 58 (Sigma) for 2/16 pairs or 0.25% Triton X-100 for blastocysts, washed in PHEM buffer and fixed with 0.2% glutaraldehyde in PHEM buffer (all steps at 30°C). Glutaraldehyde was neutralized with 0.1 M-lysine (Sigma) for 10 min and the cells labelled with TROMA-1 followed by an anti-rat immunoglobulin conjugated to 10 nm gold particles (Janssen). Cells were postfixed with 3.5% glutaraldehyde in PBS, embedded in TAAB Embedding Resin (TAAB), stained with uranyl acetate and lead citrate, sectioned on a Reichert Ultramicrotome and finally viewed under a Phillips 300 electron microscope.

Immunoblotting

Eggs and embryos were washed three times in M2 (without BSA), lysed in 10 μ l of double-strength SDS sample buffer (Laemmli, 1970) and boiled immediately for 90 s. Proteins were separated using 10% PAGE according to Laemmli (1970) on a microgel apparatus (Raven). The proteins were transferred electrophoretically onto nylon (Biodyne) membrane and the cytokeratins were detected autoradiographically after incubation of the membrane in TROMA-1 antibody (undiluted) followed by biotinylated anti-rat Ig and 35 S-StrepAvidin (Amersham). 5% milk protein in PBS was used to block nonspecific protein binding and PBS with 0.1% Tween was used for all washing steps. Sample sizes of 100–700 embryos were used and autoradiographs exposed for 3–7 days. Embryos were staged directly according to morphological criteria, except for the 16-cell-stage embryos which were collected as compact morulae after culturing a population of noncompact 8-cell-stage embryos for 14–16 h in M16 + BSA.

Results

Detection of TROMA-1 antigen by immunoblotting

A polypeptide of M_r 55 000, corresponding to the ENDO A protein previously detected in early mouse embryos by immunoprecipitation (Oshima *et al.* 1983), was detected by immunoblotting as few as 100 expanded blastocysts. With greater numbers of

blastocysts, minor lower molecular weight components were detected, probably corresponding to proteolytic fragments of the cytokeratins (Fig. 1, track B). Fig. 1 also reveals that ENDO A is much less abundant in earlier embryos. Immunoblotting of separated protein samples from 500 carefully staged embryos (see Materials and methods) in each track, revealed a clear and consistent signal from a single polypeptide at 55 000 in 16-cell embryos. In 4- and 8-cell embryos the amount of 55 000 protein present is around the limit of our detection system, so that positive signals were sometimes, but not always, observed. Fig. 1 is representative and reveals a weak signal from 4-cell embryos but not from 8-cell embryos. This result is consistent with that from Oshima *et al.* (1983) who reported a weak signal from a mixed 4- and 8-cell population by immunoprecipitation of metabolically labelled (i.e. newly synthesized) protein and identified the 4-cell stage as the earliest point at which the protein is detected. Like Oshima *et al.* (1983), we never detected a signal from 2-cell embryos or eggs. Taken together, these results suggest that the synthesis and presence of cytokeratin ENDO A may coincide at the 4-cell stage, and we find little evidence to favour significant storage of unassembled protein recognized by TROMA-1. The mRNA encoding ENDO A has been detected at the 8-cell stage but not at the 2-cell stage (Duprey *et al.* 1985).

Immunocytochemical detection of cytokeratin filaments in cleavage-stage blastomeres

TROMA-1 failed to stain unfertilized eggs, fertilized eggs, and 2-cell and 4-cell embryos or their constituent cells regardless of the fixation and extraction procedures used. In contrast, filamentous staining was evident in some compact 8-cell embryos cultured *in vitro* from the 4-cell stage (Fig. 2A–C; Table 2). Examination of pairs of 8-cell blastomeres, derived by disaggregating 4-cell embryos to single cells and

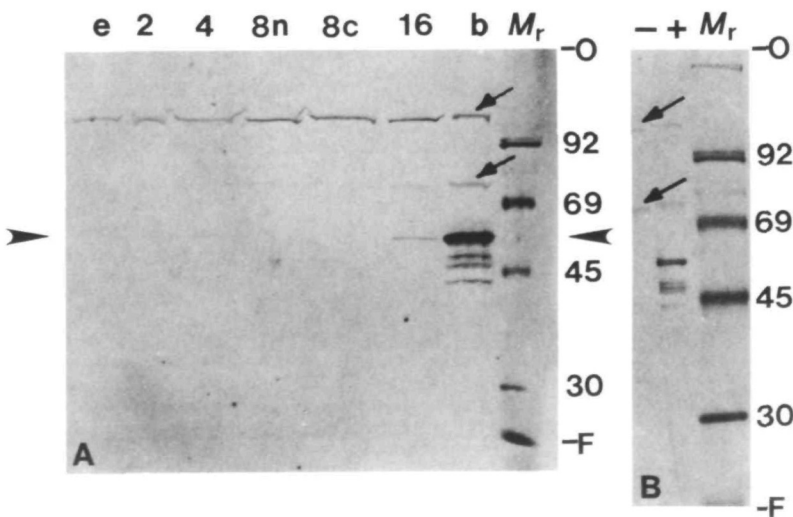


Fig. 1. (A) Separated proteins from samples of 500 embryos per track were probed with TROMA-1 and exposed for 7 days. Arrowheads indicate position of 55 000 TROMA-1 antigen. e, oocytes; 2, 4, 8 and 16, cell numbers in embryos in each track, n and c indicating noncompact and compact respectively; b, blastocyst; M_r , molecular weight markers. In (B) a lane of blastocyst proteins has been transferred to nitrocellulose and then cut in two, the two halves being processed for immunoblotting with (+) or without (-) the use of TROMA-1. It is clear that the two arrowed bands in both gels represent non-specific binding. O and F indicate the origin and front of each gel.

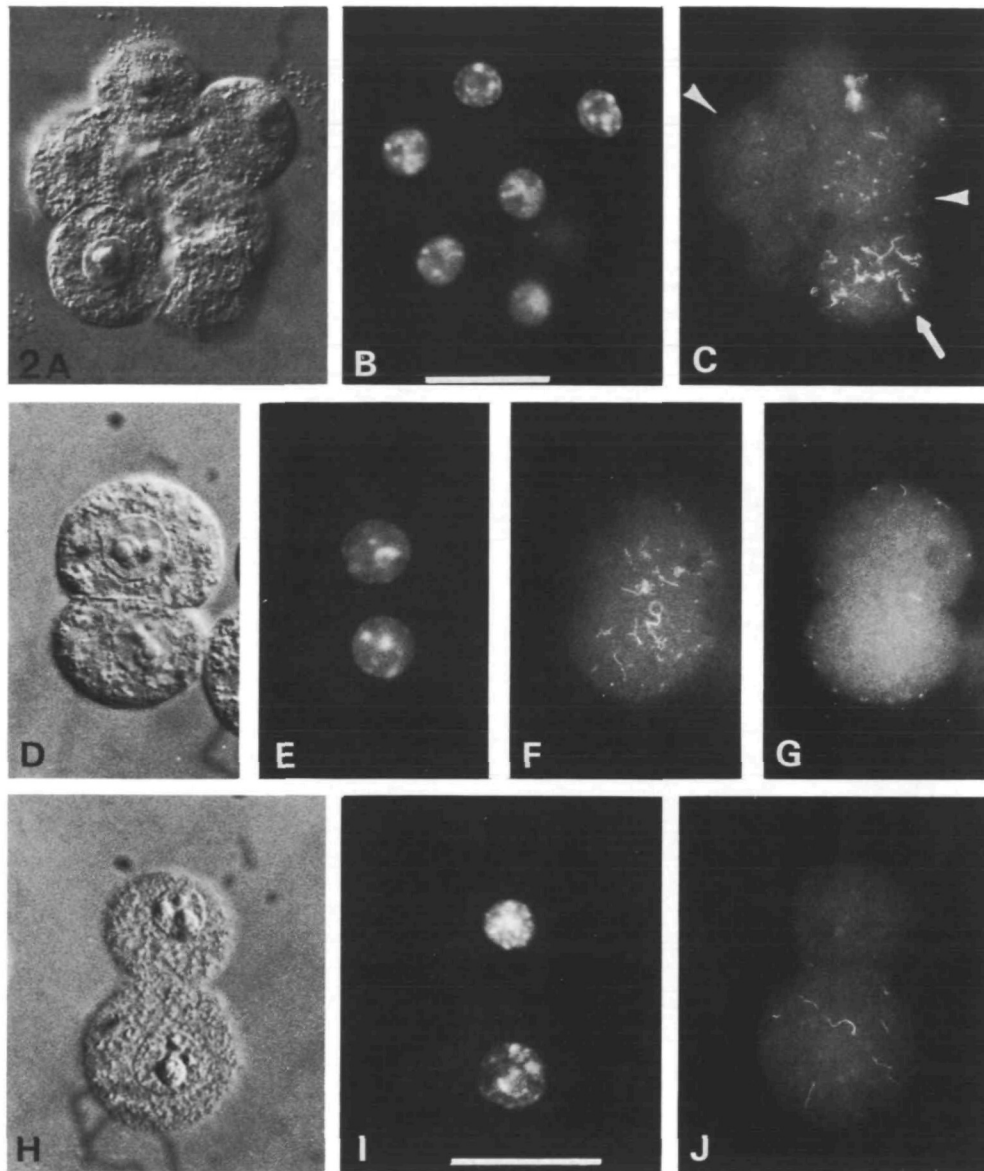


Fig. 2. Cytokeratin filaments in 8-cell blastomeres. (A,D,H) Nomarski optics. (B,E,I) Hoechst dye. (C,F,G,J) TROMA-1. (A-C) Late 8-cell embryo decompacted in Ca^{2+} -free MS + BSA. Filaments are visible in one blastomere (C, arrowed) and the beginnings of filaments in two others (arrowheads). Bar, $30\ \mu\text{m}$. (D-J) 2/8 pairs of blastomeres at 10h postdivision, showing cytoke- ratin filaments in both cells (F,G) or in one cell only (J). F and G show different levels of focus in the same pair. The pair in D-G is flattened and that in H-J is noncompact. Bar, $30\ \mu\text{m}$.

Table 2. Cytokeratin filaments in late 8-cell and 8- to 16-cell embryos

Embryo age (h post div. to 8 cells)	No. embryos	Mean cell no.	Filaments (%)		
			embryos	1/8 cells*	1/16 cells*
10	25	8.5	13 (52)	22 (12)	3 (12)
12	16	12.2	9 (56)	13 (21)	20 (15)

Embryos were timed from the 4- to 8-cell transition and zonae were removed at 0-2h postcavitation. Embryos were decompacted before being placed into chambers.

* Blastomere developmental age judged by nuclear size; 1/8 cells had large nuclei whereas nuclei of early 1/16 cells were relatively small.

allowing them to divide *in vitro* (Fig. 2D–J), revealed that some contained TROMA-1 positive filaments 4–6 h after their formation (Fig. 3). Filaments were detected in some pairs prior to intercellular flattening (Fig. 2H–J) and before polarization of the cell surface (as assessed by the labelling pattern with Rh–Con A), and thus their appearance does not appear to be tightly coupled to, or dependent on, either of these events. TROMA-1 staining of embryos fixed immediately after recovery from the mouse confirmed that filaments first appear at the 8-cell stage *in vivo* also.

To follow the assembly of filaments during the fifth and sixth cell cycles, individual blastomeres were disaggregated from late compact 8-cell embryos, cultured through division to 2/16 pairs, and the pairs (or 4/32 cell clusters derived from them) were sampled and stained at intervals thereafter (Figs 3, 4). The incidence of assembled filaments increased progressively until 100% of clusters were positive by the mid to late 32-cell stage (Fig. 5).

The earliest site of appearance of filaments was close to the plasma membrane, both in regions where cells were not in contact (Fig. 2G) and adjacent to contact regions (Fig. 4C,H). Filaments were particularly marked in pairs of 2/16 blastomeres in which the polarized cell was enveloping the apolar cell, and were concentrated at the leading edges of the enveloping cell. The early filaments were not obviously polarized in their distribution, and neither did they seem to form a complete network, short runs of independent filaments being evident. As filament assembly increased quantitatively, so longer runs of

filament bundles were evident and a network formed (Fig. 5). Nonetheless, this remained largely cortical.

The distribution of cytokeratin filaments between outside and inside cells

Earlier work has suggested that cytokeratin filaments are a specific feature of the outside, polarized trophoblast cells and are absent from the inside, apolar cells of the early ICM (see Introduction). Apolar ICM progenitor cells are formed at two stages. Asymmetric cleavage of some of the polarized 8-cell blastomeres gives rise to apolar daughter cells, positioned inside the embryo, as well as to polarized outside cells. The progeny of these apolar cells provide, on average, 75% of the final total of ICM cells, with progenitor cells of the remaining 25% being formed by a similar asymmetric division of some of the polarized 1/16 cells (Fleming, 1987). The outside, polarized cells resulting from these two rounds of division contribute their progeny to the trophoblast. Once the two allocations of ICM progenitors have been made, no further significant contribution from outside cells occurs, and neither do the progeny of the apolar inside cells contribute significantly to the trophoblast (Dyce *et al.* 1987). These phenotypic, positional and lineage relationships apply for both intact embryos and clusters of cells. We investigated whether apolar cells derived from polarized 8- and 16-cell blastomeres contained filaments.

2/16 pairs obtained early after division of polarized 1/8 blastomeres were stained with concanavalin A, which provides an indication of which cells are

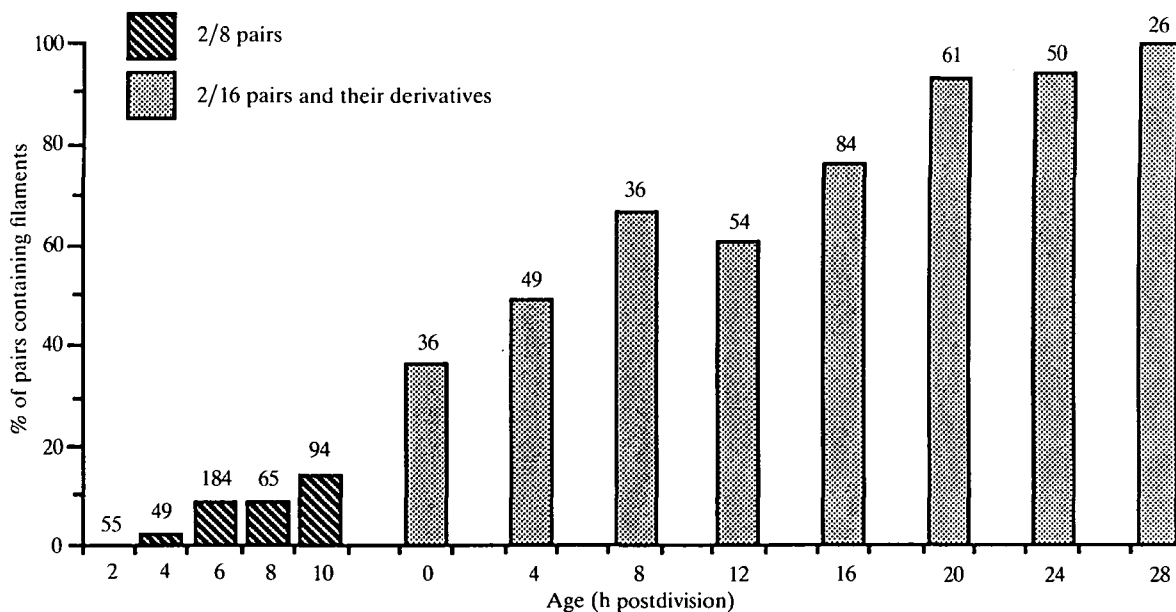


Fig. 3. Incidence of cytokeratin filaments in timed 2/8 pairs, 2/16 pairs and derivatives of 2/16 pairs. The number of pairs examined in each group is indicated above the bars. 2/16 pairs divide to 4/32 clusters at 10–14 h; thus, by 28 h, cells are equivalent to late 32- to early 64-cell stage.

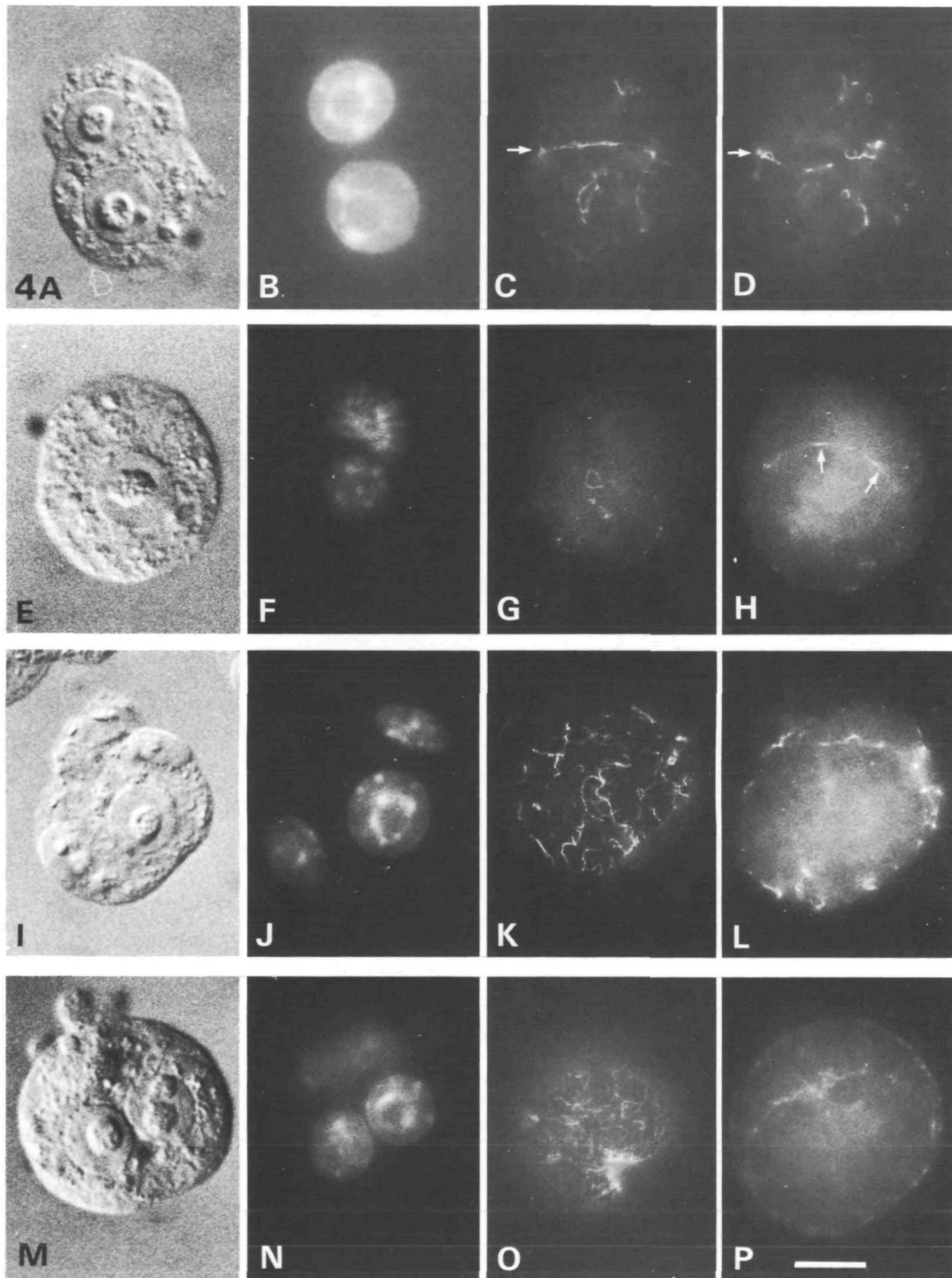


Fig. 4. Cytokeratin filaments in 2/16 pairs and 4/32 clusters. (A,E,I,M) Nomarski optics. (B,F,J,N) Hoechst dye. (C,D,G,H,K,L,O,P) TROMA-1. The two TROMA-1 staining patterns for each pair represent focal planes at the surface (C,G,K,O) and more centrally (D,H,L,P) in the pair. (A–D) Flattened 2/16 pair. A ring of filaments is visible round the contact zone (arrow) as well as filament ‘threads’ and ‘rings’ in both cells (C,D). (E–H) Enveloped 2/16 pair in which the outer cell is mitotic. Filaments are present in the outside cell (*en face* in G) and in the contact zone (arrowed) between the outside and inside cell (H). (I–L) Cluster of two outside cells at the 32-cell stage and an inside 1/16 cell. A filament network is forming in the outer cells (K) and filaments outline the boundary between the outside and inside cells (L). (M–P) 4/32 cluster of two outside and two inside cells. A filament network is becoming established in the outer cells (O) and again outlines the inner cells (P). Bar, 15 μ m.

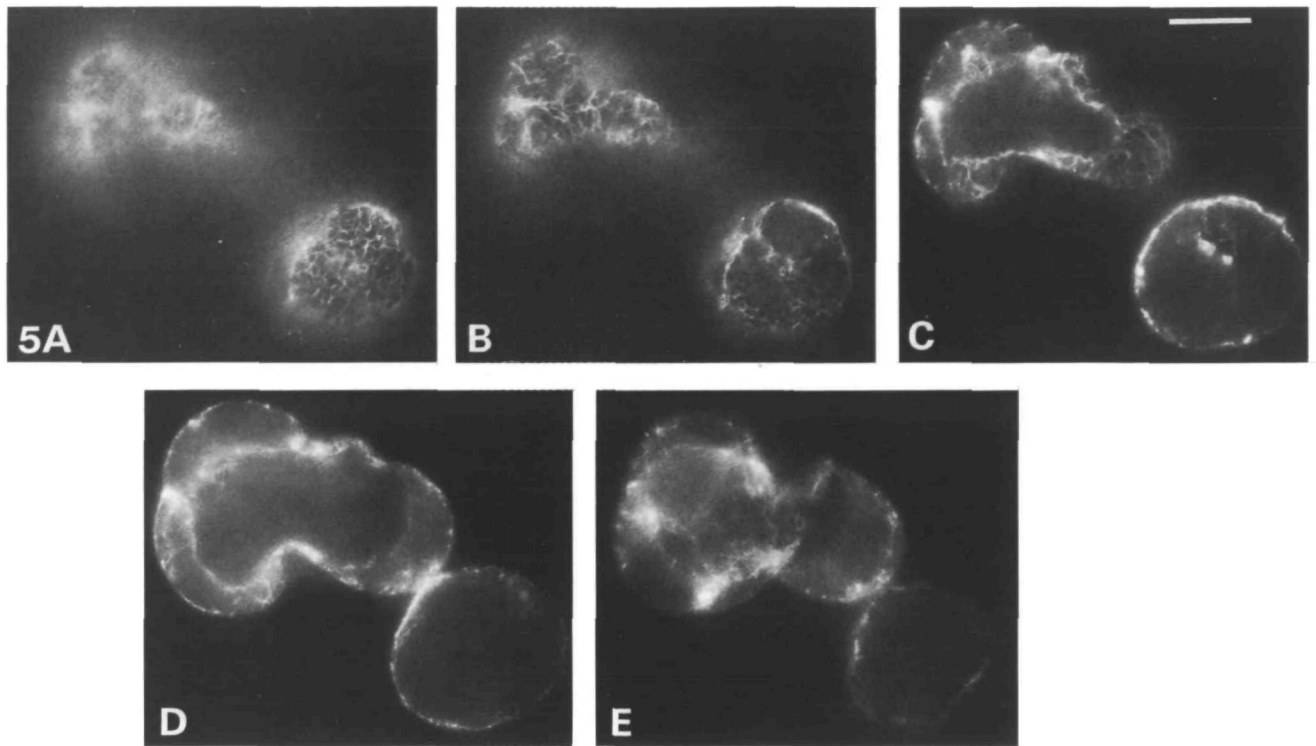


Fig. 5. Cytokeratin filament networks in 4/32 clusters. TROMA-1 staining of two 4/32 clusters at 24 h post-division, at five different planes of focus through the specimens. The upper left cluster contains three outside cells and one inside cell and the lower right cluster has two outside and two inside cells. Both clusters are cavitated and show very extensive filament networks in their outer cells. Bar, 20 μ m.

Table 3. Cytokeratin filaments in polarized (P) and apolar (AP) cells of early (2–4 h old) 2/16 pairs

Type of pair	No. pairs	% of pairs with filaments			
		both cells	one cell only		total
			polarized	apolar	
AP/P	136	34	7	3	43
P/P	21	62	0	0	62

Surface polarity is assessed by Rh-Con A labelling.

Summarizing data for polarized and apolar cells gives 46% polarized and 37% apolar cells with filaments.

polarized, and subsequently with TROMA-1 (Table 3). It was clear that filaments reactive with TROMA-1 are present in many apolar cells, as well as in polarized cells, although the number of filaments and their organization into networks was generally greater in the polarized than in the apolar cells. All pairs in which both cells were polarized also had filaments in both cells. At 6 h or later into the 16-cell stage, polarized cells had started to envelop the apolar cells (Ziomek & Johnson, 1981), which made unambiguous scoring of filament distribution in the apolar cells very difficult, although the staining appeared to be reduced (Fig. 4E–H). Enveloped pairs

were therefore exposed to concanavalin A to label the outer polarized cells, and then incubated for 20 min in trypsin–EDTA, a treatment that caused many, but not all, pairs to pull apart. The distribution of filaments in those pairs that decompacted is recorded in Table 4. It is clear that many fully or partially enveloped apolar cells possessed filaments, although again their quantity and organization was not as great as in the enveloping polarized cells.

The presence of intermediate filaments reactive with TROMA-1 in both cells of enveloped 2/16 pairs was confirmed at the electron microscope level (Fig. 6C–E). The arrays seen in both the enveloping and enveloped cells are not extensive in comparison with those seen in mature trophectoderm (Fig. 6A), but can be identified in some sections of extracted specimens with the aid of the TROMA-1 antibody and a gold-labelled second antibody. Similar rudimentary cytokeratin filaments were occasionally found in cells of the ICM in the blastocyst (Fig. 6B).

The data from 2/16 pairs gave clear evidence of cytokeratin filaments in apolar enveloped cells. It was important to show that apolar cells derived from the intact embryo also contained filaments, since it could be argued that transitory exposure of the apolar cells in the cell couplets had induced filament assembly. Zona-free 8-cell embryos were therefore observed *in*

Table 4. Cytokeratin filaments in decompacted 2/16 pairs

Age of pair (h post div.)	Type of pair	No. pairs	Pairs of filaments (%)		
			both cells	one cell only	total
4 h control	2oc	56	45	16	61
	en	35	43	14	57
	Total	91	44	15	59
8 h control	2oc	22	59	5	64
	en	37	54	19	76
	Total	59	56	14	71
8 h trypsin*	2oc	27	52	15	67
	en	55	53	20	73
	Total	82	52	18	71

Pairs were labelled briefly with Rh-Con A to allow later distinction between outside and inside cells, and then decompacted.

2oc, two outside cells; en, enveloped and enveloped.

* Pairs decompacted by 20 min incubation in trypsin/Ca²⁺-free medium.

In each group, including the trypsin-treated one, some pairs were fully enveloped at the time of fixation (2, 29 and 22 pairs, respectively). Such pairs have been excluded from the table due to the difficulty in scoring them accurately.

in vitro as they divided to the 16-cell stage, and were then cultured to the mid 16-cell stage (2–3 h post-recompaction after division), the 32-cell stage (16–17 h), or the early expanding blastocyst stage (20–21 h). Embryos were exposed briefly to Rh-PNA, to label the polarized cells on the outside of the embryo, and then disaggregated. Blastocysts timed from the earliest appearance of a blastocoel as being 3 or 12 h old were treated similarly. Their zonae had been removed just after cavitation. The results are shown in Fig. 7. A substantial proportion of embryos showed inside cells containing filaments (Fig. 8). As was found for apolar and enveloped cells derived from pairs of blastomeres, the blastomeres from the inside of whole embryos tended to have fewer filaments and these were poorly organized. Also consistent with the findings for 2/16 pairs, the proportion of outside cells with filaments increased over the 16- and 32-cell stages. In addition, when disaggregation of 16-cell embryos resulted in pairs of cells linked by a midbody, most had filaments in both or neither cells (see Table 3). In many outside cells the filaments formed a submembranous cap with a clear ring boundary that coincided with the limits of peanut lectin staining, indicating that the filaments were concentrated in the outer or apical regions of the cells (Fig. 8D–G) and particularly in association

with the site of the previous junctional region between adjacent polarized cells. The same TROMA-1 staining patterns were observed in the absence of staining with peanut lectins. Some perinuclear staining was also evident in cells from expanding blastocysts.

ICMs were also isolated from blastocysts by immunosurgery and they showed evidence of filaments. However, it was clear that at least some of the TROMA-1 reactivity was associated with the debris of dead overlying trophectodermal cells, and this impression was confirmed by staining freshly isolated ICMs with TROMA-1 prior to fixation or in nonpermeabilized embryos fixed in paraformaldehyde, when patches of fibrillar stain on the ICM cells were evident. To determine whether there were any intracellular filaments in the ICM cells, freshly isolated ICMs were incubated for 30 min in 2 × trypsin/EDTA in M2 + BSA, a treatment shown to remove debris and external TROMA-1 reactivity of ICMs. ICMs treated in this way were fixed and permeabilized prior to staining for internal filaments. Cells containing small numbers of filaments were detected (Fig. 7) and usually were clustered together and superficially located within the ICM (Fig. 9A). When isolated ICMs were cultured *in vitro*, they developed an intense network of filaments within 16–20 h (Fig. 9B,C) coincident with their transformation to a trophectodermal phenotype.

The origin of cytokeratin filaments in inside cells

The evidence presented above shows that some, but not all, apolar/inside cells derived from 2/16 pairs, 4/32 clusters, intact 16- and 32-cell morulae and expanding blastocysts contain cytokeratin filament systems, although the number and degree of organization of the filaments is not as great as in the polarized/outside cells, and fewer inside cells possess filaments than do outside cells. In principle, inside cells could assemble filaments *de novo*, and/or could acquire assembled filaments (or the capacity to assemble filaments) by inheritance from their parent cell, whether this was a polarized 1/8 cell, a polarized 1/16 cell or an apolar 1/16 cell dividing to give two apolar 1/32 cells.

Circumstantial evidence suggests that an apolar 1/16 cell could inherit filaments (or the capacity to assemble them) from its parent 1/8 cell, since 21% of mitotic 8-cells contain filaments (data not shown), and newly formed 2/16 pairs tend to contain cells in which either both or neither contain filaments (see earlier). However, since filament-containing 8-cell blastomeres are a minority, attention was concentrated on the division of polarized 1/16 cells to give two 32-cell blastomeres. Pairs of 2/16 blastomeres were harvested 11–13 h after their formation from

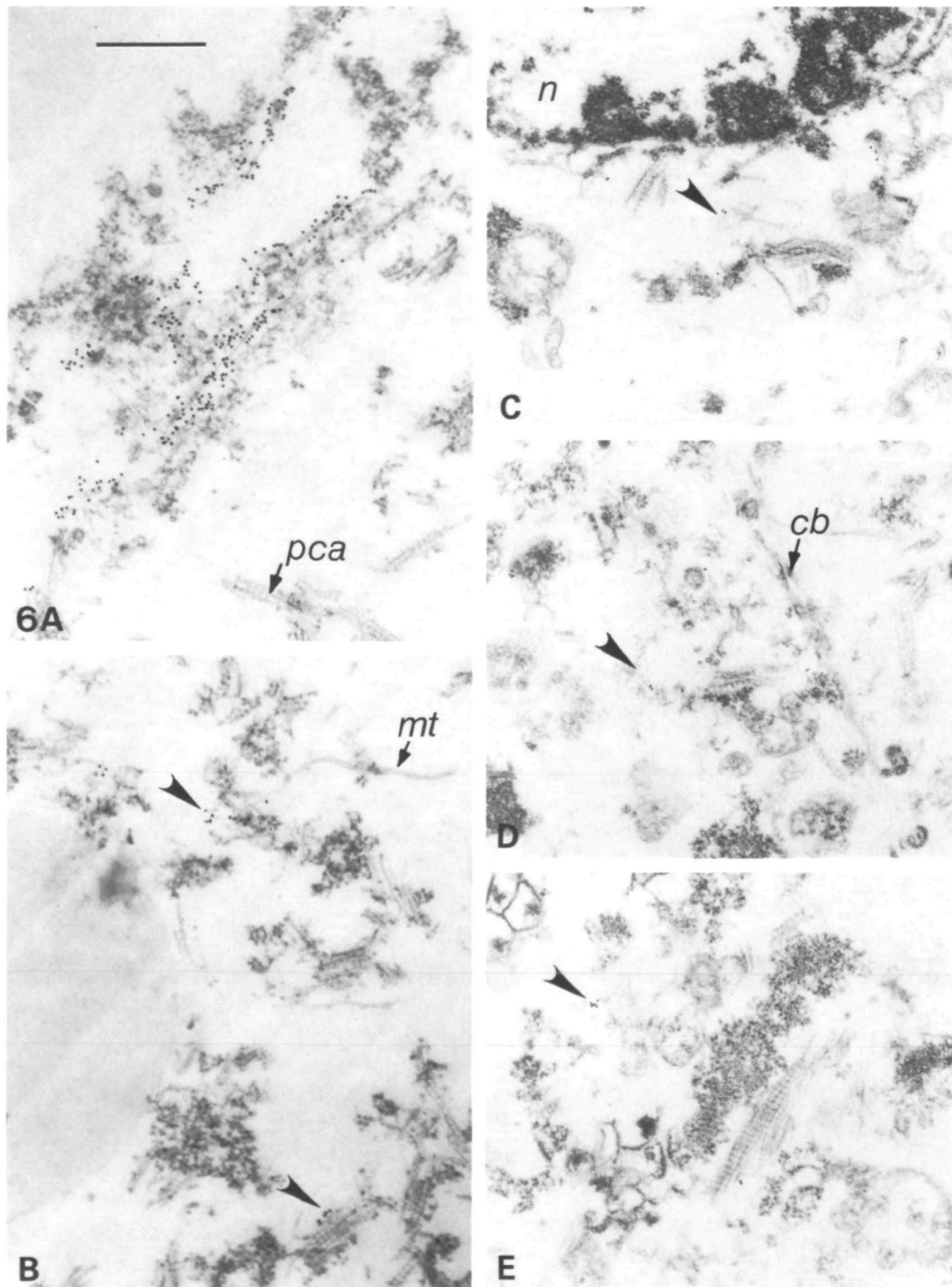


Fig. 6. Identification of cytokeratin filaments in a 12 h blastocyst (A,B) and an enveloped 9 h 2/16 pair (C-E). A mature cytokeratin network is present in the blastocyst trophoblast (A) whereas only occasional small clusters of filaments (arrowheads) are seen in ICM cells (B) and outer (C) and inner (D,E) cells of the 2/16 pair. The samples have been detergent extracted and then subjected to immunogold labelling using the TROMA-1 antibody to aid the identification of filaments. Although the clusters of filaments arrowed in B-D are small, they provide preferential sites for TROMA-1 labelling. Note the absence of gold labelling on other cell structures such as 'paracrystalline arrays' (*pca*) or microtubules (*mt*). *n*, nucleus; *cb*, cell boundary (inner cell to left, outer cell to right). Bar, 0.5 μ m.

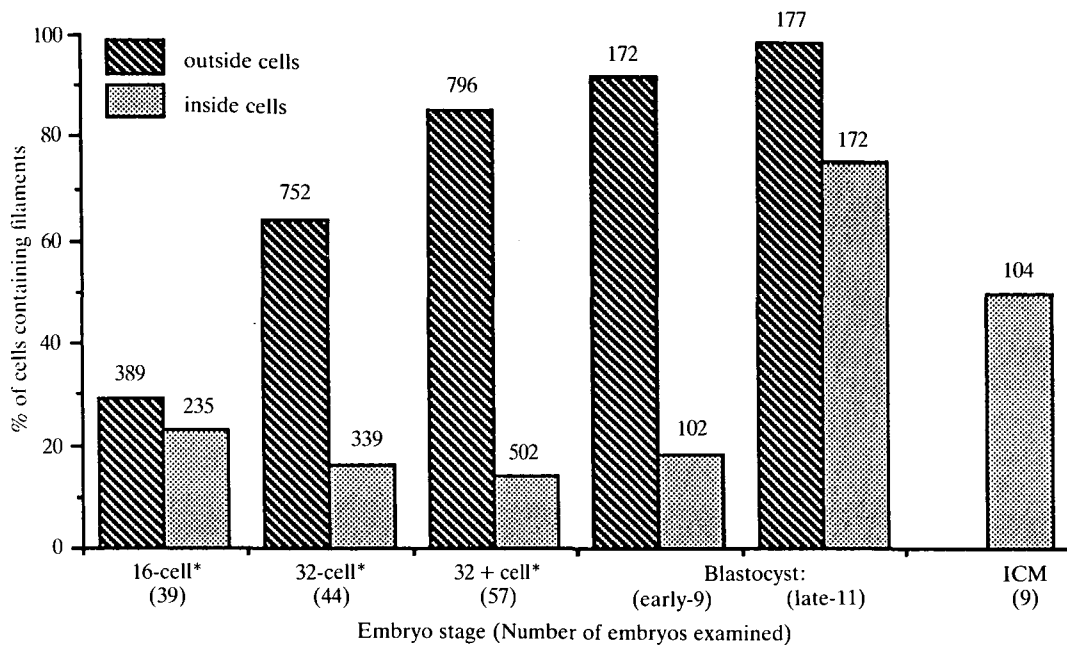


Fig. 7. Cytokeratin filaments in cells of disaggregated embryos. Embryos were incubated in Rh-PNA prior to disaggregation to label outside cells selectively. The number of cells examined in each group is shown above the bars. * Timed from recompaction after division from 8 cells (2, 16–17 and 20–21 h respectively). Early and late blastocysts were taken 0–3 h or 12 h postcavitation, respectively. ICMs were isolated by immunosurgery from blastocysts 3–5 h postcavitation. To facilitate disaggregation, late blastocysts were treated with CCD (see Materials and methods). CCD treatment of early blastocysts did not significantly affect the proportions of outside and inside cells with filaments (not shown). ICMs were disaggregated in 2× trypsin/EDTA in Ca²⁺-free medium.

1/8 cells, i.e. at around the time that the next round of cell division takes place (Garbutt, Chisholm & Johnson, 1987). Data in Table 5 show that the proportion of mitotic cells containing cytokeratin filaments was lower than for interphase cells. Moreover, the filament network characteristic of interphase polarized cells was rarely evident, a few scattered filaments and aggregates of material reactive with TROMA-1 being scattered around the cell. It seems clear that at the 16- to 32-cell transition, the organization of the cytokeratin filament network is modified substantially during mitosis. This modification may involve a true rearrangement of cytokeratin protein or could reflect a changed or masked TROMA-1 binding site.

In order to determine more directly whether those inside cells in the 32-cell embryo that contained filaments had been derived from outer 16-cells at the preceding division, we undertook a lineage analysis. Compact 16-cell embryos were labelled with red fluorescent latex beads (Fleming & George, 1987) and washed. The latex is endocytosed by, and labels internally, only outside 16-cell blastomeres and their progeny (Fleming, 1987). Labelled embryos were then cultured to the 32-cell stage (11–15 h), and were then incubated in Rh-PNA which again labels the surface of outside cells only. Embryos were then disaggregated in trypsin-EDTA and the constituent

cells stained for cytokeratin. Each PNA-negative (inside) 32-cell was then scored as being either non-latex-labelled and therefore derived from an inside 16-cell blastomere, or latex-labelled and therefore derived from an outside 16-cell blastomere (Fig. 10). The results are shown in Table 6. Of the 13% of inside 32-cells scored as being cytokeratin positive, 75% were latex labelled and thus derived from outer 16-cell blastomeres. Indeed, only 4% of inside 32-cells derived from inside 16-cells contained filaments, compared with 34% of inside 32-cells derived from outside 16-cells. Moreover, the latter group of cells usually had more extensive filaments than the former and the filaments were organized into a widespread network.

Biosynthetic requirements for filament assembly

In an attempt to determine the biosynthetic requirements for filament assembly, 2/8 blastomeres were placed in either puromycin or α -amanitin at various times after their formation by division from 1/4 blastomeres. The cells were cultured until they were 18 h postdivision to 8-cells (mid 16-cell equivalent), when they were analysed for evidence of assembled filaments. The results are shown in Table 7. It is clear that suppression of mRNA synthesis from the early 8-cell stage does not block the assembly of filaments, although the number and extent of filaments was

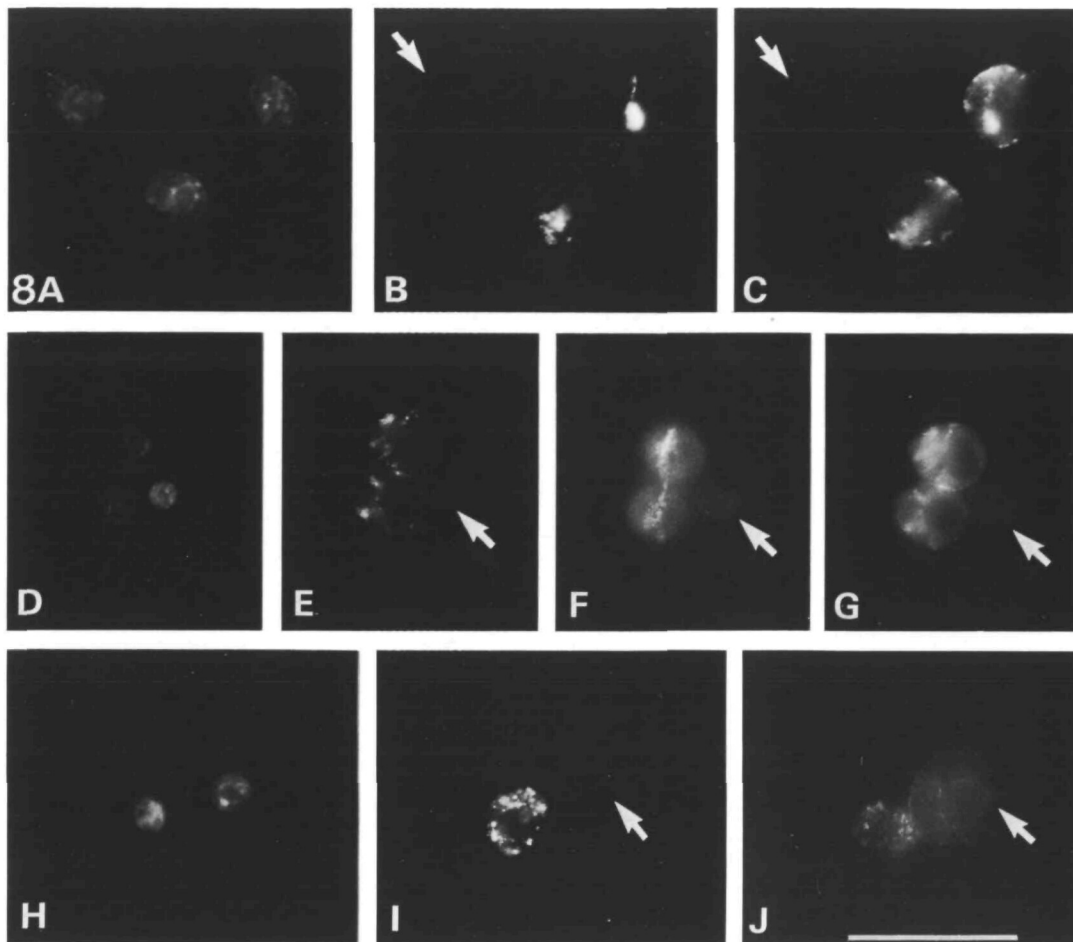


Fig. 8. TROMA-1 labelling patterns in cells of disaggregated 32-cell embryos. Embryos were labelled with Rh-PNA before disaggregation to selectively label outer cells. (A,D,H) Hoechst dye. (B,E,I) Rh-PNA. (C,F,G,J) TROMA-1. (A-C) Two outside cells and one inside cell (arrowed). The inside cell lacks Rh-PNA labelling (B) and does not stain with TROMA-1. The two outside cells show extensive TROMA-1 reactivity. (D-G) Two outside cells and one inside cell (arrowed). The inside cell shows very weak TROMA-1 reactivity (F,G). Note the 'ring' of filaments in each of the two outer cells (F). (H-J) One outside and one inside cell (arrowed). The inside cell contains cytokeatin filaments around the nucleus and the cell periphery. Perinuclear staining is unusual in blastomeres of *in vitro* cultured 32-cell embryos. Bar, 30 μ m.

reduced slightly. In contrast, proximate protein synthesis does seem to be required for successful assembly of filaments.

Discussion

The assembly of cytokeatin filaments requires the copolymerization of representatives of two types of cytokeatin, one acidic and one neutral/basic (Steinert, Idler & Zimmerman, 1976; Milstone, 1981; Eichner, Sun & Aebi, 1986; Steinert, Stevens & Roop, 1985). If only one type is present, aggregates but not filaments may form (Eichner *et al.* 1986). Moreover, cytoplasmic pools of intermediate filament monomer seem to be small in most cells, newly formed monomer being incorporated rapidly into polymer (Blikstad & Lazarides, 1983; Fulton & Wan,

1983). In the early embryo, ENDO A, the neutral/basic cytokeatin recognized by the TROMA-1 antibody, combines with the acidic cytokeatin ENDO B to form filaments. The earliest time at which synthesis of ENDO A and B has been detected is the 4- to 8-cell stage (Oshima *et al.* 1983), and this corresponds with the detection of ENDO A at the 4-cell stage by immunoblotting reported here. Messenger RNA encoding ENDO A has been detected at the 8-cell stage (Duprey *et al.* 1985), and not at the 2-cell stage, but 4-cell embryos have not been probed directly for mRNA. The absence of any major effect on filament assembly of applying α -amanitin to early 8-cell embryos argues that by this stage sufficient mRNA is present to support cytokeatin synthesis. The absence of detectable filaments prior to the 8-cell stage, coupled with the sensitivity of assembly to puromycin

and the observation that both synthesis and presence of the protein have not been detected earlier than the 4-cell stage, may suggest that the control of filament

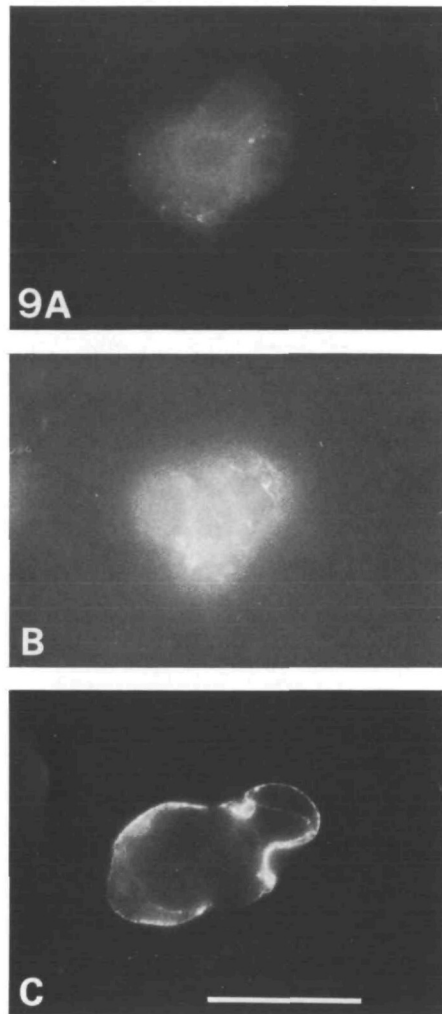


Fig. 9. TROMA-1 staining of isolated ICMs. ICMs from early blastocysts incubated in $2\times$ trypsin for 30 min before fixation. (A) Freshly isolated ICM. Some filaments are visible. (B) ICM from early blastocyst cultured for 8 h *in vitro* before fixation. (C) ICM cultured for 24 h *in vitro* before fixation. Outer cells show extensive filament networks. Bar, 15 μ m.

Table 5. Cytokeratin filaments in mitotic outside cells of 2/16 pairs at 11–13 h postdivision from 1/8 cells

Type of pair	No. pairs	Filaments (% outside cells)	
		interphase	mitotic
Outside + inside cell	119	120/198 (61)	17/40 (43)
Two outside cells	90	66/165 (40)	2/15 (13)

Clusters in which one or both cells had already divided to 2/32 cells are excluded from the data.

For both types of pair, the differences in the incidence of filaments between interphase and mitotic cells are statistically significant (χ^2 tests, $P < 0.05$).

assembly resides at the level of biosynthesis of monomers. However, Lehtonen and his colleagues have suggested that a cytokeratin-like molecule may be present in mouse embryos in advance of the 4-cell stage, but that the protein is packaged in insoluble paracrystalline arrays or aggregates in a form that is not recognized by an antibody such as TROMA-1 (Lehtonen *et al.* 1983; Lehtonen, 1985). We have also detected particulate material in eggs and early embryos (unpublished observations) using polyclonal antisera to bovine hoof prekeratin that stain cytokeratins in eggs and embryos of *Xenopus laevis* (Franz *et al.* 1983). Thus, it is possible that some cytokeratin protein is present as an insoluble store of modified monomer or aggregate in the oocyte, and that this store is also used in the early stages of assembly, perhaps requiring the synthesis of other proteins for their release or assembly. The molecular nature of any stored cytokeratin(s) and its relationship, if any, to the cytokeratins assembled into filaments from the 8-cell stage onwards remains to be established (see Table 1).

The initiation of assembly of filaments appears not to occur in all cells simultaneously, but in a cell autonomous manner over three cell cycles. Moreover, during this period there is a progressive divergence of inside and outside cell populations in terms both of the proportion of each cell type in which assembled filaments are observed, and in the extent and pattern of assembly seen. In light of previous reports (see Introduction), the elaboration of extensive filament networks in the outside, presumptive and definitive trophoctodermal cells is not surprising. However, the detection of assembled filaments in inside, presumptive and definitive ICM cells was unexpected. The relatively low levels of assembled filaments in inside cells, in conjunction with the technical difficulty of detecting them in inside cells *in situ*, explains this discrepancy with earlier findings. The results do however mean that the differences between the two developing cell lineages are quantitative rather than qualitative.

Inside cells could possess filaments as a result of synthesizing them, assembling them from pre-existing protein, by inheriting them or by a combination of these processes. Observations on the distribution of cytokeratin in mitotic and immediately postmitotic 8- and 16-cell blastomeres, when taken with the results of the lineage analysis of those inside 32-cell blastomeres that contain assembled filaments, suggest that the inheritance of filaments (or of the capacity to assemble filaments) may explain the low and lingering

presence of inside cells that are positive for cytokeratin filaments. Other types of cell vary in their handling of cytokeratin networks during division. Many show varying degrees of disassembly with the distribution of aggregate or soluble monomer to both daughter cells (Horwitz, Kupfer, Eshar & Geiger, 1981; Franke, Schmid, Grund & Geiger, 1982; Lane, Goodman & Trejdosiewicz, 1982; Brown, Anderton & Wylie, 1983) but mature, nonstratified epithelial cells tend to retain a network of filaments that

interacts with the cell surface and may place restraints on division planes preventing, for example, differentiative divisions (Lane *et al.* 1982). The pattern observed in mitotic 8- and 16-cell blastomeres resembles that of an immature epithelial type, and indeed we have observed more stable filament networks in mitotic mature trophectoderm (unpublished data). It is possible that as the epithelial nature of the trophectoderm matures progressively (Fleming, 1986), so the stability during division of its

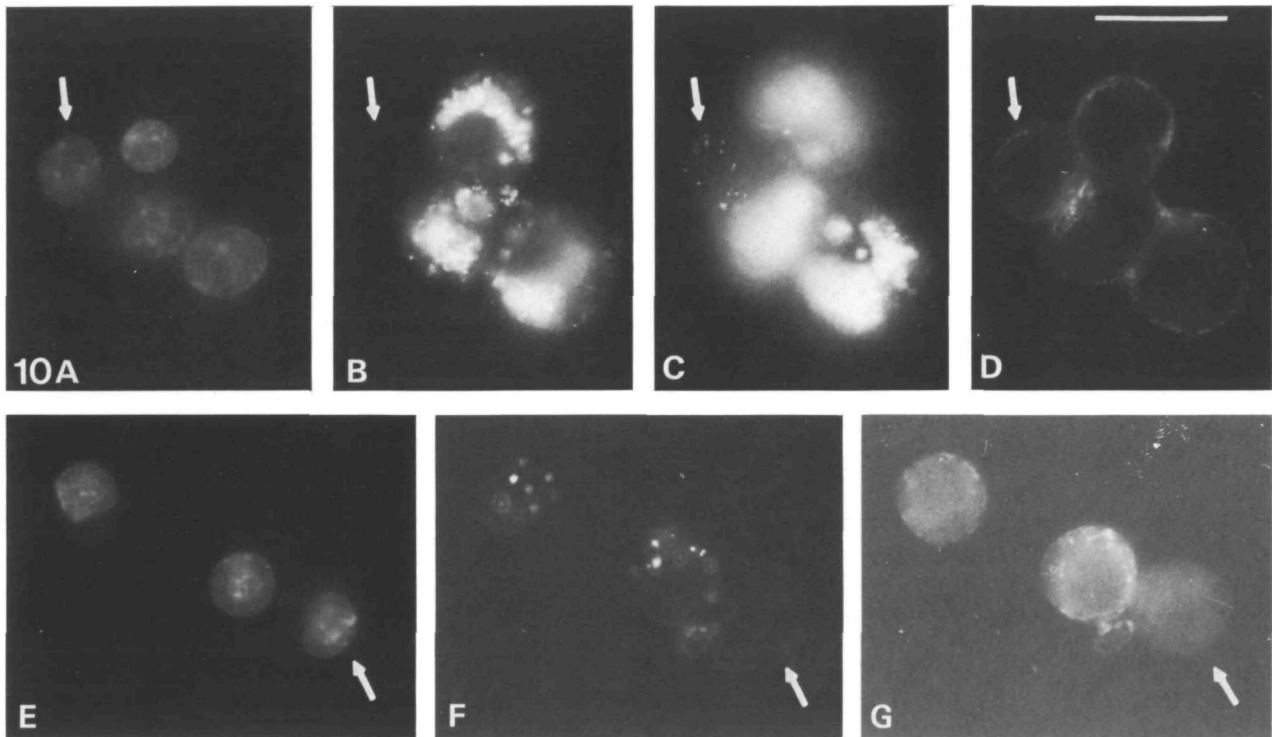


Fig. 10. TROMA-1 staining patterns in 32-cell embryos labelled with R-latex. (A,E) Hoechst dye. (B,C,F) Rh-PNA/R-latex. (D,G) TROMA-1. (A-D) Cluster of three outside cells and one inside cell (arrowed). Rh-PNA extensively labels the surface of the three outer cells (B) but is absent on the inside cell. At a different level of focus (C), it can be seen that the inside cell contains intracellular R-latex particles which are not visible in outside cells due to the high intensity of Rh-PNA fluorescence. (D) The outside cells and the inside cell contain subcortical cytokeratin filaments. (E-G) Three inside cells. Two are derived from outside cells and show R-latex labelling (F) and cytokeratin filaments (G). The third (arrowed) lacks R-latex labelling (F) and is thus derived from an inside 1/16 cell. It contains no filaments (G). Bar, 30 μ m.

Table 6. Origins of filament-containing inside cells in embryos of about 32 cells

Cell no.	No embryos scored	Mean cell no.	% cells scored	% cells with filaments			
				outside cells	total inside cells	inside from inside	inside from outside
<32	13	28.7	83	187/200 (94)	14/108 (13)	5/76 (7)	9/32 (28)
32	5	32.0	78	69/77 (90)	1/47 (2)	0/41 (0)	1/6 (17)
>32	14	38.0	69	191/214 (89)	24/154 (16)	5/108 (5)	19/46 (41)
total	32	33.3	75	447/491 (91)	39/309 (13)	10/225 (4)	29/84 (35)

Embryos were labelled with R-latex at the 16-cell stage (2-3 h post-recompaction) and later with Rh-PNA at 13-17 h post-recompaction, immediately before disaggregation, to label outside cells selectively at both stages with different markers. Combined results from four experiments.

Table 7. Effects of α -amanitin and puromycin on filament appearance in 2/8 pairs

Treatment	No. pairs	No. cells divided (%)	No. pairs with filaments (%)	Cells with filaments (%)		
				1/8 cells	1/16 cells	total
0h control	73	0	0	0	—	0
18h control	45	84 (93) [+1 mitotic]	22 (49)	1 (17)	44 (26)	45 (26)
α -amanitin 0–18h	33	43 (65) [+3 mitotic]	13 (39)	7 (30)	16 (19)	23 (21)
Puromycin* 0–18h	46	0	0	0	—	0
5–18h	51	0	13 (25)	19 (19)	—	19 (19)
10–18h	58	56 (48) [+1 mitotic]	15 (26)	NS	NS	33 (19)
16–18h	41	73 (89) [+1 mitotic]	21 (51)	NS	NS	38 (25)

* Cell pairs were placed into puromycin at the appropriate time and harvested at 18 h postdivision. NS, not scored.

intermediate filament network increases. Since the expression of totipotency in outer trophectoderm cells requires them to divide differentially (Johnson, 1986), the maturation of a stable, tangentially arrayed network of cytokeratin filaments could constrain the cells to divide conservatively, and thus effectively amount to the commitment of these cells to a restricted fate as trophectoderm cells.

The lineage studies on ICM cells suggest that the longer an inside cell remains internally, the more likely it is to lose the few assembled filaments that it has. Thus, inside 32-cells derived as a result of the differentiative division of polarized 8-cells show a much lower incidence of assembled filaments than do inside 32-cells derived from the later division of polarized 16-cells. Since the classification of the cells of the ICM into two groups on the basis of their time of allocation is also reflected in their molecular organization, it is possible that they may have different fates within the ICM. For example, primary endoderm cells express cytokeratin filaments, and we are therefore enquiring whether they are derived primarily from the second allocation of inside cells.

If both inside and outside cell populations contain cytokeratin-positive cells, we must ask why the outside cells progress to elaborate more complex networks whilst the inside cells tend to dismantle their networks? Ultimately, it seems that the two populations differ in their content of mRNA encoding ENDO-A (Johnson, 1979; Duprey *et al.* 1975), but it is not clear when or how this state is achieved. Since we know that prolonged exposure of inside cells results in the acquisition of cytokeratin mRNA and protein and in the transformation of phenotype to trophectoderm (Handyside & Johnson, 1978 and

Fig. 8 of this paper), it is tempting to speculate that the patterns of cell interaction within the morula are influencing the synthesis and assembly of cytokeratins. We know that patterns of cell contact do affect the spatial organization within the blastomeres (Ziomek & Johnson, 1980; Johnson & Ziomek, 1981), including the redistribution of cytoskeletal elements (Johnson & Maro, 1983; Houlston, Pickering & Maro, 1987). The calcium-dependent cell–cell adhesion system, of which the principle molecular component in the embryo is uvomorulin/cadherin, is involved in mediating this contact-dependent response (Shirayoshi, Okada & Takeichi, 1983; Johnson, Maro & Takeichi, 1986). It is also well established that contact between other types of cells can influence the content and degree of assembly of cytoskeletal monomers as well as the expression of the genes encoding them (Ben Ze'ev, 1984, 1985; Ungar, Geiger & Ben Ze'ev, 1986). It is thus not unreasonable to suggest that the elaboration of the cytokeratin network in outside cells and its corresponding decline in inside cells, relates to the differing contact patterns of the two cell populations. We are currently investigating this possibility, and the role that the cell–cell adhesion system of the embryo might play in the process.

In conclusion, our experiments confirm the notion that the cytokeratins may provide a useful marker system for studying the underlying molecular controls in early development, but suggest that the regulation of their expression may involve continuing cell interactions that modulate gene expression *via* their effects on the state of organization of the cytoskeletal system of the cell. Whether the cytokeratins provide a

representative example of selective gene expression in the early mouse embryo remains to be determined.

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