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Anomalous Patterns in Cultured Cell Monolayers

Abstract. *Gridlike patterns of differing cell density were observed in evenly seeded cell monolayers. Such patterns were obtained in five of six cell lines tested, suggesting widespread occurrence. The mechanism appears to involve small, transient temperature changes related to incubator tray structure. The very short time course of appearance of the patterns implicates attachment rather than growth as the critically affected factor. Impaired adhesion or directed sedimentation resulting from thermally induced microcurrents in the medium are the two most likely mechanisms.*

Achieving a uniform distribution of cells in a culture vessel is of primary importance in cell culture. Such basic considerations as plating efficiency and contact inhibition of cell growth are directly related to the density with which suspended cells settle out and attach to the culture dish. In addition, in many cell lines function at high cell densities differs from function at low densities. Mullin *et al.* (*1*) found that confluent but not subconfluent cultures of LLC-PK_1 cells actively accumulated α-methyl-D-glucoside and that the development of morphological polarity in this line (appearance of apical microvilli) was also correlated with culture density. Cereijido *et al.* (*2*) found that monolayers of MDCK cells seeded at superconfluent densities developed transepithelial resistance more rapidly when the cells were derived from confluent cultures than when they were derived from subconfluent cultures. The investigators suggested that information for synthesizing tight junctions was not present in the subconfluent cultures. Similarly, cilia development in rat liver cells (*3*) and the appearance of a 60,000-dalton cell surface protein in endothelial cell cultures (*4*) have been correlated with high cell density. In many nonepithelial cell lines, attainment of cell confluence and density-dependent growth inhibition is associated with decreased transport activity. Decreased transport of K^+, phosphate, nucleosides, and sugars have all been observed as cultures become confluent (*5*). The existence of cell populations of nonuniform density could thus introduce a significant error into investigations of a number of parameters.

During a routine check of cell growth on collagen-coated Nuclepore filters in plastic petri dishes, we observed a gridlike growth pattern. Areas of heavy cell growth surrounded evenly spaced patches of low cell density. The growth pattern appeared to correspond to the structure of the incubator tray on which the petri dishes had been placed during incubation, with areas of low cell density corresponding to holes in the tray and areas of higher density corresponding to the metal framework (Fig. 1, A and B). This correspondence between cell growth pattern and incubator tray design was previously observed with HeLa cells by Puck (*6*). The phenomenon was attributed to an extreme sensitivity of cell growth to small transient temperature differences presumed to be created between holes in the tray and the framework when the incubator door was opened.

To examine the mechanism of the observed patterning of cell growth in more detail we suspended trypsinized LLC-PK_1 cells in Eagle's minimum essential medium (Flow) containing 10 percent fetal bovine serum (Flow) and seeded them at different densities into 60-mm-diameter Falcon petri dishes (*7*). The cultures were incubated at 37°C in a humidified 5 percent CO_2 atmosphere in a water-jacketed incubator (capacity, 0.15 m^3) with perforated aluminum shelves. At the end of each experiment the cells were fixed with absolute methanol for 30 minutes and stained with Giemsa. For experiments in which cell density was assayed by DNA analysis, cells were grown on collagen-coated Nuclepore filters. Halved filters were

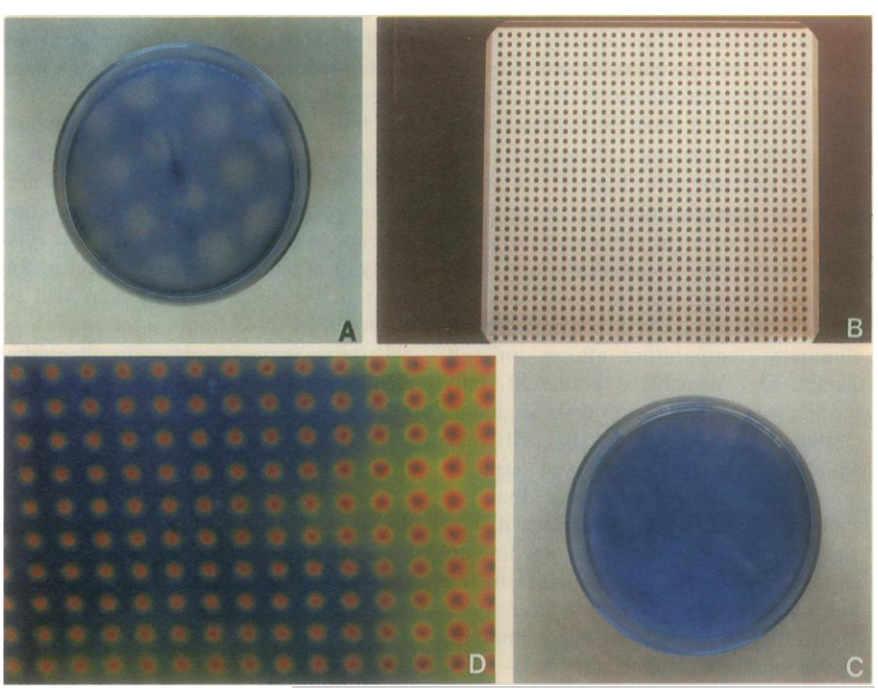

Fig. 1. (A) Patterned cell growth in a 60-mm-diameter petri dish. (B) Tray on which the dish was placed during incubation. (C) Petri dish not exhibiting patterning. This is typical of dishes resting on glass plates during incubation. (D) Sheet of liquid crystals (sensitivity, 35° to 40°C) on an incubator tray that has just been removed from an incubator at 39°C (color spectrum 35° to 40°C: black, brown, red, yellow, green, blue, and black).

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placed in 1.0*M* perchloric acid, and the DNA was hydrolyzed at 78°C for 45 minutes and measured spectrophotometrically by the technique of Burton (*8*).

To determine whether there was a temperature difference between the metal framework of the tray and the holes, we placed 30 by 30 cm sheets of thermophotochromic liquid crystals (Parker Liquid Crystals, Edmund Scientific), temperature-sensitive in the range of 30° to 40°C, on a tray in the incubator (set at 37°C). Temperature was allowed to equilibrate with the incubator door shut and then the door was opened. The drop in temperature was both greater and faster over the holes than over the metal, although the difference never exceeded 4°C (Fig. 1D). After the door was shut, about 10 minutes were required to reach a uniform temperature distribution. We then placed a sheet of crystals sensitive over the range of 35° to 36°C on a tray with the incubator set at 35.5°C. No difference in temperature could be detected between holes and framework when the incubator was left to equilibrate for several hours.

The patterning appears to be independent of the substratum, since it was observed both when cells were seeded on collagen-coated Nuclepore filters and directly on uncoated plastic petri dishes. Patterning was also obtained in Falcon T75 culture flasks. We tested LLC-PK_1 cells from confluent and subconfluent cultures and observed the phenomenon in each case. Patterning was not affected by varying incubator capacity (0.08 or 0.15 m^3) or tray design (size and shape of holes and hole/metal ratio). No patterning was observed when petri dishes were placed on a 3-mm-thick glass plate lying on the incubator tray (Fig. 1C). A sheet of liquid crystals indicated a uniform temperature over the surface of the glass plate when the incubator door was closed or opened briefly.

The time course of pattern appearance eliminates temperature effects on cell growth as a possible explanation. Patterning was obtained with cells seeded at confluent density (2.1×10^5 cells per square centimeter) and fixed after 1 hour, well within the 24-hour doubling time of the LLC-PK_1 line. Petri dishes were rotated 16 hours after seeding to expose new areas of dish surface to the holes in the tray; 24 hours later these dishes displayed patterns corresponding to the initial placement.

Eliminating temperature effects on cell growth as an explanation for the patterning implicates an initial uneven cell distribution on the culture dish. The rapidity of the appearance of pattern elements

Table 1. Dome (*12*) and pattern formation in epithelial (E) and fibroblastic (F) cell lines.

Cell line	Cell type	Dome formation	Pattern formation
LLC-PK_1	E	Yes	Yes
MDCK	E	Yes	Yes
LLC-RK_1	E	No	Yes
RK_{13}	E	No	Yes
BALB/c-3T3	F	No	No
Primary hamster embryo	F	No	Yes

1 cm in diameter eliminates the possibility that the cells migrate from areas of the dish lying over the holes. Possible explanations include directed sedimentation of the cells by thermally induced microcurrents in the medium and preferential adhesion of the cells in response to temperature differences across the culture dish.

Cells grown on trays in an incubator set at 34°C also displayed patterning, and the density (measured by DNA analysis of whole monolayers) of cells grown on glass plates at 34°C was comparable to the density of cells grown at 37°C. This suggests that patterning may not result from the lower temperature over the holes per se, but from the more rapid and pronounced temperature shifts over the holes relative to the metal framework. An inhibition of cellular adhesion to substrate by low temperatures has been noted by a number of investigators (*9–11*); however, the effects of nonuniform temperature shifts appear to have been neglected. Moreover, little effect has been observed at temperatures above 30°C, and both Attramadal (*9*) and Nath and Srere (*11*) noted the ready reversibility of inhibition upon raising the temperature. In the present study, pattern formation appears to be determined during a relatively brief period (< 1 hour) after placement in the incubator but can still be observed 24 to 48 hours later.

Patterns were observed in all epithelial lines tested (LLC-PK_1, LLC-RK_1, MDCK, and RK_{13}) and in primary hamster embryo fibroblasts, but not in BALB/c-3T3 cells (Table 1). This indicates that pattern formation is not peculiar to the LLC-PK_1 line, to dome-forming epithelia, or to epithelial lines in general but can occur in cultured cells irrespective of cell type. Concerning the detection of patterning in cell cultures, two points are worth noting:

1) The appearance of patterns is not perfectly reproducible, even when apparently identical conditions have been used. The presence of large objects in the incubator may alter pattern formation, possibly by interfering with air convection currents.

2) Cell density at staining is important for detecting patterning; when overall density is too low or too high, patterning is not easily discernible. Even when patterning is not visibly detectable after staining with Giemsa, there may be functional differences in cell populations corresponding to incubator tray structure. During trypsinization of apparently confluent cultures in Falcon tissue-culture flasks, cells were observed detaching from areas corresponding to the holes in the tray appreciably earlier than detachment of the rest of the monolayer (*12*).

A cell line known to give rise to a heterogeneous population of cells in culture will "segregate" the cell types in response to continuous temperature gradients (*13*). This is a striking example of the potential effect of the temperature gradients produced by conventional incubator trays.

The phenomenon is of obvious importance in studies involving electrophysiological measurements across cultured epithelial monolayers—a rapidly growing field (*14*). Cell density was recently implicated as an important factor in transmonolayer resistance (*15*). The presence of patches of differing cell density could thus add a substantial artifact to all electrical measurements.

While it is difficult to obtain perfectly even seeding under the best of conditions, the grossly uneven densities seen in patterning can be easily avoided. In view of the widespread appearance of patterning in various cell lines, we suggest that laboratories engaged in cell culturing institute routine checks for this phenomenon.

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7. Confluent density for the LLC-PK_1 line in 60-mm dishes is approximately 2.1×10^5 cells per square centimeter. For experiments lasting 36 to 48 hours seeding density was 1.5×10^4 cells per square centimeter and for experiments lasting 1

to 6 hours seeding density was 8.8×10^4 to 2.1×10^5 cells per square centimeter.

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The Carboxy Terminus of the Precursor to Vasopressin and Neurophysin: Immunocytochemistry in Rat Brain

Abstract. *A pituitary glycopeptide whose amino acid sequence was previously identified has now been recognized as the final portion of the precursor to arginine vasopressin and its associated neurophysin. Immunocytochemical techniques with antiserums against this 39 amino acid peptide and vasopressin were used to study their distribution in the rat central nervous system. The peptide is located in vasopressin-synthesizing cells in the neurosecretory magnocellular nuclei. Positively stained fibers project from the magnocellular nuclei through the median eminence to the posterior pituitary. Studies of the homozygous Brattleboro rat, which is known to be deficient in the production of vasopressin and its related neurophysin, also show the absence of immunoreactivity to this peptide. These immunocytochemical data strongly indicate that the peptide is synthesized with vasopressin.*

Until recently, the full structure of the precursor of vasopressin remained unknown, although it was hypothesized that neurophysin was contained within it. A 39 amino acid glycopeptide isolated from human pituitary by Seidah *et al.* (*1*) was a likely candidate for the missing portion of the vasopressin and neurophysin precursors. The peptide was isolated from the posterior pituitary and generally was found in the appropriate sites (*1*, *2*), its molecular weight was consistent with that predicted from precursor labeling experiments (*3*), and it was also glycoprotein (*3*). Furthermore, a computer search revealed that similar molecules had been isolated from pig, sheep, and ox pituitaries (*4*), indicating phylogenetic conservation and potential biological importance.

We now report that this glycopeptide and vasopressin are indeed present in the magnocellular vasopressin pathways projecting to the posterior pituitary. These results are consistent with the findings of Land *et al.* (*5*) on the sequence of complementary DNA to the messenger RNA of bovine arginine vasopressin (AVP) and neurophysin. Their report shows that the glycopeptide is located at the extreme carboxy terminus of the precursor, with neurophysin occupying the middle domain and vasopressin being adjacent to the leader sequence at the amino terminus. The production of vasopressin and neurophysin involves cleavage at pairs of basic residues, whereas cleavage of the carboxy terminal glycopeptide involves cleavage at a single arginine residue (*5*). This is known to occur for a large number of precursor molecules, including the pro-opiomelanocortin molecule (*6*). We therefore propose the name "carboxy terminus of propressophysin" (CPP) for this glycopeptide, which previously was termed "human pituitary glycopeptide."

Human CPP was extracted as described by Seidah *et al.* (*1*), coupled by carbodiimide to egg albumin, and injected into rabbits. This produced high-titer, specific antiserum. Immunocytochemistry was then carried out in rat brain and pituitary (*7*). Tissue sections were incubated with antiserum to CPP or vasopressin diluted 1:1000 in 0.3 percent Triton in phosphate-buffered saline. The tissue was prepared for immunocytochemistry by a modification of the peroxidase-antiperoxidase technique (*7*, *8*). The specificity of the immunoreactivity was systematically evaluated by performing blocking experiments with the following peptides (50 μM): AVP, oxytocin, β-endorphin, dynorphin, methionine-enkephalin, leucine-enkephalin, and adrenocorticotropic hormone. None of these peptides was effective in inhibiting the immunocytochemical reactions with antiserum to CPP. CPP itself effectively blocked CPP antiserum staining in concentrations as low as 10 nM. The antiserum to AVP is specific for AVP and is blocked by 20 nM AVP, but it is not blocked by oxytocin at concentrations up to 50 μM (*9*). Normal male Sprague-Dawley rats, male homozygous Brattleboro rats, and colchicine-treated (50 μg in 50 μl, intracerebroventricularly) animals of both types were studied.

In both normal and colchicine-treated Sprague-Dawley rats, CPP immunoreactivity was detected in the supraoptic nucleus (Figs. 1A and 2A), nucleus circularis, paraventricular nucleus (Fig. 1B), suprachiasmatic nucleus (Fig. 1D), median eminence (Fig. 1C), and posterior pituitary (Fig. 2C). A subset of cells in these nuclei was stained. Evaluation of the general anatomic staining patterns in the supraoptic (Figs. 1A and 2A) and paraventricular (Fig. 1B) nuclei strongly indicates that the CPP antiserum staining was similar to that seen with vasopressin (Fig. 2B). Indeed, using a set of serial 5-μm sections stained with antiserum to CPP or AVP, we found CPP- and AVP-like immunoreactivities in the same neurons in the supraoptic (Fig. 2, A and B) and paraventricular nuclei. Furthermore, antiserum to CPP stained many of the parvocellular elements in the suprachiasmatic nucleus (Fig. 1D). This nucleus is not known to contain oxytocin but does contain vasopressin and its related neurophysin (*10*). Fibers were seen coursing from the supraoptic and paraventricular nuclei to the median eminence (Fig. 1C), where they formed heavy bundles in the internal layer. Analysis of the posterior pituitary (the projection target for the internal layer of the median eminence) showed heavy staining (Fig. 2C); no staining was found in the intermediate or anterior lobes. Thus we conclude that the antiserums to CPP and AVP stained the same cells in the hypothalamus and the same fibers in the hypothalamus and pituitary.

The common location of two peptides does not necessarily imply a common precursor. Other substances have been identified in AVP cells of the magnocellular system, yet they are not related to the biosynthesis of AVP and its related neurophysin (*9*, *11*). One such substance, dynorphin, stains exactly the same cells as vasopressin (*9*). Yet homo-

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