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Synthesis and phosphorylation of uvomorulin during mouse early development

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Summary

The cell adhesion molecule, uvomorulin, is synthesised in both the 135×10^3 M_r precursor and 120×10^3 M_r mature forms on maternal mRNA templates in unfertilized and newly fertilized mouse oocytes. Synthesis on maternal message ceases during the 2-cell stage to resume later on mRNA encoded presumptively by the embryonic genome. Uvomorulin is detectable by immunoblotting at all stages upto the blastocyst stage, but shows variations in its total amount and processing with embryonic stage. Whilst only trace levels of phosphorylated uvomorulin are detectable in early and late 4-cell embryos, uvomorulin in 8-cell embryos is phosphorylated.

Key words: mouse, uvomorulin, compaction, phosphorylation

Introduction

Compaction of the eight cell stage mouse preimplantation embryo involves the polarization of blastomeres and the increased apposition of their basolateral membranes (Ducibella and Anderson, 1975; Lehtonen, 1980; Ziomek and Johnson, 1980; Johnson and Ziomek, 1981b; Magnuson et al., 1977; Wiley and Eglitis, 1981). Cell divisions subsequent to this process give rise to distinct cell populations from which differentiated cell lineages within the embryo arise (Johnson and Ziomek, 1981a). Antibodies raised against the cell adhesion molecule uvomorulin prevent increased cell adhesion and disturb the incidence and axis of polarization (Hyafil et al., 1980, 1981; Johnson et al., 1986; Shirayoshi et al., 1983; Vestweber and Kemler, 1984), suggesting that uvomorulin plays an important role in these events.

Uvomorulin (Gallin et al., 1983; Peyrieras et al., 1983) is a member of the cadherin family of cell adhesion molecules, which functions through calcium-dependent homophilic association between molecules on adjacent cells. It is a transmembrane glycoprotein, M_r 120×10^3, which is homologous to L-CAM isolated from liver (Bertolotti et al., 1980; Gallin et al., 1983). Like other members of the cadherin family, uvomorulin is synthesized as a larger precursor molecule of 135×10^3 M_r, which must be cleaved correctly for expression of its adhesive function (Ozawa and Kemler, 1990). Uvomorulin is thought to attach to the actin cytoskeleton via proteins known as catenins which associate with its cytoplasmic domain and were initially identified by co-precipitation with uvomorulin during immunoprecipitation (Ozawa et al., 1989, 1990). Uvomorulin has potential phosphorylation sites located in its cytoplasmic domain and L-CAM has been demonstrated to be phosphorylated on both serine and threonine residues (Cunningham et al., 1984).

Compaction is controlled at a posttranslational level: exposure to protein synthesis inhibitors from the mid 4-cell stage fails to prevent the process and may even advance it (Kidder and McLachlin, 1985; Levy et al., 1986). A potential role for protein phosphorylation at compaction, possibly involving Protein Kinase C, has been suggested following perturbation of compaction using agents that affect this kinase (Bloom, 1989; Bloom and McConnell, 1990; Winkel et al., 1990). Since uvomorulin has potential phosphorylation sites and since phosphorylation may be important in compaction, we have examined the phosphorylation status of uvomorulin during compaction. We show that uvomorulin is phosphorylated in 8-cell embryos immediately prior to the onset of compaction but not at earlier stages.

Materials and methods

Recovery and handling of embryos

MF1 female mice (3-4 weeks; Olac, Bicester, UK) were superovulated by intraperitoneal injections of 5 i.u. of pregnant mares' serum gonadotrophin (PMS, Intervet) and human chorionic gonadotrophin (hCG, Intervet) 48 hours apart. To obtain embryos, females were paired individually overnight with HC-CFLP males (Interfauna) and inspected for vaginal plugs the next day as an indication of successful mating. Embryos were flushed from oviducts in medium H6, a Hepes-buffered equivalent of modified T6 medium (Nasr-

Esfahani et al., 1990), containing 4 mg/ml bovine serum albumin (BSA), and cultured in T6 plus BSA at 37°C in 5% CO_2 in air under oil. Unfertilized and fertilized oocytes were removed from dissected oviducts by release into warmed H6 medium plus BSA, and exposed briefly to 0.1% hyaluronidase (Sigma) to remove cumulus cells. Late 2-cell and early 4-cell embryos were flushed from oviducts at 46-50 hours post-hCG. All manipulations were carried out using pre-warmed medium on heated microscope stages.

Labelling of embryos

[^{35}S]methionine: embryos at selected stages were cultured in 50 μl drops of T6+BSA containing 1.5 mCi/ml [^{35}S]methionine (1/10 dilution of SJ204, Amersham International) for 2 hours, washed twice in medium without label, and once in H6+PVP (Polyvinylpyrrolidine, 6 mg/ml).

[^{32}P]orthophosphate· embryos were recovered and cultured in phosphate-free T6+BSA medium (KH_2PO_4 omitted and osmolarity corrected with NaCl). Cohorts of embryos were incubated in 50 μl drops of phosphate-free T6+BSA containing 1 mCi/ml of [^{32}P]orthophosphate (1/10 dilution of PBS13, Amersham International) for 1 hour and washed three times in phosphate-free H6+PVP.

Immunoprecipitations and immunoblotting

Immunoprecipitations were performed on groups of embryos using an affinity-purified rabbit antibody to uvomorulin (Hyafil et al , 1981). Control immunoprecipitations were carried out using non-immune rabbit serum Embryos were lysed in 10 μl buffer (0.15 M NaCl; 10 mM Tris pH7.5; 1 mM EDTA; 25 μg/ml PMSF) containing 2% sodium dodecyl sulphate (SDS). The resulting lysate was diluted 1/20 in buffer containing 0 55% NP-40 to give a final concentration of· 0.5% NP-40; 0.1% SDS; 0.15 M NaCl; 10 mM Tris pH7.5; 1 mM EDTA; 10 mg/ml PMSF (I.P. buffer). The lysate was spun to pellet the insoluble material and the supernatant incubated with 80 μg (1/100 dilution) of antibody for 30 minutes at room temperature. The supernatant was incubated with 50 μl of a 50% suspension of protein A sepharose beads (Pharmacia) in I.P buffer for 50 minutes at room temperature. The beads were washed twice in I.P buffer and the protein removed from the beads using Laemmli sample buffer at 65°C for 15 minutes. Samples were stored at −20°C before electrophoresis.

Protein 'western' blotting was performed according to Towbin et al. (Towbin et al , 1979). Proteins were transferred to nitrocellulose paper by electrophoresis overnight at 15 V, 0.2 A After transfer, blots were blocked in Tris-buffered saline (TBS) plus 2% dried milk powder (Marvel) for 30 minutes then incubated with antibody at 200 μg/ml for 1 hour in the above buffer plus 0.5% Tween20 (blot buffer). After 3 washes over 30 minutes in blot buffer, antibody binding was detected by incubation in ^{125}I-Protein A (Amersham International) at 0.4 μCi/ml in blot buffer Washed blots were dried and exposed to X-ray film as below. Exposed films were scanned using a Joyce Loebl scanning densitometer

Gel electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (Laemmli, 1970). 7.5% acrylamide gels were used throughout. Gels of labelled samples were fixed in methanol/acetic acid (20%/7% v/v), treated with Amplify (Amersham International) for 15 minutes, dried and exposed to pre-flashed X-ray film (Fuji RX) at −80°C with intensifying screens.

Results

Presence and biosynthesis of uvomorulin

In order to determine whether the increase in adhesiveness of blastomeres at compaction might be related to an increase in the amount of uvomorulin present in embryos, samples of equal numbers of embryos from different stages were analysed by western blotting. In all post-fertilization stages, a single protein was recognized of 120×10^3 M_r, i.e. the expected position of uvomorulin (arrowhead in Fig. 1). In unfertilized oocytes only, a second immunoreactive species was recognized, of slower mobility corresponding to an apparent relative molecular mass of 135×10^3. An uvomorulin precursor migrating in this postion has been detected previously in the embryonal carcinoma line PCCA Aza (Peyrieras et al., 1983).

The amount of uvomorulin detected was dependent on the stage of embryo development examined. Its level rose after fertilization and then decreased in 2-cell embryos. The level rose again in 4-cell embryos followed by an apparent decrease in 8-cell embryos before reaching high levels at the blastocyst stage. These results were confirmed in each of three replicate blots and the relative amount of uvomorulin was quantified by densitometric scanning (Fig. 2). The rise in uvomorulin levels after fertilization could not be accounted for by processing of the 135×10^3 M_r protein to its 120×10^3 M_r form, since combining the values obtained from both the precursor 135×10^3 M_r band and

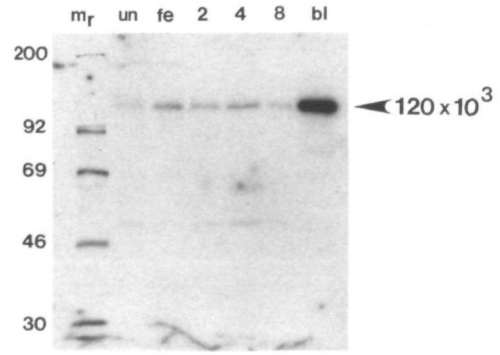

Fig. 1. Western blot analysis of uvomorulin levels in preimplantation mouse embryos. m_r, relative molecular mass markers, un, unfertilized oocytes, fe, 1-cell embryos; 2, 2-cell embryos, 4, 4-cell embryos; 8 8-cell embryos (compact), bl, blastocysts. The result was confirmed in three separate replicate experiments. Arrowhead indicates the position of uvomorulin at 120×10^3 M_r. Samples of 250 embryos from each stage were separated by SDS-PAGE on 7.5% acrylamide gels, transferred electrophoretically to nitrocellulose and probed with a polyclonal antiserum raised against uvomorulin and visualized with ^{125}I-protein A.

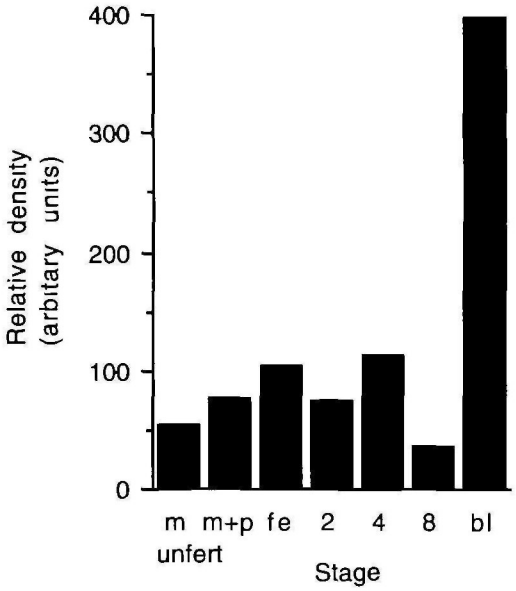

Fig. 2. Graphical representation of the densitometric analyses from a single representative "western" blotting experiment. The autoradiogram was scanned on a Joyce Loebl densitometer and the density values represent the peak heights for each stage The value for unfertilized oocytes is presented both as the value obtained for the 120×10^3 M_r band only (m) and the combined value of the 120×10^3 M_r and 135×10^3 M_r readings (m+p) The graph represents a single experimental replicate (which was repeated three times) and was calibrated using the reading for blastocyts as the maximal density.

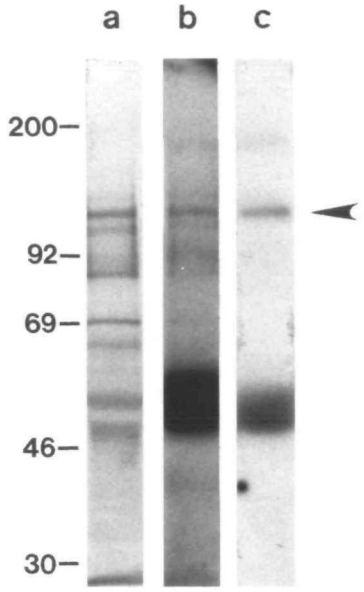

Fig. 3. Immunoprecipitate of [^{35}S]methionine-labelled (a) 8-cell embryos and (b) blastocysts, the latter having also been western blotted; (c), western blot of unlabelled blastocysts. Arrowhead indicates the position of uvomorulin at 120×10^3 M_r. Samples of 300 embryos were separated by SDS-PAGE on 7 5% acrylamide gels either with or without having previously been immunoprecipitated (see Materials and methods) The samples were then transferred to nitrocellulose and lanes b and c were probed with antiserum. Arrowhead indicates the position of uvomorulin at 120×10^3 M_r.

the mature 120×10^3 M_r uvomorulin band in unfertilized oocytes did not result in the level observed in fertilized oocytes (Fig. 2).

To determine whether the variations in the amount of uvomorulin could be explained by synthetic changes during these early stages, [^{35}S]methionine incorporation into immunoprecipitatable uvomorulin was examined. When proteins immunoprecipitated from embryos labelled internally with [^{35}S]methionine were separated by SDS-PAGE, a triplet of bands was observed at around 120×10^3 M_r. The identification of uvomorulin as the middle band of the this triplet was determined by comparing the immunoprecipitate from [^{35}S]methionine-labelled 8-cell embryos (Fig. 3,lane a) with an immunoblot of total blastocyst lysate (Fig. 3,lane c) and an immunoblot of the immunoprecipitable protein from [^{35}S]methionine-labelled blastocysts (Fig. 3,lane b). The upper protein of the triplet at 135×10^3 M_r may correspond to the uncleaved precursor form labelled in the 2 hour [^{35}S]methionine pulse but present at too low a level to be detected by western blotting. The lower band corresponds to the unglycosylated form of uvomorulin similarly not detected in western blots (Gallin et al., 1983; Peyrieras et al., 1983). Having determined the relative position of uvomorulin in immunoprecipitates, we were able to investigate the comparative levels of incorporation of [^{35}S]methionine at various stages. Two bands corresponding to 120 and 135×10^3 M_r were evident at all stages. The 135×10^3 M_r band predominated in unfertilized oocytes (Fig. 4,lane f), but the mature 120×10^3 M_r form was the major species in fertilized oocytes (Fig. 4,lane e) and 8-cell embryos (Fig 4,lane c). Very little [^{35}S]methionine incorporation could be detected in either form of uvomorulin in late 2-/early 4-cell embryos (Fig. 4,lane d), however the level had increased by the early 4-cell stage (Fig. 5,lane d).

Phosphorylation of uvomorulin at compaction

The phosphorylation of a 120×10^3 M_r protein was detected in immunoprecipitates from ^{32}P-labelled 8-cell (Fig. 5,lane c) but not 4-cell embryos (Fig. 5,lane b: arrowed). This phosphoprotein co-migrated with the protein previously identified as uvomorulin from immunoprecipitates of [^{35}S]methionine internally labelled 4- and 8-cell embryos (Fig. 5, lanes d and e). In most instances, phosphorylation of the 135×10^3 M_r form was observed in proportion to the 120×10^3 M_r phosphoprotein.

To define more precisely the relationship between the phosphorylation of uvomorulin and compaction, ^{32}P incorporation into uvomorulin was compared in newly formed 4-cell (Fig. 6,lane b), late 4-cell (timed as ten hours post-division: Fig. 6,lane c), early precompact 8-cell (Fig. 6,lane d) and late compact 8-cell embryos (Fig. 6,lane e). Whilst both populations of 4-cell embryos had only trace incorporation of ^{32}P-label into uvomorulin, both early and late 8-cell embryos showed

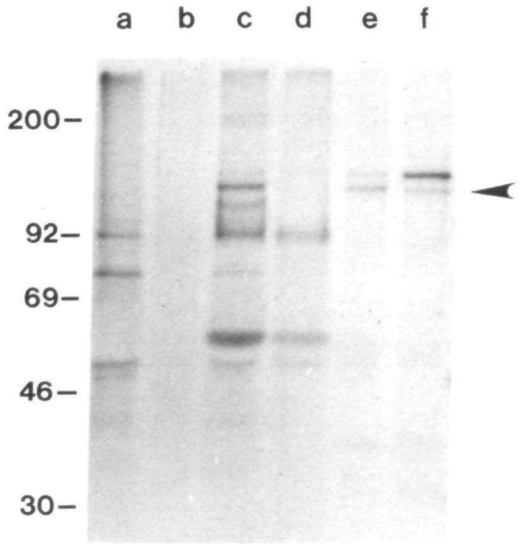

Fig. 4. [^{35}S]methionine incorporation into immunoprecipitates of embryos of various stages· (a), normal rabbit serum control for 8-cells; (b) normal rabbit serum control for unfertilized oocytes; (c), 8-cell embryos, (d), late 2- to 4-cell embryos; (e), fertilized oocytes, (f), unfertilized oocytes. Arrowhead indicates the position of uvomorulin at 120×10^3 M_r. This result was confirmed in three separate experimental replicates. Samples of 300 embryos were pulse-labelled with [^{35}S]methionine and the incorporation of the label was detected by immunoprecipitation (see Materials and methods). The samples were analysed by SDS-PAGE on 7.5% acrylamide gels.

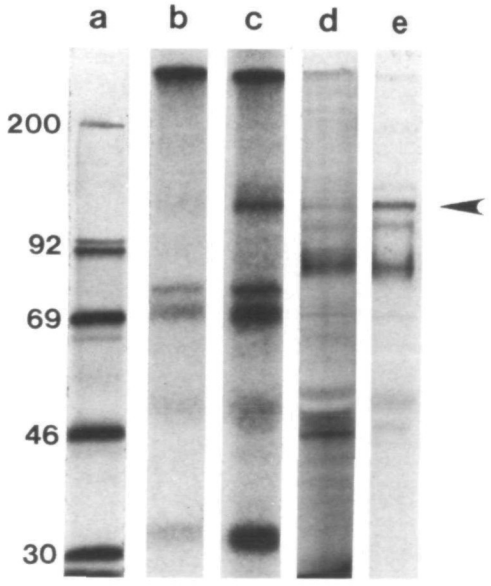

Fig. 5. Identification and determination of phosphorylation state of uvomorulin in embryos by immunoprecipitation with a polyclonal antiserum to uvomorulin: (a) relative molecular mass markers, (b) ^{32}P-labelled 4-cell embryos; (c) ^{32}P-labelled 8-cell embryos; (d) [^{35}S]methionine-labelled 4-cell embryos, (e) [^{35}S]methionine-labelled 8-cell embryos. This result was confirmed in three separate experimental replicates.

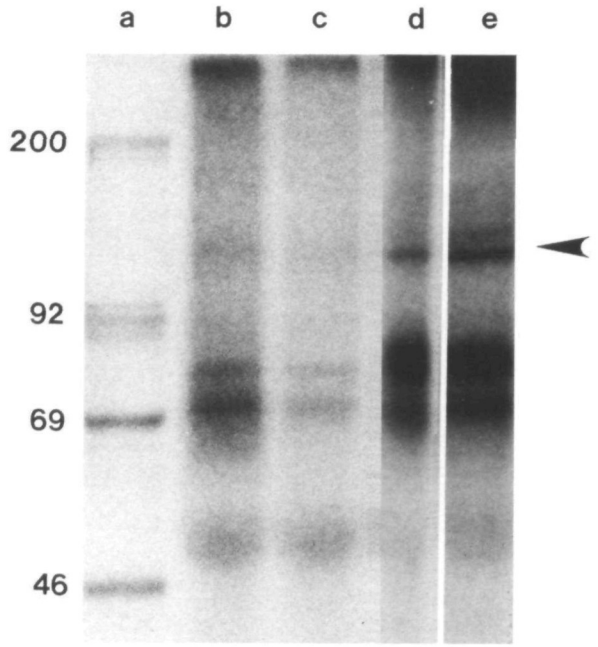

Fig. 6. Analysis of the timing of uvomorulin phosphorylation: (a) relative molecular mass markers; (b) early 4-cell embryos; (c) late 4-cell embryos, (d) early (non-compact) 8-cell embryos; (e) late 8-cell embryos. Arrowhead indicates the position of uvomorulin at 120×10^3 M_r. Samples of 250 embryos were pulse-labelled for 1 hour with [^{32}P]orthophosphate and the incorporation of label was detected by immunoprecipitation. The samples were analysed by SDS-PAGE on 7.5% acrylamide gels This result was confirmed in three separate experimental replicates

similar levels of incorporation (Fig. 6). Embryos that were in a transitional state at the start of the labelling period (embryos containing 5 to 7 cells i.e. during the transition from the 4- to 8-cell stages), also showed incorporation of phosphate label but at a lower level than that of the early and late 8-cells (data not shown).

Discussion

The involvement of uvomorulin in the events of compaction has been demonstrated previously, embryos in which uvomorulin was neutralised showing abnormal compaction (Johnson et al., 1986; Shirayoshi et al., 1983). In addition, uvomorulin is redistributed to the basolateral membrane domain during compaction (Vestweber et al., 1987; Winkel et al., 1990). The behaviour of uvomorulin in other cell systems also suggests an important role in the development of both cell adhesion and polarisation. When uvomorulin was transfected into a non-polar cell line of mouse fibroblasts (L-cells), the cells developed extensive cell-cell contacts and redistributed the membrane protein Na^+K^+ATPase to basolateral membrane domains (McNeill et al., 1990). This basolateral localization is important for the development of vectorial transport in epithelial cells, and has been shown to occur in the

mouse embryo prior to cavitation and the accumulaton of blastocoelic fluid (Vorbrodt et al., 1977; Watson and Kidder, 1988). The redistribution of uvomorulin during polarization is thought to be effected via proteins called catenins, which associate with its cytoplasmic domain and provide a direct link with the actin-based cytoskeleton (Ozawa et al., 1989, 1990). Thus both direct and indirect evidence implicates uvomorulin as an important molecular component in the genesis of the compacted state. In this study, we have shown that the amount of uvomorulin does not increase significantly between the 1-cell zygote and the 8-cell stage. A compaction-deficient mutant of the F9 teratocarcinoma cell line has recently been described which expresses reduced levels of uvomorulin (Adamson et al., 1990). This low level of expression was offered as an explanation for the impaired capacity of the cells to compact. Compaction in F9 cells shows many similarities to that seen in embryos (Adler and Ziomek, 1989), yet it does not seem, from our results, that the increase in adhesiveness seen during compaction can be accounted for simply by a quantitative increase in uvomorulin, indeed its level falls from the 4- to 8-cell stage. By immunoblotting, we also demonstrated the presence of a higher relative molecular mass species in unfertilized oocytes that is not present in post-fertilization stages. We believe that this band represents the 135×10^3 M_r precursor of uvomorulin which must be cleaved correctly for normal expression of adhesive function (Ozawa and Kemler, 1990; Peyrieras et al., 1983). It had been reported previously that uvomorulin synthesis did not commence until the embryonic genome had been activated at the late 2-cell stage (Vestweber et al., 1987). However, we have observed incorporation of [^{35}S]methionine into uvomorulin in both unfertilized and fertilized oocytes, suggesting that synthesis on maternal uvomorulin templates does occur. The previous study employed a prolonged labelling strategy which, in our experience, is deleterious for embryos at these early stages and so may explain the earlier failure to detect uvomorulin synthesis. The ability of oocytes to synthesise uvomorulin offers an explanation for its apparent increase in fertilized oocytes observed by immunoblotting. This accumulation of the putative precursor form in unfertilized oocytes suggests that processing may be slower prethan postfertilization. The activation of translation on other maternal transcripts at fertilization has been reported previously (Braude et al., 1979; Cullen et al., 1980). Furthermore, we detected only a low level of uvomorulin synthesis at the late 2-/early 4-cell stages, probably reflecting the loss of maternal transcripts occurring at this time coupled to a delay in the synthesis of embryonic transcripts for uvomorulin (Bolton et al., 1984). The results of the immunoblot experiments show that uvomorulin itself is present throughout the period in which its synthesis first declines and then rises, suggesting that the stability of uvomorulin derived from synthesis on maternal templates is sufficient to ensure its continuing presence at least into the 4-cell stage and probably for longer (Pratt et al., 1983). These results raise the possibility that the uvomorulin synthesised from the 4-cell stage onwards may differ in some way from that carried over from the oocyte, although there is no evidence at present to suggest the presence of more than one gene encoding uvomorulin or of the existence of variants due to alternative splicing (Sorkin et al., 1988).

A clear difference that does distinguish uvomorulin at the 4-cell and 8-cell stages is its phosphorylation status. Phosphorylation of uvomorulin was first observed clearly in recently formed 8-cell embryos, both in the mature and uncleaved form. At this stage, no overt sign of compaction can be observed but the process of intercellular signalling associated with the initiation and orientation of polarity is known to be occurring (Johnson and Ziomek, 1981b). Cell adhesive properties, association with catenins and the ability to induce a polarized phenotype have all been demonstrated to be dependent on a functional cytoplasmic domain in uvomorulin (Jaffe et al, 1990; McNeill et al., 1990; Ozawa et al., 1990), the location of the phosphorylation sites. It is therefore possible that the phosphorylation status of uvomorulin might affect any or all of these properties. Indeed, a role for phosphorylation at compaction has been proposed recently, based on the identification of proteins whose phosphorylation status changes in a manner temporally related to the events of compaction (Bloom and McConnell, 1990). Agents that affect elements in the cellular protein phosphorylation cascades, such as activators and inhibitors of protein kinase C, have also been shown to cause dramatic effects on compaction (Bloom, 1989; Winkel et al., 1990). We are currently investigating the effects of these and other agents on the phosphorylation status of uvomorulin in order to define more precisely its relationship to the control of the onset of compaction.

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