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Somatic cell cloning in polyester stacks

(Chinese hamster ovary cells/antibody-secreting hybridomas/tissue culture/somatic cell genetics)

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ABSTRACT **Single somatic cells, including fibroblasts, myelomas, and hybridomas, proliferate normally when trapped between a plastic dish and a disc of polyester cloth. Contact between the overlay and the plastic for 8–16 days results in identical colony patterns on the cloth and the plate. When several cloth discs are simultaneously stacked over Chinese hamster ovary cells, three or four high-resolution colony copies can be generated from a single master dish. The colonies on the cloth can be analyzed by radiochemical methods [Esko, J. D. & Raetz, C. R. H. (1978) *Proc. Natl. Acad. Sci. USA* 75, 1190–1193] or by "replica plating" to a new disc. The use of polyester cloth, singly or in stacks, has several major advantages over previous techniques for somatic cell replica plating, including: (*i*) broad applicability to diverse cell lines such as fragile membrane mutants of Chinese hamster ovary cells and relatively nonadherent myelomas or hybridomas; (*ii*) the possibility of generating multiple copies of the same colony population, allowing simultaneous analysis for several enzymes or cellular components; and (*iii*) superior resolution and transfer efficiency in copying colony patterns from one surface to another. The remarkable capacity of animal cell colonies to proliferate upward through "polyester stacks" may reflect chemotropic movement of individual cells and opens new approaches to somatic cell genetics.**

Certain somatic cells, such as the Chinese hamster ovary (CHO) line, can grow clonally between a plastic surface and a disc of filter paper (1). About 10^5 colonies (300 per plate) derived from a mutagen-treated population can be immobilized on filter paper and analyzed by replica-plating or *in situ* autoradiography for specific biochemical variations (1). Our laboratory has obtained mutants in CDP-choline (2, 3), phosphatidylethanolamine (4), and *myo*-inositol biosyntheses (5) with this approach, despite the lack of a positive selection. Filter paper sorting of CHO cells has also been used to obtain novel mutants altered in lysosomal hydrolases (6), amino acid transport (7), ultraviolet sensitivity (8), and glycoprotein synthesis (9).

Despite the inherent simplicity and biochemical specificity of filter paper cloning, the approach is not applicable to all cell lines. Even some CHO mutants fail to proliferate under filter paper, and functionally differentiated lines, such as antibody-producing hybridomas or hormone-sensitive pituitary tumor cells, are killed by filter paper overlay. These considerations have led us to develop a superior cloning technology, based on the extraordinarily high affinity of animal cells for certain types of polyester cloth.

PROCEDURES AND RESULTS

Materials. [*methyl*-^{14}C]Choline was obtained from New England Nuclear. Lysophosphatidylcholine derived from egg lecithin, poly-L-lysine hydrobromide (molecular weight average, >70,000), and other chemicals were purchased from Sigma. Ham's F-12 medium, Dulbecco's modified Eagle's medium, nonessential amino acid mixture (100× concentrate), trypsin, Mycostatin, Fungizone, and fetal bovine serum were products of GIBCO. Plastic tissue culture dishes and flasks were manufactured by Falcon.

Routine Handling and Growth of Cell Lines. CHO cells (CHO-K1) were generally cultured at 37°C in F-12 medium supplemented with 10% (vol/vol) fetal bovine serum in 5% CO_2/95% air as described (1, 2), except that the growth medium was replaced no more often than once a week. Isolation of mutant 58, deficient in CDP-choline synthetase, previously was achieved by *in situ* autoradiography (2, 3). Because mutant 58 is temperature-sensitive, routine passage of this strain was carried out at 33°C. A mouse myeloma line (SP2/0) and several antibody-secreting hybridomas derived from it by fusion with mouse spleen cells (10, 11) were obtained from C. Hayes, and these cells were cultured in Dulbecco's modified Eagle's medium/10% serum with 7.5% CO_2/92.5% air at 100% humidity. Because myeloma cells adhere poorly to normal tissue culture plasticware, the subculturing of SP2/0 and its derivatives did not require trypsin treatment. Under the conditions described below, SP2/0 and related strains did not require a macrophage feeder layer (11) to form macroscopic colonies from single cells.

Preparation of Filter Paper, Glass Beads, and Polyester Cloth. Whatman no. 50 filter paper was sterilized in glass Petri dishes for 50 min in a distilled-water autoclave and allowed to air dry for 2 hr in a laminar flow hood. Glass beads (4 mm; Scientific Products) were washed with acid and sterilized overnight at 125°C (dry heat). Polyester discs (PeCap HD7-1 super or HD7-17) were cut from sheets obtained from Tetko (Elmsford, NY) and were made to fit exactly into 100-mm-diameter tissue culture dishes. Used polyester discs (see below) could be recycled by overnight exposure to concentrated HCl at 25°C (10 ml per disc), followed by one rinse with concentrated HCl and extensive washings with phosphate-buffered saline (12), water, and ethanol. After air drying, the polyester discs were placed into a glass Petri dish, interleaved with Whatman no. 50 discs to keep the cloths flat, and sterilized in a distilled water autoclave for 50 min.

Clonal Growth of CHO Cells Between a Plastic Dish and Polyester Cloth. Just prior to use, all plastic tissue culture dishes are treated with poly-L-lysine by placing 5 ml of poly-L-lysine (1 mg/100 ml in phosphate-buffered saline) in each 100-mm dish, incubating for 60 min at 25°C, and aspirating as much of the liquid as possible with a sterile pipet. Next, 15 ml of F-12 medium supplemented with 10% fetal bovine serum is added to the dish, and it is seeded with several hundred single CHO-

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Abbreviation: CHO, Chinese hamster ovary.

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K1 cells. The plate is incubated at 37°C in 5% CO_2/95% air at 100% humidity. After 1 day, a closely fitting disc of polyester (HD7-1 super) is floated above the cells and weighed down by pouring on a single layer of sterile 4-mm glass beads (1, 2). In this configuration the cells proliferate rapidly without loss of plating efficiency. After 8 additional days (or 16 days at 33°C), during which time the medium is exchanged once, the disc is removed. Both the plate and the disc are rinsed briefly with phosphate-buffered saline, fixed for 1 hr in 10% trichloroacetic acid, and then stained with 0.05% Coomassie blue G dissolved in methanol/water/acetic acid, 45:45:10 (vol/vol). Following this, the plates and discs are destained rapidly with several portions of the same solvent. An excellent colony pattern is present both on the plastic and on the overlay (Fig. 1 *Upper Left*) with essentially 100% transfer efficiency. At this stage parental CHO colonies are 2–4 mm in diameter.

CHO cells bind to polyester so tightly that almost all of the colony material transfers to the cloth unless the plastic is first treated with poly-L-lysine. When this is done, a fraction of each colony (5–10%) remains behind on the plastic to allow storage at 28–33°C and retrieval of viable cells at a later date. The high transfer efficiency is especially important for the analysis of smaller mutant colonies, which sometimes fail to transfer if filter paper is used as the overlay. If the master plate must be stored for a week for subsequent retrieval of viable cells, it is also rinsed with phosphate-buffered saline after removal of the polyester cloth but then is immediately filled with 15 ml of growth medium containing Mycostatin (20 units/ml) and Fungizone (2.5 μg/ml). After 3 days at 33°C to allow cell growth and filling in of the empty centers (Fig. 1 *Upper Left A*), the plate is shifted to 28°C. Overlay with a new disc is not necessary during storage, although secondary colonies may form, particularly at 33°C. Mutant candidates are retrieved by local trypsin treatment (1, 2) or scraping (13).

Upward Proliferation of CHO Cells Through Polyester Stacks. When CHO colonies immobilized on a single overlay of polyester cloth are stained, the intensity of the stain (Coomassie blue) may be greater on the upper surface (i.e., the one not in contact with the plastic). This is caused by accumulations of cells that had penetrated the cloth, perhaps in search of nutrients.

To examine this phenomenon further, about 300 cells of the CHO-K1 line are seeded into a 100-mm poly-L-lysine-treated tissue culture dish as above. After 18 hr at 37°C, the cells are overlayed with four discs of polyester cloth (HD7-17) evenly sandwiched against the bottom with beads. After 10 days (with one medium change) at 37°C, the beads are removed, and the discs are fixed and stained with Coomassie blue G (Fig. 1 *Upper Right*). The cells grow up and through the cloth so that excellent colony copies are present on each of the four levels. If more than four discs are used, progressively fewer colonies reach the uppermost layers, but some can penetrate as many as six or seven discs.

When used in stacks, large-pore cloth such as HD7-17 (17-μm openings) is slightly better than the finer HD7-1 super (1-μm). Interestingly, HD7-17 is ineffective as a single overlay, because the formation of satellite colonies is not inhibited. In all cases the resolution of the colony pattern is the least sharp on the uppermost copy, independent of the number of discs used.

Disc-to-Disc Replica Plating in Polyester Stacks. When a polyester disc containing several hundred CHO colonies is put in contact with a new plastic dish in order to generate a replica plate, the downward transfer of cells to the new plastic surface is sparse and uneven. With filter paper (1) this copying maneuver works fairly well, possibly because the cells have less affinity for the paper. When a polyester disc with several hundred colonies is put in contact with a clean polyester disc, extensive transfer occurs after 2–3 days at 37°C. The best results are obtained if the new cloth is placed on top of the original and both discs are held together against the bottom of the dish with glass beads. As in the case of the stacks, the preference for upward growth may reflect chemotropic movement of individual cells, but whatever the explanation it yields much better colony copies than any previous method.

The remarkable clarity of the colony patterns generated by disc-to-disc transfer and the application of the method for the isolation of auxotrophs is illustrated by the following. About 1,000 mutagen-treated (1, 2) CHO cells are seeded into a 100-mm poly-L-lysine-treated tissue culture dish containing 15 ml of F-12 supplemented with 10% fetal bovine serum and mixed lysolipids (lysophosphatidic acid, lysophosphatidylethanolamine, and lysophosphatidylcholine, each at 25 μM). After 1 day at 37°C, the cells are overlayed with three discs of HD7-17. After 10 additional days at 37°C, with one change of growth medium at day 7, the three discs are removed. The top layer is discarded. The master dish is stored at 33°C and then 28°C. The lower two discs, designated A and B (similar to *A* and *B* of Fig. 1 *Upper Right*), are incubated on top of glass beads (1) and growth medium for 2 days at 37°C. Disc A is incubated without lysolipids; disc B is incubated with supplement. Next, the discs are placed in a new 100-mm plate containing 15 ml of growth medium with or without supplement and are covered with a new cloth overlay, designated A′ and B′. After 2 days at 37°C, disc A′ is stained with 0.15% Ponceau G, R, 2R (acid red 26) in methanol/water/acetic acid, 45:45:10 (vol/vol), and disc B′ is stained with Coomassie blue G as before. At each step involving transfer of a disc from one Petri dish to another, it is desirable to rinse the disc to remove loose cells. This is done most easily by placing the disc in a Petri dish containing 20 ml of phosphate-buffered saline and gently shaking under sterile conditions in a laminar flow hood. To ensure good cell viability the discs must not be allowed to dry. The optimal colony density is about 200 per 100-mm dish. With mutagen-treated (1, 2) cells the plating efficiency, independent of overlay, is about 20%.

Identification of auxotrophs is achieved by superimposition and transillumination of A′ (stained red) over B′ (stained blue). Normal CHO colonies, which grow under both conditions independently of the supplement, appear to be purple (red over blue); mutants unable to grow in the absence of the lysolipid supplement are blue (Fig. 1 *Lower*). The arrow indicates the position of such a mutant, and the entire figure represents an enlargement of an original colony pattern of approximately 4 × 4 cm. Retrieval of viable mutant cells from the master dish (which is stored as above) is achieved by local trypsin treatment or scraping (1, 13).

The advantages of the disc-to-disc method for "replica plating" animal cells include not only its remarkable resolution but also its redundancy. Mutants can be recognized not only by comparison of A′ and B′ but also by superimposition of A and A′. However, the latter requires that nongrowing (or possibly lysing) mutant colonies remain attached to disc A during the process of generating A′. This is not always the case.

Clonal Growth of Myelomas and Hybridomas on Polyester Cloth. The striking affinity of CHO cells for polyester cloth suggested that this material might be useful for the cloning of nonadherent lines, such as myelomas and hybridomas. Existing techniques for propagating these lines from single cells are tedious and involve the use of microtiter dishes (11) or soft agar (14). The polyester technology described here might simplify the detection and recovery of rare monoclonal antibody-producing strains from large hybridoma populations.

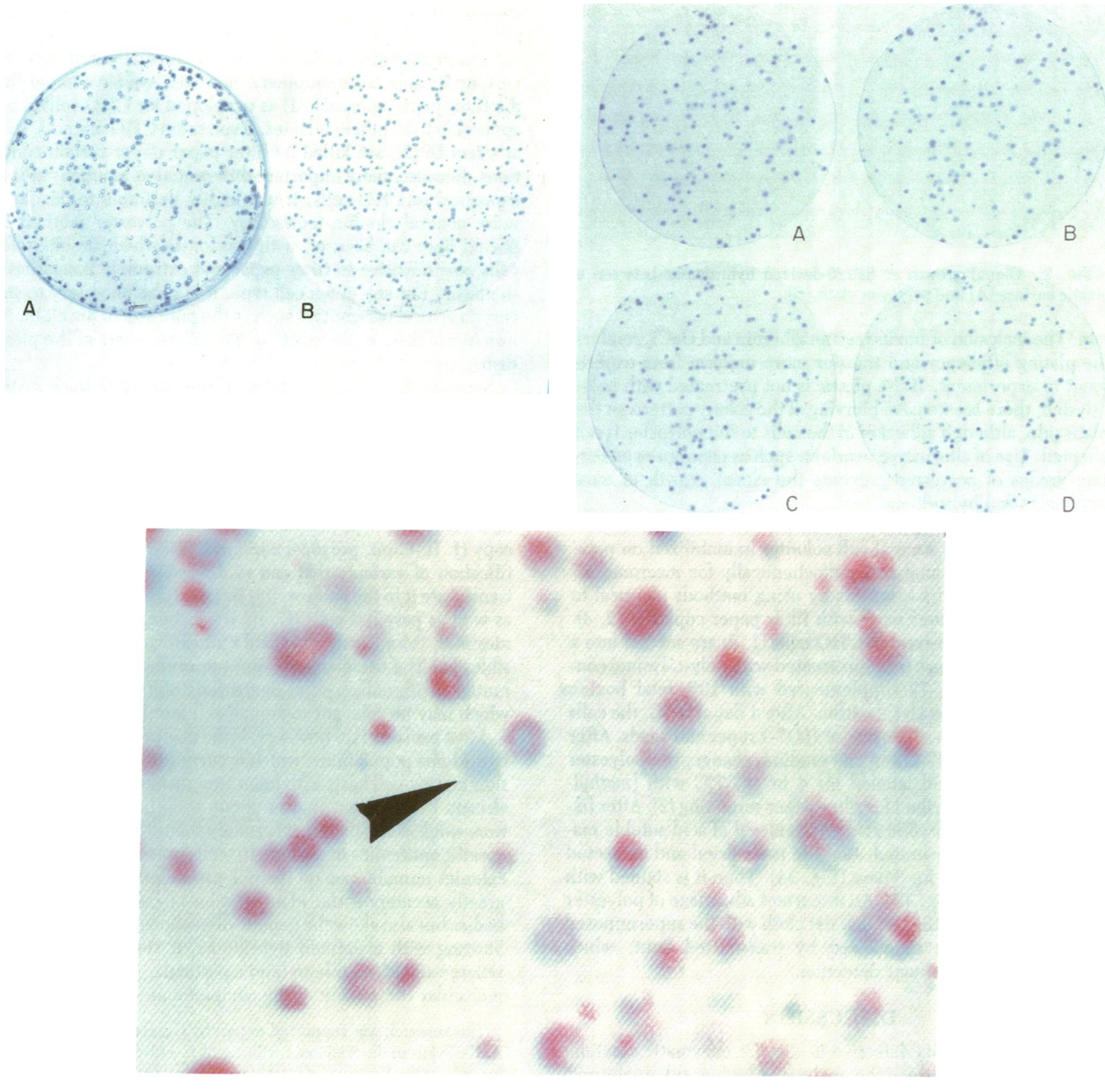

FIG. 1. (*Upper Left*) Transfer of CHO cell colonies from plastic to polyester cloth. (*A*) Stained master plate. (*B*) Stained polyester copy. A few colonies occasionally fail to transfer along the edges because the cloth may have been curled or unevenly cut. (Coomassie blue G.) (*Upper Right*) Somatic cell cloning in polyester stacks. *A* through *D* show the colony pattern at each level in the stack. *A* was in contact with the bottom; *D* was at the top directly underneath the beads. The master plate (not shown) had the same pattern as *A*, indicating 100% transfer, but enough viable cells were present for each colony to allow subsequent recovery. (Coomassie blue G.) (*Lower*) Ascending disc-to-disc "replica plating" on polyester cloth. (Acid red 26/Coomassie blue G.)

It is possible to clone SP2/0 and hybridomas derived from it between polylysine-treated plastic and single sheets of polyester (HD7-1 super). The plating efficiency under these conditions is 40–80%. About 150 cells are seeded into a 100-mm tissue culture dish (pretreated with poly-L-lysine) containing 15 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM $CaCl_2$, and bovine serum albumin at 10 mg/ml. The cells were incubated at 37°C in 7.5% CO_2/92.5% air at 100% humidity. After 1 day they were overlayed with a single layer of HD7-1 super and beads. After 13 days, during which time the medium was exchanged once, the overlay was removed and stained with Coomassie blue (as in Fig. 1 *Upper Left*). The plate was rinsed gently with phosphate-buffered saline, fixed with trichloroacetic acid, and similarly stained. Colony transfer is virtually 100%, except along the irregular edges (Fig. 2). If viable cells are to be retrieved from the master, this can be done with localized trypsin treatment or simply by shearing with a Pasteur pipette. For prolonged storage (up to 1 week), the master dish is filled with growth medium containing 10% (vol/vol) dimethyl sulfoxide and is slowly frozen to −80°C. The plate is rapidly thawed in a water bath when the desired colonies are to be retrieved.

Unlike CHO cells, the hybridomas do not penetrate more than two layers of cloth, most likely because of their slow growth

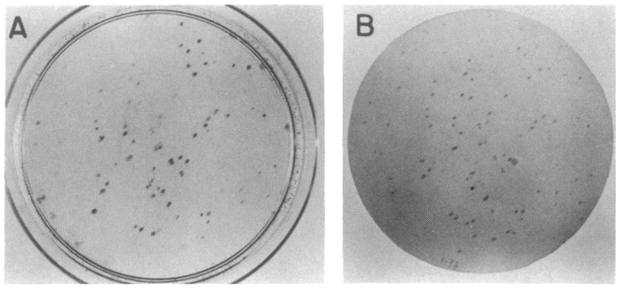

FIG. 2. Clonal growth of SP2/0-derived hybridomas between a plastic surface (*A*) and polyester cloth (*B*).

rate. The inclusion of bovine serum albumin and $CaCl_2$ renders the plating efficiency and transfer more uniform from experiment to experiment. If the plastic is not pretreated with poly-L-lysine, there is extensive blurring of the colony pattern on the plate side, although adhesion of the cells to the polyester is not affected. Use of alternative overlays, such as paper or even certain grades of polyester prevents the clonal growth of most myelomas and hybridomas.

Autoradiographic Analysis of Animal Cell Colonies Attached to Polyester Cloth. Animal cell colonies immobilized on polyester cloth can be analyzed radiochemically for macromolecular or membrane biosynthesis by using methods identical to those used in previous work with filter paper copies (1, 2, 4). About 200 mutagen-treated CHO cells (1, 2) are seeded into a 100-mm tissue culture dish (pretreated with poly-L-lysine) containing 15 ml of F-12 supplemented with 10% fetal bovine serum and 40 μM egg lysolecithin. After 1 day at 37°C, the cells are overlayed with a single disc of HD7-1 super and beads. After a total of 9 days at 37°C and one medium change, the polyester disc is removed and labeled for 4 hr at 37°C with [*methyl*-^{14}C]choline as described for filter paper screening (2). After fixation with trichloroacetic acid and removal of acid-soluble material on a Buchner funnel, the disc is air dried and subjected to autoradiography for 5 days (Fig. 3*A*). Then it is stained with Coomassie blue (Fig. 3*B*). An important advantage of polyester autoradiography is the fact that the cloth with the superimposed autoradiogram can be viewed by transmitted light, which greatly facilitates mutant detection.

DISCUSSION

Somatic cell mutants defective in specific enzymatic reactions are extremely useful for the analysis of higher eukaryotic regulation and metabolism. Despite considerable progress over the last decade (15, 16), the techniques for isolating such mutants are still limited. The lack of a universal procedure for replica-plating animal cell colonies frequently necessitates cell-by-cell analysis in microtiter dishes (17), which have a limited capacity. Despite its appealing simplicity, however, we have found that the filter paper approach (1) as developed for CHO cells is not applicable to all other cell lines. Even some CHO mutants, such as strain 58 (2), are killed by filter paper under certain conditions. Because many important differentiated functions are not expressed in CHO cells, it was highly desirable to develop a more general cloning technology. The polyester method reported here can be used with myelomas which, unlike CHO cells, are sensitive to filter paper and extremely nonadherent to plastic. For any given cell type, it may be necessary to vary certain critical parameters such as the pore size of the cloth, the number of discs in the stack, or the adhesiveness of the plastic dish.

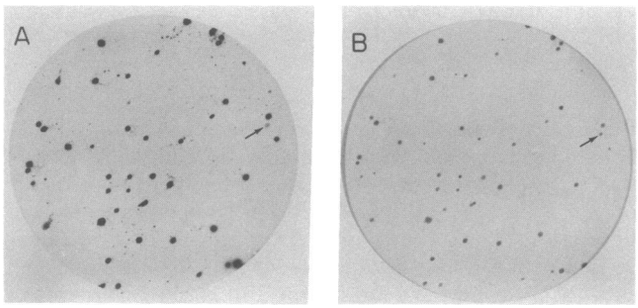

FIG. 3. Autoradiographic analysis of membrane lipid synthesis by [*methyl*-^{14}C]choline labeling of colonies immobilized on polyester cloth. (*A*) Autoradiography for 5 days. (*B*) Staining with Coomassie Blue. Arrows indicate the position of a colony with a defect in its ability to incorporate choline into acid precipitable material.

Some of the unique features of the polyester stack system deserve consideration. (*i*) With multiple copies of a colony population one can examine several steps in a pathway simultaneously. (*ii*) The presence or absence of a particular enzyme in a specific subcellular compartment can be assessed. For instance, a surface enzyme such as alkaline phosphatase (18) can be assayed first with an intact and then with a detergent-treated copy (J. R. Gum, personal communication), allowing for identification of variants that can synthesize the enzyme but not translocate it to the surface. (*iii*) Because CHO fusogens transfer as well as parental cells (C. B. Hirschberg, personal communication), the analysis of several traits for genetic linkage is possible. (*iv*) The use of a large number of stacks should select for mutants with enhanced or decreased ability to grow vertically, which may be relevant to animal cell motility.

Most provocative, however, is the prospect of analyzing large hybridoma populations, which is currently done on a relatively slow cell-by-cell basis with microtiter dishes (11). Because it has already been shown that antibody secretion can be detected from single cells (19, 20), it certainly should be possible to detect specific antibodies in the 10^3–10^4 cells present in the hybridoma colonies immobilized on the polyester cloth. This will not only greatly accelerate the identification of rare monoclonal antibodies but also allow the genetic dissection of antibody function. Starting with a defined hybridoma, it should be possible to isolate variants unable to bind a particular antigen or a specific molecular component of the complement system.

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