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Use of a replica technique to isolate muscle cell lines defective in expressing the acetylcholine receptor

(differentiation/fusion/neurobiology/somatic cell genetics/protein transport)

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ABSTRACT **We have isolated genetic variants of the C2 muscle cell line that are defective in expressing the acetylcholine receptor (AcChoR). Because the AcChoR is expressed only after C2 myoblasts have fused to form myotubes, we employed a replica technique to detect the variants. This technique yields two copies of each clone, one of which can be used for screening and the other, as a source of dividing cells. In a screening of about 10,000 clones derived from mutagenized cells, we found 2 that fused normally and expressed normal levels of acetylcholinesterase but had reduced amounts of AcChoR on their surface. One of these also had a reduced level of intracellular AcChoR, but, in the other, the amount of intracellular AcChoR was 5-fold higher than normal. Several variants were found that failed to fuse and had reduced levels of both AcChoR and acetylcholinesterase. Though we relied on ^{125}I-labeled α-bungarotoxin to distinguish wild-type from deficient clones, we found that an antiserum to the AcChoR, followed by a biotinylated second antibody and a horseradish peroxidase–avidin complex, could also be used. Therefore, it should be possible to obtain muscle cell variants defective in the expression of a variety of proteins for which specific antibodies are available.**

Genetic variants are powerful tools in studying the regulation, mechanism of action, and physiological roles of particular gene products. Although their use in mammalian cell lines has primarily been restricted to the study of metabolic enzymes (1, 2), they are potentially useful in studying such complex processes as synapse formation. Two major difficulties have prevented their use in attacking such problems: (*i*) the paucity of suitable nerve and muscle cell lines and (*ii*) the difficulty of recovering mutants with altered phenotypes that are expressed after the cells have differentiated and withdrawn from the cell cycle.

Recently, we described the properties of a mouse muscle cell line, C2, originally isolated by Yaffe and Saxel (3), that has many of the properties of primary muscle cultures (4). C2 myoblasts fuse in culture and express several proteins that are associated with the postsynaptic membrane and with the extracellular matrix at the mature neuromuscular junction (5, 6). We have attempted to isolate variants that are defective in the expression of one of these components, the acetylcholine receptor (AcChoR). To identify and recover such variants, we have modified for use with muscle cells a replica technique recently developed by Raetz *et al.* (7) for other eukaryotic cell lines. This technique generates two copies of each clone, one of which can be induced to differentiate and express the AcChoR, while the other is maintained in a dividing state.

We report here the success of this technique in yielding two variant lines of C2 that fuse normally but are deficient in the surface expression of the AcChoR on myotubes: one shows greatly diminished levels of intracellular AcChoR as well, whereas the other accumulates the protein in an internal pool. We have also isolated several variants that fail to fuse.

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MATERIALS AND PROCEDURES

Tissue Culture. The C2 cell line, established by Yaffe and Saxel (3), was maintained and grown essentially as described in ref. 4. The cells were maintained as exponentially growing myoblasts in Dulbecco's modified Eagle medium (DME medium) with 1 g of glucose (Gibco, H-16) per liter supplemented with 20% fetal calf serum and 0.5% chick embryo extract. Cultures were kept at 37°C in a humidified 92% air/8% CO_2 atmosphere. To induce fusion, the cells were grown to at least 50% confluence and then the growth medium was replaced with DME H-16 medium supplemented with 10% horse serum (fusion medium). For mutagenesis, exponentially growing cells were exposed to 0.4 mg of ethyl methanesulfonate per ml for 16 hr, resulting in a survival rate of about 10%.

Replica Copies. *Materials.* Polyester cloth (PeCap screening fabric–Swiss polyester monofilament) was obtained from Tetko (Elmsford, NY). Cloth with a mesh opening of 10 μm was routinely used, but 1-, 6-, and 17-μm cloth worked equally well. Discs were cut out with a scissors or with a 3.25-inch (diameter) punch (1 inch = 2.54 cm) from C. S. Osborne & Co. (Harrison, NJ). A small notch was cut in each disc to facilitate peeling off the dish, and the discs were labeled by pencil. They were then dipped in 100% ethanol briefly, allowed to dry, and sterilized by autoclaving as described (7) for 30 min. Glass beads were 4 mm in diameter and were obtained from American Scientific Products (Stone Mountain, GA). They were cleaned by soaking overnight in sulfuric acid with Nochromix (Godex Laboratories, New York) and then were rinsed for several hours, aliquoted in glass tubes, and sterilized by autoclaving for 30 min.

Procedures. Several hundred cells were added to 10-cm culture dishes (Falcon no. 3003) filled with 15 ml of growth medium with penicillin (100 units/ml) and streptomycin (0.1 mg/ml). After 24–48 hr of incubation at 37°C in a humidified 92% air/8% CO_2 atmosphere, a disc of polyester cloth was placed in each dish with ethanol-flamed forceps, and the disc was weighted down with a monolayer of sterile glass beads as described in ref. 7.

After 1 week of further incubation at 37°C, the medium was aspirated and the beads were removed. The dish was briefly placed upside down on a sterile paper towel to blot residual drops of medium, and the orientation of the cloth with respect to the dish was indicated by marking on the dish the position of the notch in the cloth. The dish was then placed right-side-up, and the cloth was carefully peeled off

Abbreviations: AcChoR, acetylcholine receptor; α-BuTx, α-bungarotoxin.

by holding it in place with one pair of forceps, while a corner of the notch was lifted with another pair. Sliding of the cloth relative to the dish during removal seemed to reduce drastically the number of colonies that transferred.

The cloth was placed in a 10-cm culture dish containing 15 ml of fusion medium; it floated about 2 mm beneath the surface of the medium without being weighted down. The original dish was refilled with 15 ml of growth medium and was placed either in a humidified CO_2 incubator at 30°C or a gastight chamber (GasPak 150 vented anaerobic system, available from Fisher) equilibrated with 8% CO_2 and kept in a simple temperature-controlled incubator at 30°C. At this temperature the colonies remained distinct for 6 days and fusion within the colonies was greatly reduced.

Colonies were cloned by placing a 3-mm (inner diameter) cloning ring (Bellco), with the end greased, around the clone of interest, incubating it with a few drops of trypsin solution for 1–2 min, and then removing the suspension with a Pasteur pipette. Generally about 10^4 cells were obtained from each colony.

Assays of Colonies on Cloth. Colonies on the cloth were assayed for AcChoR after 3–4 days of incubation in fusion medium at 37°C. ^{125}I-labeled α-bungarotoxin (^{125}I-α-BuTx) was added to a final concentration of 5 nM and the incubation was continued for 90 min. The unbound toxin was removed by rinsing the cloth four times with 5 ml of 20 mM sodium phosphate, pH 7.4/150 mM NaCl (P_i/NaCl) that contained 0.2% bovine serum albumin to aid wetting. After rinsing, the colonies were fixed with 1% formaldehyde/0.8% glutaraldehyde in P_i/NaCl for 3 min and then stained with copper ferrocyanide (8). Finally, the discs were hung up briefly to dry, sandwiched between sheets of SaranWrap, and autoradiographed at −70°C about 16 hr with X-Omat AR film (Kodak) backed by a Lightning Plus Cronex intensifying screen (Dupont).

The assay of colonies on the cloth by antibody binding utilized biotinylated second antibody, avidin, and biotinylated horseradish peroxidase supplied in Vectastain ABC kits from Vector Laboratories (Burlingame, CA). 3,3′-diaminobenzidine tetrachloride was from Polysciences (Warrington, PA). Cloths were rinsed with P_i/NaCl containing 0.2% bovine serum albumin, fixed with P_i/NaCl containing 1% formaldehyde and 2 mM $MgCl_2$, and then incubated for 2 hr with a 1:250 dilution of rabbit anti-AcChoR serum (9). To prevent later nonspecific binding of the second antibody (biotinylated goat anti-rabbit), a 1:40 dilution of normal goat serum was also added. Following the incubation with the first antibody, the cloths were incubated for 45 min with biotinylated goat anti-rabbit immunoglobulin diluted one drop to 15 ml of P_i/NaCl containing 0.2% bovine serum albumin. Endogenous peroxidase activity was then inactivated by incubating 5 min with methanol, 10 min with methanol containing 1% acetic acid and 10 mg of sodium nitroprusside per ml, and then another 5 min with methanol. The cloths were then incubated with an avidin–horseradish peroxidase conjugate, in 50 mM Tris·HCl (pH 7.6) for 30 min. The final incubation was with 0.5 mg of diaminobenzidine per ml/0.01% H_2O_2 in 50 mM Tris·HCl (pH 7.2) for 2 min.

Quantitative Assays. Surface AcChoRs were assayed quantitatively as described in ref. 4 by incubating myotubes with 10 nM ^{125}I-α-BuTx at 37°C for 90 min, rinsing four times with P_i/NaCl, and extracting the myotubes with 1% Triton X-100 for counting the radioactivity. As a control, myotubes were incubated with 100 nM unlabeled α-BuTx for 60 min prior to the addition of the labeled toxin.

To assay intracellular AcChoRs, myotubes grown in 1.7-cm (diameter) wells were incubated with 40 nM unlabeled α-BuTx for 90 min; the unbound toxin was then rinsed out five times with P_i/NaCl, and the myotubes were taken up in 40 μl of 1% Triton X-100/1 mM sodium tetrathionate/1 mM *N*-ethylmaleimide/1 mM phenylmethylsulfonyl fluoride/1 mM EDTA/1 mM EGTA in 10 mM Tris·HCl (pH 7.4); 20 μl of this buffer was used to rinse the well and was added to the initial extract. The samples were then centrifuged a few minutes in an Eppendorf Microfuge to remove debris, and 45 μl of the supernatant was incubated with 5–10 nM ^{125}I-α-BuTx at 37°C for 60–90 min. AcChoR–^{125}I-α-BuTx complex formation was assayed by DEAE filtration as described (10). To assay total AcChoR, the preceding procedure was followed but without the initial incubation of myotubes with unlabeled toxin.

For acetylcholinesterase assays, myotubes were similarly extracted with 1 M NaCl/0.5% Triton X-100/20 mM EDTA/20 units of Aprotinin per ml/20 μg of pepstatin per ml/1 mM benzamidine/1 mM *N*-ethylmaleimide/0.1 mg of bacitracin per ml in 20 mM Tris·HCl (pH 7.4). Ten-microliter aliquots of the extract were then assayed for acetylcholinesterase activity by the method of Ellman *et al.* (11).

Unless otherwise indicated, values cited are the average of determinations from two separate cultures. Protein levels, measured by the method of Lowry *et al.* (12), were found to vary by no more than 20% from sample to sample and were not routinely determined.

RESULTS

Replica Copying of C2 Colonies. As a first step, we sought to establish conditions for the clonal growth of C2 myoblasts. Approximately 500 myoblasts were plated on uncoated tissue culture dishes, and observation by microscope 3 days later indicated that about 50% of the cells had survived and divided. Within 1 week, these cells gave rise to colonies 2–3 mm in diameter.

To obtain replicas of C2 colonies, a disc of polyester cloth cut to fit the dish was placed on the cells 24–48 hr after plating as described by Raetz *et al.* (7). The disc was weighted down with a monolayer of glass beads, and the plate was then returned to the incubator. Seven to 8 days later, the disc was removed and both the disc and plate were stained for cells. As reported for other cell lines, the muscle cells partitioned between the cloth and the dish to form discrete colonies on the cloth (Fig. 1 *A* and *B*). The colonies on the cloth were compact and were well separated from each other at a density of about 200 colonies per 10-cm dish. A few colonies failed to transfer to the cloth; every colony that transferred, however, left enough cells on the plate to form a visible colony after several further days of incubation. Although the colonies clearly penetrated the discs (the intensity of staining was higher on the top surface), we were unable to obtain transfer to multiple discs in a stack, as has been reported for other cell lines (7). Moreover, we were unable to transfer the colonies from the cloth to a new culture dish, suggesting that C2 myoblasts adhere more strongly to the polyester discs than to the tissue culture plastic.

To induce differentiation of the cells on the polyester cloth, discs bearing unstained cells were transferred to fusion medium, which causes C2 cells grown under normal conditions to fuse and differentiate (4). Three to 4 days later the colonies were assayed for AcChoRs on the cell surface by incubation of the discs with medium containing ^{125}I-α-BuTx, followed by autoradiography. Comparison of the autoradiogram with the cloth, which was stained for cells following the incubation, showed that each colony bound toxin (Fig. 1*C*). Because the radiolabeling of the colonies was blocked by 0.1 mM carbamoylcholine (Fig. 2), we conclude that it is due entirely to toxin binding to the AcChoR. No toxin binding was observed if the cloth was assayed immediately after removal from the original culture dish, indicating that the expression of AcChoR occurs during the incubation in fusion medium.

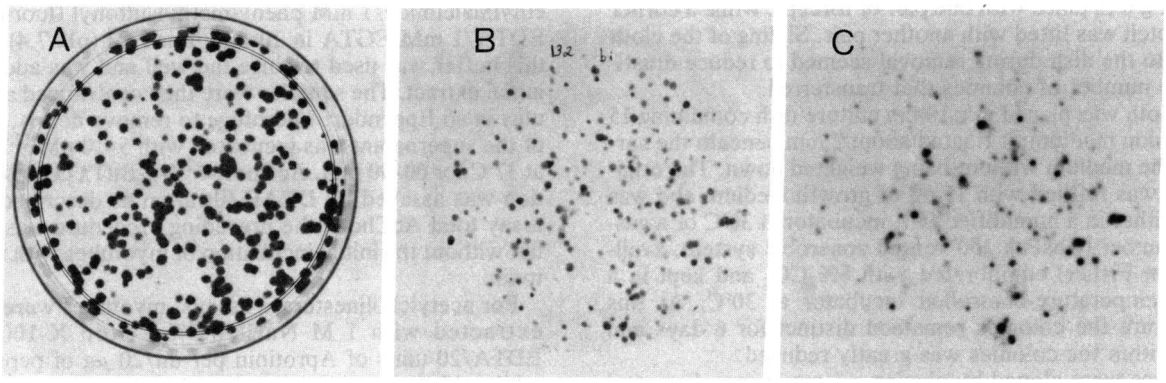

FIG. 1. Replica copying and assaying of C2 muscle cell colonies. (*A*) Tissue culture dish seeded with C2 cells and overlayed with a disc of polyester cloth 24 hr later. After 8 additional days of incubation the cloth was peeled off and the dish was placed in a 30°C incubator for 5 days. The colonies on the dish were then stained with Coomassie blue as in ref. 7. (*B*) Cloth peeled off the dish shown in *A*. The cloth was placed in fusion medium for 4 days and then assayed for ^{125}I-α-BuTx binding, fixed, and stained. (*C*) Autoradiogram of the cloth shown in *B*.

Screening for Mutants. We then sought to isolate mutants deficient in the surface expression of the AcChoR. Cells were mutagenized with ethyl methanesulfonate and grown under polyester discs as described above. After 1 week the discs were transferred to fusion medium to induce differentiation, while the original plates were held at 30°C in growth medium to prevent fusion. After 4 days in fusion medium, the colonies on the discs were assayed for their ability to bind toxin. Comparison of the discs stained for cells with the resulting autoradiograms allowed the identification of colonies deficient in the expression of the AcChoR (Fig. 3). Of about 10,000 myotube colonies screened, 100 that gave a weak or no spot on the autoradiogram were selected for further analysis. Each corresponding colony of myoblasts was located on the original dish, isolated with a cloning ring, and trypsinized. Cells from each of the colonies were then plated at clonal density and subjected to a further round of screening. Colonies that again appeared at least partially AcChoR-deficient were found on the second set of cloths in 28 cases (Fig. 4). For each of these, the recloning procedure was repeated one or two times until a homogeneous population was obtained.

Cells from these 28 clones were then grown in tissue culture dishes, induced to fuse, and quantitatively assayed for surface AcChoR by binding of ^{125}I-α-BuTx to the intact myotubes. In 4 of the 28 cases, the surface AcChoR level was <10% of the wild-type level and in 6 other instances it fell between 10% and 25% of the wild-type level. In most of the remaining cases the higher level found was not surprising since the autoradiograms had indicated a significant, even though relatively low, level of toxin binding. In a few cases the autoradiograms were deceptively negative, apparently reflecting the clones' inability to grow and differentiate on the polyester rather than their inability to express receptor.

Because one cause of the failure of the AcChoR to be expressed on the surface could be a general defect in differentiation, the cells grown in culture dishes were also checked by microscopic observation for myotube formation and were assayed for acetylcholinesterase activity. Of the 10 clones with <25% of the wild-type surface receptor level, only 2, clones 23 and 36, showed both normal fusion and acetylcholinesterase activity comparable to that seen in the wild type (Table 1). The other variants, such as clone 90, are apparently generally defective in differentiation (Table 1).

Clones 23 and 36 were analyzed further to determine if they were deficient in their intracellular level of AcChoR as well as in their surface level. For clone 36, the intracellular level was also depressed compared to the wild type, suggesting that this clone is defective in some aspect of AcChoR synthesis, AcChoR turnover, or receptor–toxin binding. Clone 23, in contrast, had an intracellular pool of AcChoR that was >5 times as large as that seen in normal cells, even though the amount of surface AcChoR was less by a factor of ≈7. Thus, this variant may be defective in transport of the AcChoR to the surface. Both of these clones are sufficiently

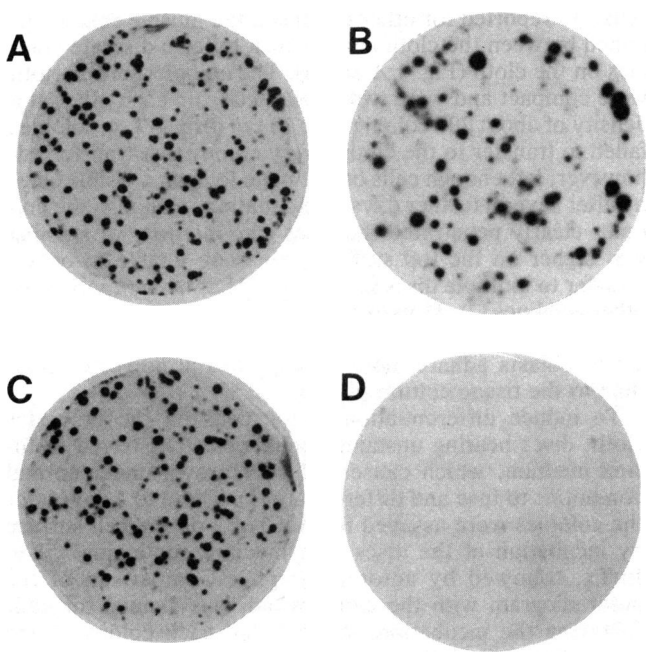

FIG. 2. Effect of carbamoylcholine. (*A*) Polyester disc bearing colonies of C2 myotubes. (*B*) The corresponding autoradiogram obtained after the cloth was incubated with ^{125}I-α-BuTx. (*C*) A second polyester disc. (*D*) The corresponding autoradiogram, obtained exactly as for *A* and *B* except that the cloth was incubated with 0.1 mM carbamoylcholine for 30 min prior to the addition of ^{125}I-α-BuTx.

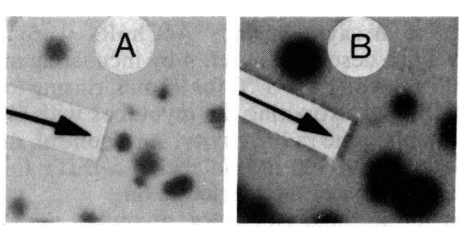

FIG. 3. Identification of an AcChoR-deficient clone. (*A*) Portion of a cloth assayed and stained. (*B*) The corresponding autoradiogram. The arrows point to the position where a colony appears on the cloth but not on the autoradiogram.

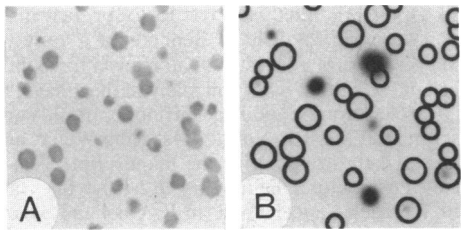

FIG. 4. Example of partial purification of an AcChoR-deficient clone. (*A*) Portion of a polyester disc assayed and stained. (*B*) The corresponding autoradiogram. The circles indicate colonies that appear on the cloth but not on the autoradioagram.

stable for extended study: clone 23 showed no revertants when 200 colonies were rescreened by the cloth assay after about 15 generations, and clone 36 appeared to revert at a rate of about 0.1% based on rescreening after 30 generations.

Screening with Antibodies. Because the method described here promises to be applicable to the selection of mutant cells deficient in other proteins for which suitable toxins are not available, we used one of our mutants to test the feasibility of screening for variant colonies with antibodies. To see if we could detect proteins on the surface of the cells, we used a rabbit antiserum prepared to AcChoR purified from denervated rat muscle (9). The binding of antibodies in this serum to AcChoRs in intact C2 myotubes had been demonstrated previously by immunofluorescence (unpublished results). We used a biotinylated second antibody followed by horseradish peroxidase-conjugated avidin to detect the antibody.

The antiserum clearly stained wild-type colonies of myotubes on the polyester cloth, but it stained colonies of the receptor-deficient clone 36 much more weakly. To demonstrate that this method could be used to distinguish mutant from wild-type colonies, we mixed cells from the wild type and clone 36 before plating at clonal density. Replica copies of the resulting colonies were obtained as described above and, following incubation in fusion medium, the colonies were both labeled with ^{125}I-α-BuTx and stained with the antiserum. A population of very faintly stained colonies was clearly distinguishable from the more intensely stained ones (Fig. 5*A*); comparison with the corresponding autoradiogram confirmed that the weakly stained colonies were in fact AcChoR-deficient (Fig. 5*B*).

DISCUSSION

We have used a replica technique to obtain genetic variants of a mouse muscle cell line. Our results are of interest for two reasons. First, the variants that we have obtained are defective in the expression of the AcChoR, a protein that is the major component of the postsynaptic membrane in muscle cells, and whose synthesis, assembly, and accumulation at the synapse are currently subjects of intensive investigation (13). Variants that are defective in specific steps of these processes should provide powerful tools in their elucidation.

Table 1. AcChoR levels and distribution in C2 variants

	% wild type				
	AcChoR			Acetylcho-	
Clone	Total	Surface	Internal	linesterase	Fusion
Wild type	(100)	(100)	(100)	(100)	+
23	206	14	522	74	+
36	6	3	13	316	+
90	7	—	—	18	−

Values are the average of determinations from at least two separate cultures, and the variation was within 5% in all cases, except the value for acetylcholinesterase activity in clone 36, for which the difference between determinations was 18%. The average values for the wild type were 55, 39, and 16 fmol per well for total, surface, and internal AcChoRs, respectively.

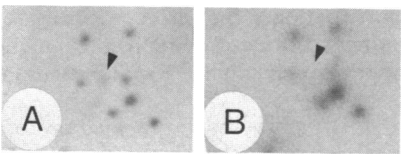

FIG. 5. Use of antibodies to assay colonies on polyester cloth. A cloth bearing both wild-type and clone 36 colonies was incubated in fusion medium and then labeled with both ^{125}I-α-BuTx and antiserum against the AcChoR. (*A*) Portion of the cloth stained for antibody. (*B*) The corresponding autoradiogram. The arrowheads indicate the position of a colony stained faintly by the antibody and showing no ^{125}I-α-BuTx binding.

Some of the clones are defective not only in their levels of AcChoR but also in their ability to fuse and express acetylcholinesterase; these variants should be useful in studying the differentiation of muscle cells. Second, the methods that we have used may be of general utility in isolating other variants of myotubes as well as variants of other types of cells that express proteins of interest only after terminal differentiation.

Our original aim was to develop methods for isolating genetic variants in proteins associated with the postsynaptic cell surface. Many of these proteins are made by muscle cells grown without nerves, but only after fusion of the cells to form myotubes (5, 6). Because myotubes do not divide, selection procedures are not possible, and cells must be screened to detect variants that arise, either naturally or after mutagenesis. Although a replica technique for eukaryotic cells using polyester cloth had been described (7), several aspects of the application of this technique to muscle cells were problematic. First, it was essential that the myoblasts be able to differentiate on the polyester cloth and that proteins of interest could be assayed on the cloth. Second, it was important that the frequency of mutations be high enough for the screening procedures required in a replica technique. Many of the variants of eukaryotic cells that have been obtained previously by screening techniques are derived from Chinese hamster ovary cells, which appear to be functionally haploid over part of their genome (14). Finally, it was important that any variants obtained be stable.

Our initial attempts to obtain mutants have focused on the AcChoR, because it can be quickly and easily assayed in intact cells and because a variety of specific immunological reagents is available for its study. Using the AcChoR as a marker we found that C2 cells grown on a polyester disc do undergo differentiation. Although we have no direct evidence for fusion on the disc, we assume that it occurs since AcChoR expression is normally coincident with myotube formation. The appearance of the AcChoR was conveniently detected on the cloth by autoradiography after exposure to ^{125}I-α-BuTx, a method that was suitably efficient for the identification of mutants. Our initial selection of variants was intentionally broad and included many dubious negatives. By eliminating small colonies and those giving a weakly positive signal, the number of "false mutants" could be easily reduced. The majority of AcChoR-defective variants also failed to fuse. A suitable double-labeling procedure (see below) that scored colonies for the presence of another protein expressed after fusion as well as for the presence of the AcChoR should allow these colonies to be eliminated from consideration.

Our first screen, of 10,000 cells mutagenized with ethyl methanesulfonate, produced 2 variants in AcChoR expression that differentiate normally by other criteria. As we can conveniently plate 200 colonies of myoblasts per dish, this frequency is only marginally satisfactory. More recent experiments, however, indicate that high levels of nitrosoguanidine increase the frequency to a more useful range (data not

shown). The two clones that are described are sufficiently stable for extended study. The replica techniques described here thus appear to be suitable for the isolation of AcChoR mutants.

The 2 variants that we have obtained are of interest because they represent two different types of defects. One does not accumulate active AcChoR; the other shows an altered pattern of distribution within the cell. These mutants need to be characterized further to locate the site of the defect. As additional mutants are obtained, the ability of myoblasts to form myotubes can be exploited for complementation analysis. The genetic approach thus may provide a valuable adjunct to recombinant DNA techniques in studying not only synthesis and assembly of the AcChoR but also mechanisms of transporting it within the cell.

Because other proteins cannot be identified so easily by specific toxins, we investigated whether antibodies could be used in the screening procedure, as has been described for prokaryotic cells (15). We used an antiserum that binds the AcChoR in intact cells in a procedure with biotinylated second antibody and a horseradish peroxidase–avidin conjugate to increase sensitivity. Colonies of mutant myotubes were clearly distinguishable from those of the wild type and could be reliably identified in mixed cultures. In preliminary experiments, we have found that antibodies against internal antigens also stain colonies after permeabilization of the cells (data not shown). Thus, it should be possible to detect mutants defective in the expression of any protein that is expressed at a sufficient level and for which specific antibodies are available. Moreover, the peroxidase method and autoradiography can be used simultaneously, thus increasing the efficiency and flexibility of possible screening protocols. A genetic approach to complex physiological and developmental processes may thus be possible with mammalian cell lines that undergo terminal differentiation as well as with whole animals.

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