

# Fibroblasts Transfected with *Torpedo* Acetylcholine Receptor $\beta$ -, $\gamma$ -, and $\delta$ -Subunit cDNAs Express Functional Receptors When Infected with a Retroviral $\alpha$ Recombinant

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**Abstract.** *Torpedo californica* acetylcholine receptor (AChR)  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -subunit cDNAs were each stably introduced into muscle and/or fibroblast cell lines using recombinant retroviral vectors and viral infection, or using SV-40 vectors and DNA-mediated cotransfection. The expressed proteins were characterized in terms of their molecular mass, antigenicity, posttranslational processing, cell surface expression, stability in fibroblasts, stability in differentiated and undifferentiated muscle cells, and ability (of  $\alpha$ ) to bind  $\alpha$ -bungarotoxin (BuTx). We demonstrated that the  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$  polypeptides acquired one, one, two, and three units of oligosaccharide, respectively. If all four subunits were expressed in the same cell, fully functional cell surface AChRs were produced which had a  $K_d$  for BuTx of  $7.8 \times 10^{-11}$  M. In contrast, subunits expressed individually were not detected on the sur-

face of fibroblasts and the  $K_d$  for BuTx binding to individual  $\alpha$  polypeptides was only  $\sim 4 \times 10^{-7}$  M. The half-lives of the  $\alpha$ -,  $\gamma$ -, and  $\delta$  subunits at 37°C were all found to be quite short ( $\sim 43$  min), while the half-life of the  $\beta$  subunit was found to be even shorter ( $\sim 12$  min). The unique half-life of the  $\beta$  subunit suggests that it might perform a key regulatory role in the process of AChR subunit assembly. One stable fibroblast cell line was established by transfection that expressed  $\beta$ -,  $\gamma$ -, and  $\delta$  subunits simultaneously. When this cell line was infected with a retroviral  $\alpha$  recombinant, fully functional cell surface AChRs were produced. The successful expression of this pentameric protein complex combining transfection and infection techniques demonstrates one strategy for stably introducing the genes of a heterologous multisubunit protein complex into cells.

THE nicotinic acetylcholine receptor (AChR)<sup>1</sup> is located in the postsynaptic membrane of the vertebrate neuromuscular junction where, upon binding by the neurotransmitter acetylcholine (ACh), a channel permeable to small cations opens. It is an intrinsic membrane glycoprotein consisting of four different subunits with molecular masses of  $\sim 40$  ( $\alpha$ ), 50 ( $\beta$ ), 60 ( $\gamma$ ), and 65 ( $\delta$ ) kD which associate as an  $\alpha_2\beta\gamma\delta$  pentamer to form functional AChR complexes (for reviews see Popot and Changeux, 1984; Karlin et al., 1986; McCarthy et al., 1986; Adams, 1987; Maelicke, 1988; Claudio, 1986, 1989). The  $\alpha$  subunit contains the binding site for ACh and the elapid snake neurotoxin,  $\alpha$ -bungarotoxin (BuTx), thus there are two ACh or BuTx binding sites per AChR complex. The subunits in this pentameric complex are tightly associated and can only be dissociated using conditions that denature the polypeptide chains. Once the subunits

have been denatured, it is not possible to reform functional molecules, a fact which has greatly impeded studies designed to determine the function of the individual subunits. With the application of recombinant DNA techniques, it is now possible to express any combination of subunits by microinjecting in vitro-transcribed AChR subunit mRNAs into *Xenopus laevis* oocytes. With such an approach it has been determined, for example, that all four subunits are required to reconstitute all of the properties of the AChR (Mishina et al., 1984). The oocyte system has become a powerful tool for characterizing many proteins, including the AChR, but this transient expression system does not lend itself to addressing many questions concerning the cell biology, development, modulation, and AChR-nerve or AChR-muscle interactions. We have established a stable expression system in cultured mammalian cells such that these and other questions concerning the biology of the AChR could be investigated.

In this paper, we describe the establishment of both fibroblast and muscle cell lines that stably express only one of the four *Torpedo* AChR subunits. These cell lines have allowed us to begin investigating the complex processes of AChR

1. **Abbreviations used in this paper:** ACh, acetylcholine; AChR, acetylcholine receptor; aprt, adenosine phosphoribosyltransferase; BuTx,  $\alpha$ -bungarotoxin; CS, calf serum; LTR, long terminal repeat; MLV, murine leukemia virus; neo, neomycin; ptk, plasmid containing the herpes simplex virus-1 thymidine kinase; sod, son-of-delta; tk, thymidine kinase.

subunit biosynthesis and assembly. We also describe the establishment of one fibroblast cell line, using transfection techniques, that stably expresses  $\beta$ ,  $\gamma$ , and  $\delta$  subunits simultaneously (all-6). When the all-6 cell line was subsequently infected with a retroviral  $\alpha$  recombinant, a new cell line was established in which functional cell surface AChRs were expressed. This result not only demonstrates one approach that can be used for establishing stable cell lines for proteins composed of multiple subunits, but it also presents a method that should be very useful for easily introducing a series of mutations into just one of the subunits of a heterologous multisubunit complex.

## Materials and Methods

### Vectors and Constructions

Restriction enzymes, linkers, and enzymes required for the various constructions were all purchased from New England Biolabs (Beverly, MA), except for DNA polymerase I (endonuclease free) and calf intestinal alkaline phosphatase, which were purchased from Boehringer Mannheim Diagnostics, Inc. (Houston, TX). A series of direct orientation retrovirus vectors (pDOL [Korman et al., 1987], pLJ [J. Schwarzbauer, Princeton University, Princeton, NJ], pEU, and pWE [Choudary et al., 1986]) were all generously provided by R. C. Mulligan (Massachusetts Institute of Technology, Cambridge, MA). In the vector pDOL, the gag, pol, and env genes of murine leukemia virus (MLV) were deleted and a cDNA can be introduced at a unique Bam HI site between the long terminal repeats (LTRs). Downstream from the cDNA are an SV-40 early promoter, neomycin resistance gene (neo<sup>r</sup>), and a pBR322 origin of replication. The neo<sup>r</sup> gene provides a dominant selectable marker which encodes G418 (a neomycin analog) resistance in mammalian cells (Davies and Jimenez, 1980) and kanamycin resistance in *Escherichia coli* (Jorgensen et al., 1979). Also included in this vector are 3.8 kb of polyoma DNA including the origin of replication and  $\sim$ 3 kb of pBR322 DNA. A cDNA can be introduced into the viral genome in place of the gag-pol protein coding region, and an independent transcriptional unit driven by the SV-40 early promoter can provide expression of the G418<sup>r</sup> gene. Another direct orientation retrovirus vector, pDOJ, was generously provided by J. Morgenstern and H. Land (Imperial Cancer Research Fund, London, UK). All of these vectors permit the simultaneous expression of a selectable marker gene and an inserted sequence (Korman et al., 1987). In all cases, the selectable marker gene is neo<sup>r</sup>. In the pDOL, pLJ, and pDOJ vectors, the AChR subunit cDNAs are under the control of the MLV LTR and the internal SV-40 promoter drives the expression of the neo gene. In the pEU and pWE vectors, the LTRs drive the expression of the neo gene and the cDNAs are under the control of an internal chicken  $\beta$ -actin promoter (Kost et al., 1983). An SV-40 expression vector was constructed by removing the dihydrofolate reductase gene from the vector pSV2-DHFR (Subramani et al., 1981) and creating a unique Bgl II cloning site with Bgl II linkers as previously described (Claudio, 1987).

The AChR  $\alpha$ -,  $\beta$ -, and  $\delta$ -subunit cDNAs were obtained from our *Torpedo californica* electric organ  $\lambda$ gt10 cDNA library (Claudio, 1987) and the  $\gamma$  clone was isolated previously (Claudio et al., 1983). The  $\alpha$ -cDNA clone is  $\sim$ 1,760 bp long with  $\sim$ 80 bp of 5' untranslated sequences and 300 bp of 3' untranslated sequences. No upstream ATG sequences or polyadenylation sequences are present in this clone. Bam HI linkers were ligated to the Eco RI ends of the  $\alpha$  clone and it was inserted into the Bam HI site of pDOL. The  $\beta$ ,  $\gamma$ , and  $\delta$  cDNAs that would be inserted into the pDOL vector were digested with appropriate restriction enzymes such that no upstream ATG sequences and no polyadenylation sequences were present in the inserts. The  $\beta$  cDNA extended from  $-2$  bp to the Fnu DII site 150 bp after the translation stop codon (insert  $\sim$ 1,670 bp). The  $\gamma$  clone extended from the Nco I site at  $-1$  bp to a Sau 3AI site 40 bp 3' of the translation stop codon (insert  $\sim$ 1,560 bp). The  $\delta$  clone extended from the Sac I site at  $-8$  bp to the end of the clone (insert  $\sim$ 1,770 bp). The ends of the DNA fragments were made flush by filling in with the Klenow fragment of DNA polymerase I, Bcl I linkers (No. 1009) were phosphorylated with polynucleotide kinase and ligated to the termini, and the fragments were inserted into the Bam HI cloning site of pDOL. The clones were treated as follows for insertion into pLJ, pEU, pWE, and pDOJ: the  $\alpha$  insert was removed from pDOL by digestion with Bam HI, the  $\beta$  insert was removed from pSS-2 (Claudio, 1987) by digestion with Eco RI, the  $\gamma$  insert extended from the Nco I site at  $-1$  bp

to the Pvu II site 110 bp 3' of the translation stop codon, and the  $\delta$  insert extended from the Sac I site at  $-8$  bp to the end of the clone. Bcl I linkers were ligated to the termini of inserts that were not compatible with cloning into the Bam HI sites of the vectors. The pSV2- $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$  constructions are described elsewhere (Claudio et al., 1987): in brief, full-length  $\alpha$  and  $\beta$  clones were used,  $\gamma$  extended from Nco I to Pvu II, and  $\delta$  extended from Sac I to the Eco RI linker.

### Cell Lines

Murine NIH3T3 fibroblast cells and  $\varphi$ -2 cells (Mann et al., 1983) were obtained from R. C. Mulligan and maintained in DME and 10% calf serum. Murine fibroblast L cells deficient in thymidine kinase (tk<sup>-</sup>) and adenine phosphoribosyltransferase (Ltk<sup>-</sup>aprt<sup>-</sup>) were obtained from R. Axel (Columbia University, New York) and maintained in DME supplemented with 10% calf serum. The rat fusing muscle cell line, L6 (Yaffe, 1968), was obtained from D. Schubert (Salk Institute, La Jolla, CA) and maintained in DME plus 10% FCS. The mouse fusing cell line C2 (Yaffe and Saxel, 1977; Blau et al., 1983) was obtained from L. Rubin (Rockefeller University, New York) and maintained in DME plus 20% FCS and 0.5% chick embryo extract (Gibco Laboratories, Grand Island, NY). The mouse nonfusing muscle cell line BC<sub>3</sub>H-1 (Schubert et al., 1974) was obtained from P. Taylor (University of California at San Diego, San Diego, CA) and maintained in DME plus 10% FCS. Expression of endogenous AChRs in the different muscle cell lines was induced as follows: 75% confluent L6 cells were changed to DME containing 2% horse serum; 70% confluent C2 cells were changed to DME containing 5% FCS; confluent BC<sub>3</sub>H-1 cells were changed to DME containing 2.5% FCS. *Torpedo* AChR subunit-specific antisera and antisera directed against purified AChR complex were prepared in rabbits as previously described (Claudio and Raftery, 1977).

Transfections into Ltk<sup>-</sup>aprt<sup>-</sup> cells were performed using the calcium phosphate precipitation procedure of Graham and van der Eb (1973) as modified by Wigler et al. (1979). 5  $\mu$ g each of pSV2- $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -, plus 50 ng of a plasmid containing the herpes simplex virus-1 tk (ptk) gene (Wigler et al., 1977) were introduced into  $5 \times 10^5$  Ltk<sup>-</sup>aprt<sup>-</sup> cells on 10-cm plates as described (Claudio et al., 1987). tk<sup>+</sup> transformants were selected in DME containing 10% calf serum (CS) and 15  $\mu$ g/ml hypoxanthine, 1  $\mu$ g/ml aminopterin, and 5  $\mu$ g/ml thymidine ( $1 \times$  HAT). Individual colonies were isolated using cloning cylinders and grown into separate cell lines (all-6 is described in this paper). Two other cell lines, in which only pSV2- $\delta$  and ptk or pSV2- $\gamma$  and ptk were cotransfected into Ltk<sup>-</sup>aprt<sup>-</sup> cells, are referred to as L-pSV2- $\delta$ 2 and L-pSV2- $\gamma$ 5, respectively. A 3T3 cell line expressing *Torpedo*  $\gamma$  was established by cotransfecting 10  $\mu$ g of pSV2- $\gamma$  and 0.1  $\mu$ g of pSV2-neo onto  $5 \times 10^5$  NIH3T3 cells, letting the cells grow to confluency, and then splitting them 1:20 into DME containing 10% CS and 0.6 mg/ml G418 (Gibco Laboratories). Two colonies which could not be cleanly separated from one another were pooled to establish the 3T3-pSV2- $\gamma$ 1,2 cell line.

Transfection of  $\varphi$ -2 cells, harvest of virus, infection of cells, and selection in G418 were performed as follows: 10  $\mu$ g of retroviral recombinant plasmid DNA was transfected onto  $2 \times 10^6$   $\varphi$ -2 cells using the procedure of Graham and van der Eb (1973) as modified by Parker and Stark (1979). 18 h after the glycerol shock, culture medium was collected and filtered through a 0.45- $\mu$ m acrodisc (Gelman Sciences, Inc., Ann Arbor, MI). 1 ml of supernatant containing 8  $\mu$ g/ml polybrene (Aldrich Chemical Co., Milwaukee, WI) was added to  $10^6$  NIH3T3, L6, C2, and BC<sub>3</sub>H-1 cells in 10-cm plates and incubated for 2.5 h at 37°C. DME plus the appropriate serum was added and the cultures were incubated for 2-3 d until confluent and then split 1:20 into DME containing serum and 0.6 mg/ml G418. Either several hundred colonies were pooled to make mass cultures (3T3-DOL- $\alpha$ mass, 3T3-DOL- $\beta$ mass) of individual colonies were isolated using cloning cylinders and grown into separate cell lines (3T3-DOJ- $\alpha$ 3, 3T3-DOJ- $\beta$ 6 C2-DOL- $\alpha$ 4, C2-DOL- $\beta$ 1, L6-DOL- $\alpha$ 2, L6-DOL- $\beta$ 4).

For an efficient infection of BC<sub>3</sub>H-1 and Ltk<sup>-</sup>aprt<sup>-</sup> cells, we found it was necessary to treat BC<sub>3</sub>H-1 cells with 0.05  $\mu$ g/ml tunicamycin (Calbiochem-Behring Corp., La Jolla, CA) and Ltk<sup>-</sup>aprt<sup>-</sup> cells with 0.3  $\mu$ g/ml tunicamycin for 18 h before infection (Rein et al., 1982). The following protocol was used for infection of all-6 cells (Ltk<sup>-</sup>aprt<sup>-</sup> cells with integrated copies of the plasmids pSV2- $\beta$ -,  $\gamma$ -,  $\delta$ -, and ptk). A 10-cm dish of  $\sim$ 10<sup>6</sup> all-6 cells in DME containing 10% CS and  $1 \times$  HAT was treated for 18 h at 37°C with 0.3  $\mu$ g/ml tunicamycin. Medium was removed, cells were infected with 1 ml of culture supernatant collected from a stable pDOJ- $\alpha$   $\varphi$ -2 producer cell line, and grown until confluent. Cells were next split 1:20 into DME supplemented with 10% CS,  $1 \times$  HAT, and 0.6 mg/ml G418. Seven colonies were isolated using cloning cylinders and grown into separate stable cell

lines (all-6- $\alpha$ 1, all-6- $\alpha$ 2, etc.). These cell lines were maintained in DME containing 10% CS and  $1 \times$  HAT.

### Rescue of Integrated Proviral Sequences

The retrovirus vectors used in this study all contain features that allow the efficient introduction of DNA sequences into mammalian cells and allow the recovery of the inserted sequences as molecular clones. The procedures for recovering integrated sequences were essentially as described (Cepko et al., 1984). A subclone of COS-1 cells (Gluzman, 1981), COS M6 (Horowitz et al., 1983), was maintained in DME plus 10% FCS. Confluent dishes of NIH3T3 cells containing an integrated copy of the retrovirus recombinant and COS M6 cells were each split 1:2 and plated at a 1:1 ratio in a 10-cm dish containing DME, 10% CS, and 10% FCS. Very densely packed cells were washed three times in DME, and then in 2 ml of 50% DME and 50% polyethylene glycol 1000 (J. T. Baker Chemical Co., Phillipsburg, NJ) was added to the cells, and the cells were incubated at room temperature for 1–3 min (depending on the batch of polyethylene glycol). The polyethylene glycol solution was rapidly removed by aspiration, cells were washed twice with 10 ml of DME and then twice with 10 ml of DME plus serum, and the cells were returned to the incubator for 1–3 d with daily media changes. For the isolation of DNA, cells were washed twice with PBS, and a Hirt supernatant (Hirt, 1967) was then prepared, extracted once with phenol, twice with phenol/chloroform (1:1), twice with chloroform, and finally precipitated with 2 vol of isopropanol at  $-20^{\circ}\text{C}$ . This DNA was used to transform *E. coli* HB101 cells and the transformed bacteria were then plated on agarose plates containing 50  $\mu\text{g}/\text{ml}$  kanamycin sulfate. Colonies were picked, grown in small liquid cultures, and plasmids were isolated and mapped by digestion with various restriction enzymes.

### Labeling and Immunoprecipitations

Confluent 10-cm dishes of cells were washed twice with PBS, and then incubated for 15 min at  $37^{\circ}\text{C}$  with media containing all amino acids except those which would be added radiolabeled. Dishes were washed once with PBS, then 2 ml of the above media containing 200  $\mu\text{Ci}$  each of [ $^3\text{H}$ ]leucine and [ $^3\text{H}$ ]isoleucine (Amersham Corp., Arlington Heights, IL), 400  $\mu\text{Ci}$  of [ $^3\text{H}$ ]leucine, or 400  $\mu\text{Ci}$  of [ $^{35}\text{S}$ ]methionine (Amersham Corp.) were added, and cells were incubated for 20 min at  $37^{\circ}\text{C}$ . In the cold room, plates of cells were washed twice with  $4^{\circ}\text{C}$  PBS and then lysed with 700  $\mu\text{l}$  of lysis A buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.02%  $\text{Na}_3\text{N}$ , 0.5% NP-40, 1 mg/ml hemoglobin) containing fresh 2 mM PMSF, and 2 mM *N*-ethylmaleimide. The cells were scraped into 1.5-ml Eppendorf tubes, vortexed a few times over a 10-min period at  $4^{\circ}\text{C}$ , spun 10 min at  $4^{\circ}\text{C}$ , and the supernatants were collected. SDS was added to 1% and NP-40 was added to 5%. Lysates were precleared by incubating them for 15 min at  $4^{\circ}\text{C}$  with 50  $\mu\text{l}$  of protein A-Sepharose (diluted twice in lysis A buffer) followed by a 30-s spin in an Eppendorf centrifuge. Rabbit polyclonal antiserum directed against SDS gel-purified *Torpedo* AChR subunits (anti- $\alpha$ -,  $\beta$ -,  $\gamma$ -, or  $\delta$ -) (Claudio and Raftery, 1977) was added to the supernatants and the samples were rocked overnight at  $4^{\circ}\text{C}$ . 50  $\mu\text{l}$  of 2 $\times$  diluted protein A-Sepharose was added and the samples were rocked a minimum of 3 h at  $4^{\circ}\text{C}$ . Samples were next spun in an Eppendorf centrifuge for  $\sim$ 1 min, the supernatants were removed, and the resins were washed twice in lysis A buffer containing 0.5 M NaCl and then washed three times in lysis A buffer. After the final spin, the supernatants were removed and 35  $\mu\text{l}$  of 2 $\times$  gel loading buffer (4% SDS, 20% glycerol, 0.125 M Tris, pH 6.8, 0.01% bromphenol blue) containing fresh 10 mM DTT was added to the pellets. The resins were removed by centrifugation and the supernatants were loaded onto 10% discontinuous SDS-polyacrylamide gels (Laemmli, 1979). Gels were fixed for 30 min in 25% methanol and 10% acetic acid, then soaked in Amplify (Amersham Corp.) for 30 min, dried on a gel dryer, and put on X-Omat AR film (Eastman Kodak Co., Rochester, NY) at  $-70^{\circ}\text{C}$  with an intensifying screen.

### Cell Surface vs. Internal Expression of Individual Subunits

All experiments were performed at  $6^{\circ}\text{C}$ . To test for subunits expressed on the surface of cells, confluent 60-mm dishes of 3T3-DOL- $\alpha$ mass, 3T3-DOL- $\beta$ mass, L-pSV2- $\gamma$ 5, and L-pSV2- $\delta$ 2 cells were first incubated for 10 min with DME supplemented with 5% CS (CS/DME) followed by a 2-h incubation with 0.5 ml CS/DME containing 70-fold diluted anti-AChR (CHI-103) antiserum. The anti-AChR antiserum is a polyclonal serum which is prepared in rabbits and directed against affinity column purified *Torpedo*

*californica* AChR. Analysis of this serum by either immunoblotting or immunoprecipitation of metabolically labeled AChR subunits revealed that the antiserum recognized each of the four AChR subunits. After incubation with the antiserum, cells were washed twice for 7.5 min (2.5 ml per wash) with CS/DME, and then twice for 7.5 min with PBS containing 0.2% gelatin (PBS/gelatin). Cells were incubated 60 min in 0.5 ml of PBS/gelatin containing  $2 \times 10^5$  cpm of  $^{125}\text{I}$ -protein A (sp act,  $>30 \mu\text{Ci}/\mu\text{g}$ ; ICN Biochemicals, Irvine, CA), washed twice for 13 min with 2.5 ml of PBS/gelatin, washed once for 13 min with 2.5 ml of CS/DME, lysed with 1% Triton X-100, and counted in a gamma counter. For L-pSV2- $\gamma$ 5 cells, all washes and incubations were performed with PBS containing 2% filtered nonfat dried milk (Carnation) instead of PBS/gelatin. During the incubations at  $6^{\circ}\text{C}$  and subsequent washes, all cell types remained intact and attached to the culture dishes.

Tests for the expression of subunits on the inside of cells were performed in parallel with those for surface expression. Confluent 35-mm dishes of cells were permeabilized by treating them for 10 min in PBS/gelatin supplemented with 0.03% Saponin (Sigma Chemical Co., St. Louis, MO). The L-pSV2- $\gamma$ 5 cells were then fixed for 30 min with 3% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in PBS at room temperature, rinsed twice for 5 min with PBS, once for 5 min with PBS containing 50 mM ammonium chloride, and once for 10 min with  $6^{\circ}\text{C}$  CS/DME containing 0.03% Saponin. Plates of cells were then treated exactly as described above for intact cells except that 0.03% Saponin was included in all of the incubations and washes. For both permeabilized and intact samples, appropriate control cells (NIH3T3 for  $\alpha$ - and  $\beta$ -expressing cell lines, or sodium butyrate-treated Ltk<sup>-</sup>aprt<sup>-</sup> cells for  $\gamma$ - and  $\delta$ -producing lines) were analyzed in parallel.

### Glycosylation Studies

Confluent 10-cm dishes of cells were metabolically labeled with [ $^{35}\text{S}$ ]methionine ( $^{35}\text{S}$ -TRANS; ICN Biochemicals) for 20 min as described above. In some experiments, cells were first incubated for 2 h with 2.5  $\mu\text{g}/\text{ml}$  tunicamycin (Calbiochem-Behring Corp.), then metabolically labeled in the continued presence of tunicamycin. Cells were lysed by incubating for 5 min at room temperature in 740  $\mu\text{l}$  of lysis B buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.02%  $\text{Na}_3\text{N}$ , 1% Triton X-100) containing 1% SDS. Triton X-100 was added to a final concentration of 5%, followed by immunoprecipitation with the appropriate polyclonal anti-subunit antiserum. Protein A-Sepharose was added to immune complexes as described above, pelleted in an Eppendorf centrifuge, and the pellets were washed four times with lysis B buffer containing 0.1% SDS, then once with 0.1 M sodium acetate, pH 5.5. Subunits were dissociated from the pellets in 0.1 M sodium acetate containing 0.4% SDS, and aliquots of the samples were then adjusted to 0.1% SDS in 0.1 M sodium acetate before digestion overnight at  $25^{\circ}\text{C}$  with  $0.5 \times 10^{-3}$  U of endoglycosidase H (Miles Scientific Div., Naperville, IL). Samples were either processed directly for SDS-PAGE or they were first reimmunoprecipitated with appropriate antiserum before gel electrophoresis.

### Protein Transfer and $^{125}\text{I}$ -BuTx Binding

Eight 10-cm dishes each of confluent 3T3, confluent 3T3-DOL- $\alpha$ mass, 50% confluent L6, and 50% confluent L6-DOL- $\alpha$ 2 cells were washed with  $4^{\circ}\text{C}$  PBS and then lysed in 300  $\mu\text{l}$  of lysis A buffer containing 5% Triton X-100 and 0.2% SDS. Cells were scraped from dishes with a rubber policeman, transferred to Eppendorf tubes, and spun for 8 min at  $4^{\circ}\text{C}$ . Supernatants were pooled for each cell type, precleared with protein A-Sepharose, and incubated overnight with anti- $\alpha$  antiserum followed by a 3.5-h incubation with protein A-Sepharose. Pellets were washed as described above and  $\alpha$  protein was eluted in 2 $\times$  gel loading buffer containing a final concentration of 5% SDS and 100 mM DTT. Samples were electrophoresed on SDS gels, transferred to Zetabind filters, and labeled with  $^{125}\text{I}$ -BuTx as described (Wilson et al., 1984).

### $^{125}\text{I}$ -BuTx Binding Studies

Two 10-cm dishes of 3T3-DOJ- $\alpha$ 3 cells were grown at  $37^{\circ}\text{C}$  until confluent, and then the medium was supplemented with 10 mM sodium butyrate and the cells were grown at  $26^{\circ}\text{C}$  for 2 d. Cells were solubilized in lysis A buffer, spun for 10 min at  $4^{\circ}\text{C}$  in an Eppendorf centrifuge, and the lysates were incubated for 24 h at  $4^{\circ}\text{C}$  with different concentrations of  $^{125}\text{I}$ -BuTx in the presence or absence of 40  $\mu\text{M}$  unlabeled BuTx. Anti- $\alpha$  antiserum was added to each vial and incubated at  $4^{\circ}\text{C}$  for 2 h followed by a 1-h incubation at

4°C with protein A-Sepharose. Immunoprecipitates were collected and washed as described under Labeling and Immunoprecipitations and then counted in a gamma counter.

To determine the percentage of  $\alpha$  polypeptides capable of binding  $^{125}\text{I}$ -BuTx, one 10-cm dish of 3T3-DOJ- $\alpha$ 3 cells was grown and solubilized as described above. The lysate was incubated with 100 nM  $^{125}\text{I}$ -BuTx for 24 h at 4°C, precipitated with anti- $\alpha$  antiserum and protein A-Sepharose, and counted in a gamma counter. After the sample was counted, it was electrophoresed on SDS gels, transferred to Zetabind filters, and overlaid with anti- $\alpha$  antiserum and  $^{125}\text{I}$ -protein A. By comparison with known amounts of *Torpedo* electric organ AChR run on the same gel, the amount of  $\alpha$  polypeptide could be determined. This was quantitated by densitometrically scanning the autoradiograph band or by cutting out the gel band and counting it in a gamma counter.

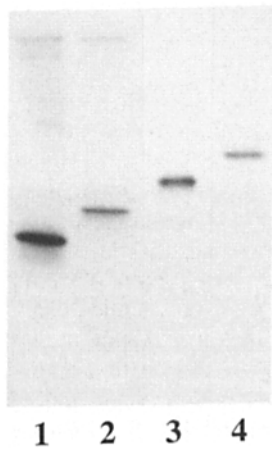
Cell surface expression of AChRs was determined as follows. All-6- $\alpha$ 1-7 cells were grown in 35-mm dishes at 37°C until confluent, then the medium was supplemented with 10 mM sodium butyrate and the cells were grown at 28°C for 10 d. Dishes were labeled in 750  $\mu\text{l}$  of PBS containing 0.03% BSA and 0.35 nM  $^{125}\text{I}$ -BuTx (1,000 cpm/fmol) for 140 min, washed three times in 4 ml of PBS/BSA, solubilized in 1 ml of 1% Triton X-100, and counted in a gamma counter.

## Results

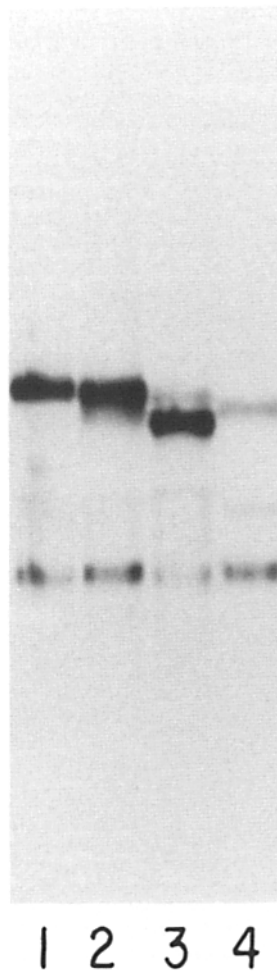
### Expression of $\alpha$ and $\beta$ Polypeptides with Retroviral Vectors

Colonies and mass cultures of NIH3T3 cells infected with the pDOL- $\alpha$ , - $\beta$ , - $\gamma$ , or - $\delta$  recombinants were metabolically labeled with [ $^3\text{H}$ ]leucine for 20 min, solubilized, immunoprecipitated with subunit-specific antisera, electrophoresed on SDS-polyacrylamide gels, and processed for autoradiography. We were able to easily detect  $\alpha$ - and  $\beta$ -polypeptide chains which comigrated on gels at precisely the same positions as native *Torpedo californica*  $\alpha$  and  $\beta$  polypeptides isolated from electric organ (Fig. 1, lanes 1 and 2, respectively). However, no  $\gamma$  or  $\delta$  protein could be detected in either mass cultures or in several clonal cell lines, even though our polyclonal antisera are known to recognize epitopes expressed on both denatured and native AChR (Claudio and Raftery, 1977, 1980).

To determine why no  $\gamma$  or  $\delta$  subunits were produced, we isolated mRNA from several cell lines to determine the amounts and sizes of the different mRNAs. RNA was prepared (Chirgwin et al., 1979) from mass cultures of pDOL- $\alpha$ , - $\beta$ , - $\gamma$ , or - $\delta$  infected 3T3 cells and six clonal  $\delta$ -3T3 cell lines. The RNA was run on formaldehyde-agarose gels, transferred



**Figure 1.** Four metabolically labeled fibroblast cell lines each stably expressing one of the four *Torpedo* AChR subunits. 10-cm dishes of  $\sim 10^7$  cells were labeled for 20 min with 400  $\mu\text{Ci}$  of [ $^3\text{H}$ ]leucine, solubilized, immunoprecipitated with subunit-specific antisera, and electrophoresed on SDS-polyacrylamide gels. (Lane 1) 3T3-DOL- $\alpha$ mass; (lane 2) 3T3-DOL- $\beta$ mass; (lane 3) L-pSV2- $\gamma$ 5; and (lane 4) 3T3-DOJ- $\delta$ 2. The films were exposed at  $-70^\circ\text{C}$  with an intensifying screen for 2 d (lanes 1 and 2) or 4 d (lanes 3 and 4).



**Figure 2.** RNA blot of 3T3-DOL- $\alpha$ mass, 3T3-DOL- $\beta$ mass, 3T3-DOL- $\gamma$ mass, and 3T3-DOL- $\delta$ mass cells. RNA was prepared (Chirgwin et al., 1979) from the four cell lines and 10  $\mu\text{g}$  of each was run on formaldehyde-agarose gels, transferred to nitrocellulose paper, and probed with a nick-translated ( $5 \times 10^6$  cpm) neo fragment using standard procedures (Maniatis et al., 1982). Because pDOL contains two promoters, two RNA transcripts are seen: one initiating from the 5' LTR ( $\sim 6$ -kb transcript) and one initiating from the internal SV-40 promoter ( $\sim 3.3$ -kb transcript) with both transcripts terminating at the 3' LTR. (Lane 1) 3T3-DOL- $\alpha$ mass; (lane 2) 3T3-DOL- $\beta$ mass; (lane 3) 3T3-DOL- $\gamma$ mass; and (lane 4) 3T3-DOL- $\delta$ mass. The film was exposed at  $-70^\circ\text{C}$  with an intensifying screen for 2 d.

to nitrocellulose paper, and probed with a nick-translated neo fragment using standard procedures (Maniatis et al., 1982). Because pDOL contains two promoters, two RNA transcripts are expected: one initiating from the 5' LTR and one initiating from the internal SV-40 promoter, with both transcripts terminating at the 3' LTR. As shown in Fig. 2, the smaller ( $\sim 3.3$  kb) transcripts (SV-40 transcripts) produced in each of the cell lines were identical in size, which is the expected result if the recombinants had integrated correctly into genomic DNA. Because the cDNA inserts were essentially the same size and the SV-40 transcripts produced were identical in size, we would have expected that each of the LTR transcripts would also be identical in size. As shown in Fig. 2, large ( $\sim 6$  kb)  $\alpha$  and  $\beta$  transcripts were produced (lanes 1 and 2, respectively), but the  $\gamma$  and  $\delta$  transcripts were smaller than expected (lanes 3 and 4, respectively). These results suggested that either the  $\gamma$  and  $\delta$  integrated proviral genomes had aberrant structures or that correct recombinants had integrated but the transcripts produced from them were processed incorrectly.

Features of the pDOL vector allow the integrated proviral sequences to be recovered from genomic DNA as molecular clones (Cepko et al., 1984). 3T3-DOL- $\alpha$ mass, 3T3-DOL- $\beta$ mass, 3T3-DOL- $\gamma$ mass, and 3T3-DOL- $\delta$ mass cells were fused to COS cells, and DNA was isolated and used to transform HB101 bacterial cells. Five kanamycin-resistant colo-

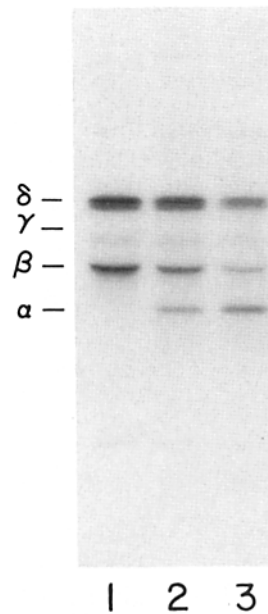
nies from each transformation were picked, liquid cultures were grown, and plasmid DNA was isolated. Restriction endonuclease mapping of the plasmids demonstrated that the  $\alpha$  and  $\beta$  cDNAs had integrated intact, whereas segments of the  $\gamma$ - and  $\delta$ -DNA sequences had been deleted (data not shown). Two of the  $\gamma$  clones had deletions that extended from the splice donor sequence in the vector (located between the 5' LTR and the site of insertion of the cDNA [Korman et al., 1987]) to a site within the cDNA resulting in the loss of  $\sim 1,100$  bp of insert. Two other clones had internal deletions resulting in the loss of  $\sim 1,400$  bp of cDNA sequence. A 300-bp deletion in the fifth clone suggested that this clone was probably excised improperly from the chromosome or that rearrangement of the plasmid occurred during the fusion-rescue or bacterial transformation steps. With  $\delta$ , three of the clones examined had a deletion of  $\sim 300$  bp which mapped to a site within the cDNA. The other two clones appeared to have integrated intact.

### Expression of $\delta$ Polypeptides with Retroviral Vectors

Examination of the  $\gamma$ - and  $\delta$ -cDNA sequences reveals that a large number of potential splice sites are present in these cDNAs. From restriction endonuclease mapping of the rescued  $\gamma$  clones, we determined that with two of the five rescued clones the donor sequence provided in the vector had been one of the splice sites. We thus engineered all of the subunit cDNAs into the vector pLJ in which the donor sequence had been mutated (Korman et al., 1987). Whereas proper  $\alpha$  and  $\beta$  polypeptides were expressed with this vector, no  $\gamma$  polypeptides were produced, and the expressed  $\delta$  polypeptide had a molecular mass  $\sim 20$  kD smaller than expected (son-of-delta; Fig. 6 E, *sod*). A number of other vector and insert constructions were tested in attempts to express full-length  $\gamma$  and  $\delta$  polypeptides: we tried an expression vector in which a legitimate intron was present (pEU); vectors were tested in which the positions of the neo and cDNA sequences were reversed (pEU, pWE [Choudary et al., 1986]); and we increased the flanking sequences of the cDNAs by using our full-length clones in some of the constructions. None of these constructions resulted in a properly sized  $\gamma$  or  $\delta$  polypeptide. A correct  $\delta$  polypeptide was finally achieved with the vector pDOJ (Fig. 1, lane 4). The major difference between this vector and pLJ is that pLJ contains a 5-bp deletion in addition to the point mutation that destroyed the donor sequence (Korman et al., 1987) whereas pDOJ only contains the point mutation. Even with pDOJ, however, we were unable to express any  $\gamma$  protein.

### Expression of $\gamma$ Polypeptides with pSV2

When  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  cDNAs were inserted into pSV2 vectors and cotransfected with tk into Ltk<sup>-aprt</sup> cells, one clonal cell line (all-11) was isolated that expressed all four subunits (Claudio et al., 1987) and a second cell line (all-6) was isolated that expressed only  $\beta$ ,  $\gamma$ , and  $\delta$  subunits (Fig. 3, lane 1). In both of these cell lines,  $\gamma$  polypeptide was produced which was recognized by anti- $\gamma$  antiserum. Thus, to produce a  $\gamma$ -only-expressing fibroblast cell line, we cotransfected pSV2- $\gamma$  and pSV2-neo into 3T3 cells (3T3-pSV2- $\gamma$ 1,2) or pSV2- $\gamma$  and ptk into Ltk<sup>-aprt</sup> cells (L-pSV2- $\gamma$ 5). The results from metabolic labeling of L-pSV2- $\gamma$ 5 cells, followed



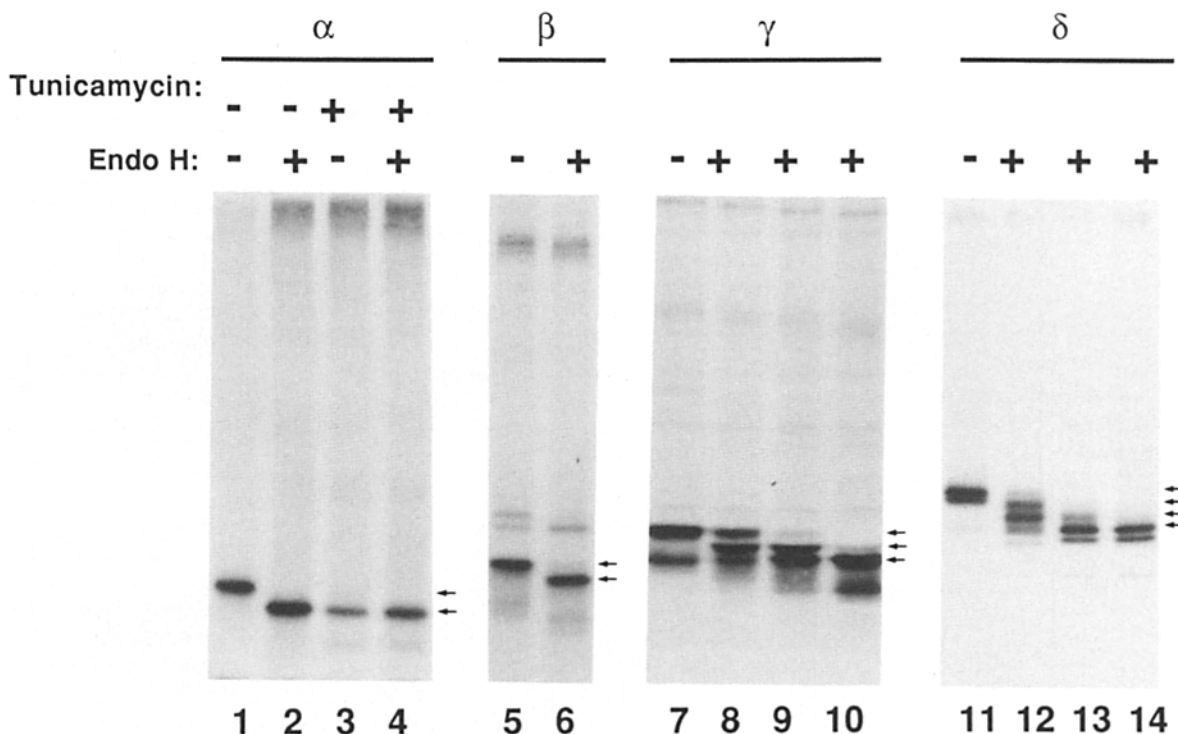
**Figure 3.** Subunit expression in all-6, all-6- $\alpha$ 1, and all-6- $\alpha$ 3 cells. The positions of AChR subunits isolated from *Torpedo californica* electroplaque tissue are marked  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ . One 10-cm dish of each cell line was grown for 2 d at 37°C in the presence of 10 mM sodium butyrate. The cells were incubated in 2 ml of leucine-depleted medium for 15 min and then 400  $\mu$ Ci of [<sup>3</sup>H]leucine was added and the cells were labeled for 30 min. The cells were next washed, solubilized in a buffer containing 1% Triton X-100, incubated with a mixture of anti-*Torpedo*  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  antisera, followed by protein A-Sepharose. Immunoprecipitates were run on 10% SDS-polyacrylamide gels and treated for fluorography. The film was exposed for 5 d at -70°C with an intensifying screen. (Lane 1) all-6 cells; (lane 2) all-6- $\alpha$ 1 cells; and (lane 3) all-6- $\alpha$ 3 cells.

by immunoprecipitation with anti- $\gamma$  antiserum and electrophoresis on SDS gels, are shown in Fig. 1, lane 3.

Although we have now expressed  $\gamma$  polypeptides in over ten different cell lines and we produce functional AChRs in those lines in which all four subunits are expressed, the  $\gamma$  polypeptide always migrates  $\sim 3$  kD smaller than  $\gamma$  polypeptides isolated from *Torpedo* electric organ. We believe that this slightly altered migration is due to differences in post-translational modifications between *Torpedo* electrocytes and other environments rather than to some difficulty with the clone for the following reason: when a  $\gamma$ -cDNA clone isolated from a different laboratory was used as a template to prepare mRNA in vitro and the RNA (or the polyadenylated RNA fraction from electric organ) was microinjected into *Xenopus* oocytes, the  $\gamma$  polypeptides produced migrated on SDS gels  $\sim 3$  kD smaller than expected (Mishina et al., 1984). Thus, two independent clonal isolates (isolated from two different laboratories) as well as RNA isolated from electric tissue, encode polypeptides that migrate on SDS gels  $\sim 3$  kD smaller than expected (whether expressed in oocytes or in mammalian cells).

### Posttranslational Processing

Several posttranslational modifications of the AChR are known to occur, including glycosylation, phosphorylation, disulfide bond formation, and fatty acylation. Although we have shown that the pharmacological and physiological properties of *Torpedo* AChRs are properly reconstituted in mouse fibroblast cells when all of the subunits are present (Claudio et al., 1987), the observation that the  $\gamma$ -polypeptide chain does not migrate correctly on SDS gels would suggest that it has not been processed entirely correctly. One possible explanation for the altered electrophoretic mobility is altered glycosylation. Nomoto et al. (1986) analyzed the oligosac-



**Figure 4.** Tunicamycin and endoglycosidase H treatment of *Torpedo* subunits expressed individually in fibroblasts. For each subunit, the upper arrow marks the position of the fully glycosylated subunit, the lowest arrow indicates the fully deglycosylated (or nonglycosylated) subunit, and the intermediate arrows indicate partially deglycosylated subunits. Dishes of cells were labeled for 20 min with [<sup>35</sup>S]methionine (with or without tunicamycin pretreatment), solubilized, and immunoprecipitated with subunit-specific antisera. Aliquots of the immunoprecipitates were treated with endoglycosidase H for different lengths of time, or mock treated in buffer without enzyme. 3T3-DOL- $\alpha$ mass cells (lane 1) mock treated for 19 h, (lane 2) endoglycosidase H treated for 19 h, (lane 3) tunicamycin treated and then mock treated for 19 h, and (lane 4) tunicamycin treated and then endoglycosidase H treated for 19 h. 3T3-DOL- $\beta$ 6 cells (lane 5) mock treated for 15 h and (lane 6) endoglycosidase H treated for 15 h. L-pSV2- $\gamma$ 5 cells (lane 7) mock treated for 7 h and (lanes 8-10) endoglycosidase H treated for 20 min, 40 min, or 7 h, respectively. L-pSV2- $\delta$ 2 cells (lane 11) mock treated for 19 h and (lanes 12-14) endoglycosidase H treated for 15 min, 3 h, or 19 h, respectively. The lowest band seen in lanes 7-10 is an artifact arising from incubation of the  $\gamma$  subunit in 0.1 M sodium acetate buffer, pH 5.5. Films were exposed at  $-70^{\circ}\text{C}$  with an intensifying screen for the following times:  $\alpha$ , 10 d;  $\beta$ , 9 d;  $\gamma$ , 26 h;  $\delta$ , 7 d.

charides obtained after digestion of each *Torpedo* subunit and obtained data that were consistent with there being one, one, two, and three units of oligosaccharides for the  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  subunits, respectively. Using our four different subunit-expressing fibroblast cell lines, we treated intact cells with the glycosylation inhibitor, tunicamycin, or treated immunoprecipitated subunits from these cells with endoglycosidase H (Fig. 4). We determined that each subunit was glycosylated (compare Fig. 4, lanes 1, 5, 7, and 11 with Fig. 4, lanes 2, 6, 10, and 14) in our cell lines.

Using incomplete digestion with endoglycosidase H, we further determined that there were one, one, two, and three units of asparagine-linked oligosaccharides for the  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  subunits, respectively (note the lack of intermediates in Fig. 4, lanes 2 and 6, one intermediate in lane 8, and two intermediates in lane 12). Our results would indicate that at least the addition of the high mannose-type oligosaccharide units occurred properly in these cell lines. It is interesting to note that although the  $\gamma$  subunit contains fewer sites of glycosylation than  $\delta$  (which appears to migrate correctly), there is a greater effect on the migration of  $\gamma$  with glycosylation. Similar mobility differences between the  $\gamma$  and  $\delta$

subunits were observed using entirely different expression systems: Anderson and Blobel (1981) used a wheat germ cell-free protein synthesizing system and our laboratory (Hartman, D. S., unpublished observations) used a rabbit reticulocyte lysate system in the presence or absence of added dog pancreas rough microsomal membranes.

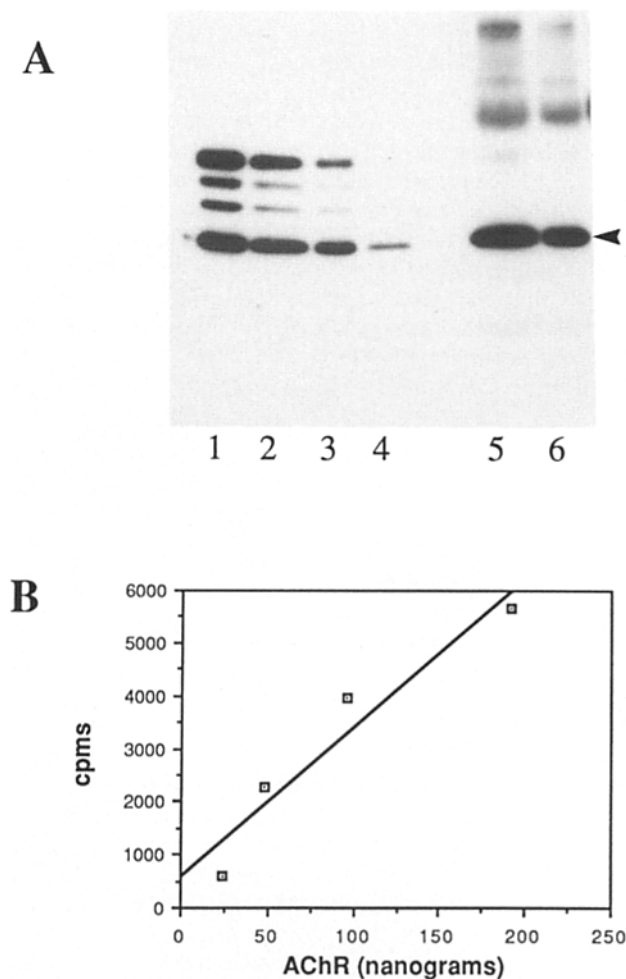
Since there are more units of oligosaccharide added to the  $\delta$  subunit than to the  $\gamma$  subunit, and the total amount of oligosaccharide is greater in  $\delta$  than in  $\gamma$  (Nomoto et al., 1986), the observation that the addition of oligosaccharide to  $\gamma$  has a more profound effect on its electrophoretic mobility compared with the mobility of the other subunits suggests that perhaps differences in the types of complex oligosaccharide added are contributing to the unexpected mobility. Thus, although all of our results would indicate that glycosylation was occurring correctly in these cell lines, subtle differences in glycosylation between *Torpedo* electrocytes and mammalian cells might result in the altered electrophoretic migration. Alternatively, differences in phosphorylation or fatty acylation, could be the cause. Whatever the explanation for the altered electrophoretic mobility on SDS gels of the  $\gamma$  polypeptide, it should be noted that fully functional AChRs

are expressed on the surface of mouse fibroblasts and *Xenopus* oocytes using these clones.

### Level of Subunit Expression

A 10-cm dish of 3T3-DOL- $\alpha$ mass cells was solubilized, the supernatant was incubated with anti- $\alpha$  antiserum, and the immune complex was precipitated with protein A-Sepharose. The precipitate was washed and proteins were eluted and electrophoresed on SDS-polyacrylamide gels along with known amounts of purified *Torpedo* AChR isolated from electric organ. Electrophoresed proteins were then trans-

ferred to Zetabind filters, overlaid with anti- $\alpha$  antiserum, overlaid with  $^{125}\text{I}$ -protein A, and autoradiographed (Fig. 5 A). A graph of the cpm of  $^{125}\text{I}$ -protein A bound to  $\alpha$  polypeptides vs. the nanograms of *Torpedo* electric organ AChR applied to the gel (Fig. 5 B) shows a linear correlation. We were thus able to quantitate the amount of  $^{125}\text{I}$ -protein A bound to  $\alpha$  polypeptides isolated from 3T3-DOL- $\alpha$ mass cells and determine the number of  $\alpha$  subunits produced in these cells by comparison with values obtained from electric organ AChR. The value we obtained indicated a steady-state level of  $\sim 200,000$   $\alpha$  molecules per 3T3-DOL- $\alpha$ mass cell. 92% of the  $\alpha$  polypeptides were precipitated after the first cycle of immunoprecipitation and 78% were eluted from the protein A-Sepharose beads, suggesting that a more accurate estimate of the number of  $\alpha$  subunits produced is  $\sim 270,000$  molecules per cell.

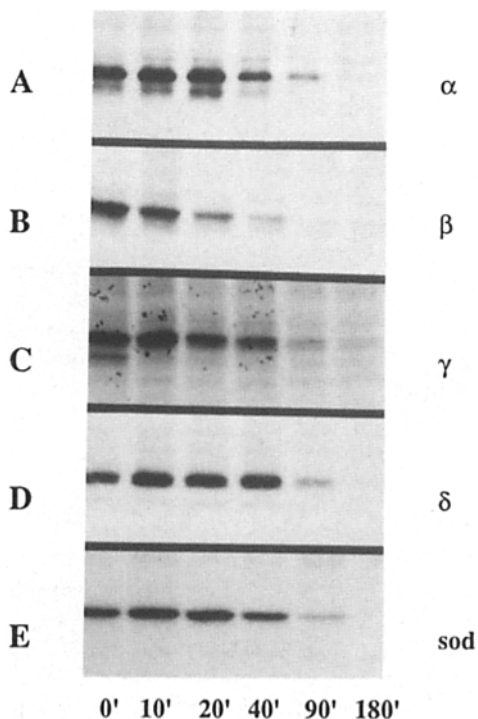


**Figure 5.** Quantitation of  $\alpha$  subunit in 3T3-DOL- $\alpha$ mass cells by immunoblot analysis. Different amounts of native *Torpedo* AChR and  $\alpha$  subunit isolated from 3T3-DOL- $\alpha$ mass cells were electrophoresed on SDS-polyacrylamide gels, transferred to Zetabind filters, incubated with a mixture of the four subunit-specific antisera, and labeled with  $^{125}\text{I}$ -protein A. The resultant autoradiograph is shown in A. Lanes 1-4 contain 192, 96, 48, and 24 ng of native *Torpedo* AChR, respectively. Lanes 5 and 6 contain  $\alpha$  subunit immunoprecipitated from  $6 \times 10^6$  and  $3 \times 10^6$  3T3-DOL- $\alpha$ mass cells. The lower band in each lane (arrowhead) represents the  $\alpha$  signal. The film was exposed at  $-70^\circ\text{C}$  with an intensifying screen for 14 h. (B) The  $\alpha$  bands in lanes 1-4 of the immunoblot shown in A were cut from the filter, counted in a gamma counter, and plotted against the ng of native AChR applied to each lane. The line drawn through the points then served as a reference for determining the amount of  $\alpha$  polypeptide expressed in 3T3-DOL- $\alpha$ mass cells.

### Half-Life of Individual AChR Subunits in Fibroblasts and Muscle Cells

Pulse-chase experiments were conducted to determine the stability of the individual AChR subunits in the absence of the other subunit types. Dishes of confluent ( $\sim 10^7$ ) 3T3 or Ltk<sup>aprt</sup> cells expressing  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and sod (son-of-delta, the 45-kD  $\delta$  fragment) polypeptides were labeled for 10 min with [ $^3\text{H}$ ]leucine, and then harvested immediately (0-min chase) or harvested after 10, 20, 40, 90, or 180 min in media containing an excess of unlabeled leucine. Fig. 6 shows the autoradiographs of pulse-chase experiments conducted on these cells (Fig. 6, A-E). The bands were scanned with a densitometer and the percent intensities were plotted against the chase times to determine the half lives (data not shown). The half lives of  $\alpha$ ,  $\gamma$ ,  $\delta$ , and sod were consistently  $\sim 40$  min and were not altered by the type of expression vector used to introduce the cDNA into cells (SV-40-derived or retrovirus-derived vectors). Although 40 min is a fairly short half-life, it is similar to that observed previously (Merlie and Lindstrom, 1983) for unassembled endogenous AChR  $\alpha$  subunits in BC<sub>3</sub>H-1 muscle cells ( $\sim 30$  min). Thus, our results demonstrated that *Torpedo*  $\alpha$ ,  $\gamma$ , and  $\delta$  polypeptides expressed in mouse fibroblast cells were no less stable than unassembled mouse  $\alpha$  polypeptides expressed in their native muscle cell environment. The half-life of the  $\beta$  subunit was quite different, however, and was calculated to be only  $\sim 12$  min.

In part, because the  $\beta$  half-life was so short in fibroblasts, we thought it necessary to perform half-life studies in muscle cells to ascertain whether the cellular environment might influence *Torpedo* AChR subunit half-lives. To test for effects of cellular environment, we performed half-life studies of *Torpedo*  $\alpha$  and  $\beta$  polypeptides expressed in undifferentiated muscle cells ( $\alpha$ -expressing L6, C2, and BC<sub>3</sub>H-1 cells and  $\beta$ -expressing L6 and C2 cells). We found no differences in subunit half-lives between fibroblasts and undifferentiated muscle cells. The results of undifferentiated C2-DOL- $\alpha$ mass cells are shown in Fig. 7 A. The presence of the remaining three subunit types might also contribute to subunit stability. This possibility was tested in two ways: (a) we determined *Torpedo* subunit half-lives in differentiated muscle cells; and (b) we determined subunit half-lives in AChR-fibroblast cell lines. Again, no differences were detected among fibroblasts expressing a single subunit, differentiated muscle cells ex-

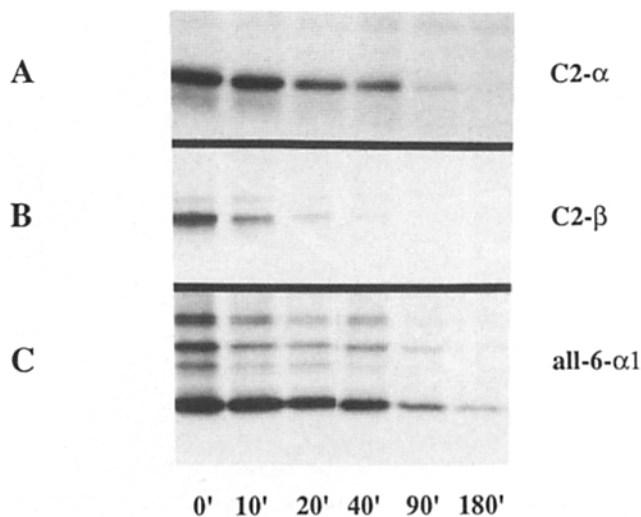


**Figure 6.** Pulse-chase experiment conducted at 37°C on 3T3 and L cells stably expressing *Torpedo*  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , or sod ( $\delta$ -subunit fragment). For each of the five cell lines, six identical 10-cm dishes of  $\sim 10^7$  cells were used. Cells were incubated in leucine-depleted, leucine- and isoleucine-depleted, or methionine-depleted medium for 15 min. Cells were next incubated in depleted medium supplemented with 400  $\mu$ Ci of [ $^3$ H]leucine, 200  $\mu$ Ci each of [ $^3$ H]leucine plus [ $^3$ H]isoleucine, or 400  $\mu$ Ci of [ $^{35}$ S]methionine for 10 min, and then chased with medium containing an excess of unlabeled amino acid. Cells were harvested after 0, 10, 20, 40, 90, or 180 min of chase, solubilized, immunoprecipitated with subunit-specific antiserum, electrophoresed on SDS-polyacrylamide gels, and treated for autoradiography. (A) 3T3-DOL- $\alpha$ mass cells labeled with [ $^3$ H]leucine and [ $^3$ H]isoleucine; (B) 3T3-DOL- $\beta$ mass cells labeled with [ $^3$ H]leucine and [ $^3$ H]isoleucine; (C) 3T3-pSV2- $\gamma$ 1,2 cells labeled with [ $^{35}$ S]methionine; (D) L-pSV2- $\delta$ 2 cells labeled with [ $^3$ H]leucine; (E) 3T3-pLJ- $\delta$ mass (sod) cells labeled with [ $^3$ H]leucine and [ $^3$ H]isoleucine. Films were exposed at  $-70^\circ\text{C}$  with an intensifying screen: 5 d for A, C, and D; 7 d for B; and 13 d for E.

pressing *Torpedo* subunits, and fibroblasts expressing all four subunits. The results of experiments conducted on differentiated C2-DOL- $\beta$ 1 and AChR-fibroblast (all-6- $\alpha$ 1) cells are shown in Fig. 7, B and C, respectively. The bands were scanned with a densitometer and the percent intensities were plotted against the chase times to determine the half-lives (data not shown). The average of three half-life experiments on *Torpedo*  $\alpha$  expressed in differentiated and undifferentiated muscle cells was 36 min. The average of five half-life experiments on  $\beta$  expressed in differentiated and undifferentiated muscle cells was 10 min. We concluded from these studies that the half-lives of the subunits were not affected by the cellular environment (fibroblasts, differentiated or undifferentiated muscle cells), and were not affected by the presence of the other subunits. The explanation for the extremely short  $\beta$  half-life was not forthcoming, but was cause for some concern.

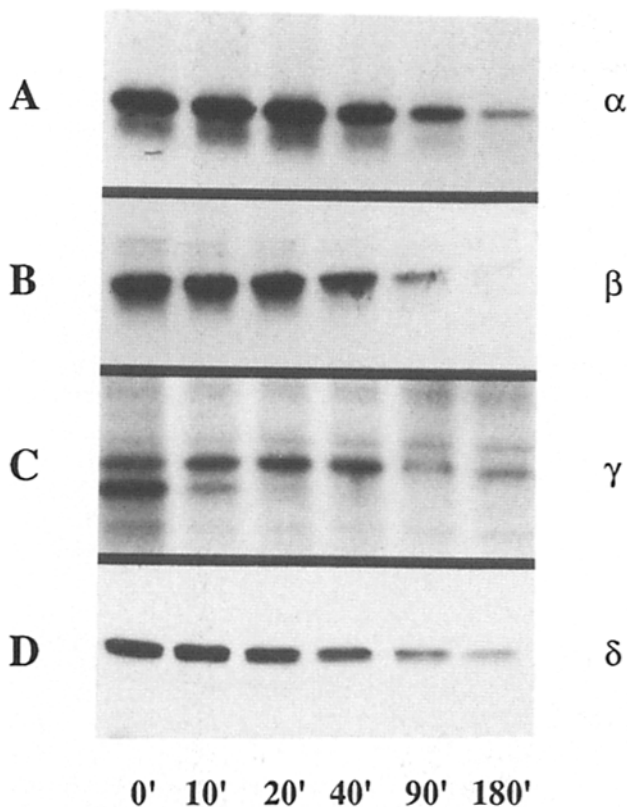
Two lines of evidence indicated that the  $\beta$  polypeptide produced was correctly synthesized and processed: (a) it was recognized by  $\beta$ -specific antisera and comigrated precisely with native *Torpedo*  $\beta$  subunits on SDS gels; and (b) our tunicamycin and endoglycosidase H experiments indicated that it was properly glycosylated. Nonetheless, subtle differences in proteins often cannot be resolved on one-dimensional gels and so it was possible that the subunit had not been synthesized correctly and was somehow marked for rapid degradation. An indirect test of this possibility was to perform pulse-chase experiments on a cell line that expressed a grossly altered polypeptide (3T3-sod) and compare the results with those obtained for the other (presumably wild-type) polypeptides. The calculated half-life of sod was  $\sim 40$  min (Fig. 6 E), not 12 min, indirectly suggesting that the short half-life of  $\beta$  was not due to an incorrectly synthesized subunit.

All of the above pulse-chase experiments were performed on cells grown at the normal temperature for mammalian cells, 37°C. The expression of *Torpedo* AChRs in mammalian cells is acutely temperature sensitive, with functional complexes only being detected at temperatures lower than 37°C (Claudio et al., 1987). After making this discovery, we repeated the pulse-chase experiments at 28°C, a temperature which produces large quantities of cell surface *Torpedo* AChRs in mouse fibroblast cells. The results of 3T3-DOJ- $\alpha$ 3, 3T3-DOJ- $\beta$ 6, 3T3-pSV2- $\gamma$ 1,2, and L-pSV2- $\delta$ 2 cells are



**Figure 7.** Pulse-chase experiment conducted at 37°C on undifferentiated mouse muscle C2-*Torpedo* $\alpha$ , differentiated C2-*Torpedo* $\beta$ , and AChR-fibroblast cells. Pulse-chase experiments were performed as described in the legend to Fig. 6. For undifferentiated muscle cells, 75% confluent C2-DOL- $\alpha$ mass cells were used. For differentiated muscle cells, C2-DOL- $\beta$ 1 cells were induced to form multinucleated myotubes and assays were performed when  $\sim 70\%$  of the cells had formed myotubes. For L cells, confluent dishes of cells were used. (A) C2-DOL- $\alpha$ mass cells were labeled with [ $^3$ H]leucine plus [ $^3$ H]isoleucine; (B) C2-DOL- $\beta$ 1 cells were labeled with [ $^3$ H]leucine plus [ $^3$ H]isoleucine; (C) AChR-fibroblast (all-6- $\alpha$ 1) cells were labeled with [ $^3$ H]leucine. Chase times (0, 10, 20, 40, 90, and 180 min) are indicated below the figure. Film exposure times ( $-70^\circ\text{C}$  with an intensifying screen) for A, B, and C were 13, 9, and 7 d, respectively.





**Figure 8.** Pulse-chase experiment conducted at 28°C on 3T3 cells stably expressing *Torpedo*  $\alpha$ ,  $\beta$ ,  $\gamma$ , or  $\delta$  subunits. Confluent dishes of cells were labeled with 400  $\mu$ Ci of [ $^3$ H]leucine and processed as described in the legend to Fig. 6. (A) 3T3-DOJ- $\alpha$ 3 cells; (B) 3T3-DOJ- $\beta$ 1 cells; (C) 3T3-pSV2- $\gamma$ 1,2 cells; (D) L-pSV2- $\delta$ 2 cells. Chase times (0, 10, 20, 40, 90, and 180 min) are indicated below the figure. Films were exposed at  $-70^\circ\text{C}$  with an intensifying screen for 3 d.

shown in Fig. 8, A–D, respectively. The half-lives of  $\alpha$ ,  $\gamma$ , and  $\delta$  were  $\sim 70$  min (compared with 43 min, the average of five experiments performed at 37°C) and the half-life of  $\beta$  was  $\sim 50$  min (compared with 12 min, the average of seven experiments performed at 37°C). Although the half-lives of each subunit increased at the lower temperature, the  $\beta$  subunit increased 4-fold while the others only increased  $\sim 1.5$ -fold. A more thorough analysis of the effects of temperature on *Torpedo* AChR expression will be described later (Paulson, H. L., and T. Claudio, manuscript in preparation). In that study we describe the effects of temperature on the expression of hybrid AChRs (formed between *Torpedo*  $\alpha$  subunits and rat [ $\alpha$ ] $\beta\gamma\delta$  subunits). We found that hybrid AChR formation increased linearly with decreasing temperature from  $\sim 20\%$  hybrids at 33°C to  $\sim 60\%$  hybrids at 24°C, with no hybrid formation occurring at 37°C. We do not know what the optimal temperature is for *Torpedo* AChR synthesis. However, we can say that temperature appears to have a more profound effect on the  $\beta$  subunit than it does on the other subunits. We do not understand why this is, nor do we understand the significance of the short  $\beta$  half-life. One intriguing possibility is that the  $\beta$  subunit performs some regulatory role in the process of subunit assembly.

### Cell Surface Expression

We wished to determine if individual  $\alpha$  subunits could be in-

serted into the plasma membrane in the absence of the other receptor subunits. Initially, we tested for surface expression by labeling intact cells with  $^{125}\text{I}$ -BuTx for 90 min, washing away the unbound toxin, and counting the cells in a gamma counter. The results of this study indicated that there were no cell surface toxin-binding  $\alpha$  subunits in 3T3-DOL- $\alpha$ mass cells. Because this assay requires that the subunit be expressed on the cell surface and be in the correct toxin-binding conformation, we also needed to test for surface expression of subunits that might be incapable of binding toxin.

Surface expression was next tested by incubating intact cells with polyclonal anti-AChR antiserum followed by  $^{125}\text{I}$ -protein A. Because antibody cross-linked AChRs are internalized more quickly than noncross-linked AChRs (Heinemann et al., 1977), we had to prevent the possible internalization of antibody-subunit complexes. Initially, we fixed 3T3-DOL- $\alpha$ mass cells with 3% paraformaldehyde and then labeled them with antiserum followed by  $^{125}\text{I}$ -protein A. Experiments with an antiserum generated against lysosomal membrane proteins (generously provided by I. Mellman [Yale University, New Haven, CT]), however, indicated that such fixation procedures also permeabilized the cells. By performing the experiments on unfixed cells at 6°C, we were able to maintain the integrity of the plasma membrane while also blocking the internalization of surface membrane proteins. 6-cm dishes of cells were cooled to 6°C, incubated with anti-AChR antiserum, washed free of unbound antiserum, and then incubated with  $^{125}\text{I}$ -protein A. The results of these experiments are shown in Table I. Intact 3T3-DOL- $\alpha$ mass, 3T3-DOL- $\beta$ mass, L-pSV2- $\gamma$ 5, and L-pSV2- $\delta$ 2 cells bound no more  $^{125}\text{I}$ -protein A than did control 3T3 or Ltk-*aprt*-cells, indicating that none of the individual subunits were expressed on the surface of these cells. When we tested AChR-fibroblast (all-11) cells under identical conditions, specific surface binding counts were easily detected demonstrating that the antiserum was capable of recognizing at least one of the subunits expressed on the cell surface (Table I). If subunit- or AChR-expressing cells were first permeabilized with 0.03% Saponin and then labeled with antiserum followed by  $^{125}\text{I}$ -protein A, counts were detected in all of the lines demonstrating that the anti-AChR antiserum was capable of rec-

**Table I.** Cell Surface vs. Cytoplasmic Expression of *Torpedo* Subunits\*

Cell line	Intact cells <sup>‡</sup> (6°C)		Permeabilized cells <sup>‡</sup> (0.03% saponin)	
	n <sup>§</sup>	specific cpm <sup>  </sup> $\pm$ SEM	n	specific cpm <sup>¶</sup> $\pm$ SEM
3T3-DOL- $\alpha$ mass	3	109 $\pm$ 282	3	12,390 $\pm$ 1,734
3T3-DOL- $\beta$ mass	4	0 $\pm$ 199	2	7,825 $\pm$ 1,154
L-pSV2- $\gamma$ 5	3	383 $\pm$ 255	3	16,615 $\pm$ 462
L-pSV2- $\delta$ 2	3	123 $\pm$ 206	4	5,460 $\pm$ 1,440
L-pSV2- $\alpha\beta\gamma\delta$ (all-11)	2	3,326 $\pm$ 142	2	21,082 $\pm$ 416

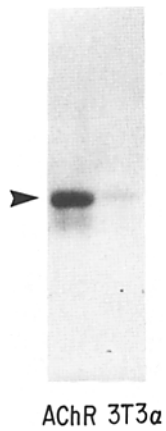
\* Cells were grown at 26°C. Intact or permeabilized cells were incubated with anti-AChR antiserum followed by  $^{125}\text{I}$ -protein A.

<sup>‡</sup> Data are expressed as the mean number of cpm per 6-cm dish  $\pm$  SEM.

<sup>§</sup> The number of samples.

<sup>||</sup> Specific cpm for the  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and all-11 cell lines represented 3, 0, 14, 3, and 42% of uncorrected counts, respectively.

<sup>¶</sup> Specific cpm for the  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and all-11 cell lines represented 31, 20, 59, 21, and 34% of uncorrected counts, respectively.



**Figure 9.**  $^{125}\text{I}$ -BuTx binding to 3T3-DOL- $\alpha$ mass cells. Shown is an immunoblot of *Torpedo* AChR and 3T3-DOL- $\alpha$ mass cells. The AChR lane contained 4  $\mu\text{g}$  of AChR isolated from *Torpedo californica* electroplaque. For the 3T3-DOL- $\alpha$ mass sample, eight 10-cm dishes of  $\sim 5 \times 10^6$  cells per dish were lysed, immunoprecipitated with anti- $\alpha$  antiserum followed by protein A-Sepharose. Samples were electrophoresed on SDS gels, transferred to Zetabind filters, and labeled with  $^{125}\text{I}$ -BuTx as described (Wilson et al., 1984). The arrow indicates the position of the  $\alpha$  subunit. The film was exposed at  $-70^\circ\text{C}$  with an intensifying screen for 2 d.

ognizing all four subunits. We concluded from these experiments that individual subunits were not expressed on the cell surface. Our results differ from those obtained by Hess and his colleagues who were able to detect individual *Torpedo*  $\alpha$  (Fujita et al., 1986) and  $\delta$  (Sweet et al., 1988) subunits on the surface of yeast cells. We do not know if the different results obtained by the two laboratories are due to the different expression systems used or due to differences in the method of assay.

### Ability to Bind BuTx

*Torpedo*  $\alpha$  subunits expressed in 3T3 and L6 cells were tested for their toxin-binding function. Dishes of cells were immunoprecipitated with anti- $\alpha$  antiserum, the immunoprecipitates were electrophoresed on SDS-polyacrylamide gels, protein was transferred to Zetabind filters, and the filters were labeled with  $^{125}\text{I}$ -BuTx. The results of toxin binding to *Torpedo* electroplaque AChR and 3T3-DOL- $\alpha$ mass cells are shown in Fig. 9.  $^{125}\text{I}$ -BuTx bound only to  $\alpha$  subunits and it bound both to the  $\alpha$  subunits expressed in 3T3-DOL- $\alpha$ mass cells and to the  $\alpha$  subunits synthesized in electroplaque tissue. Toxin binding to L6-DOL- $\alpha 2$  cells was also observed but none could be seen binding to 3T3 cells (data not shown).

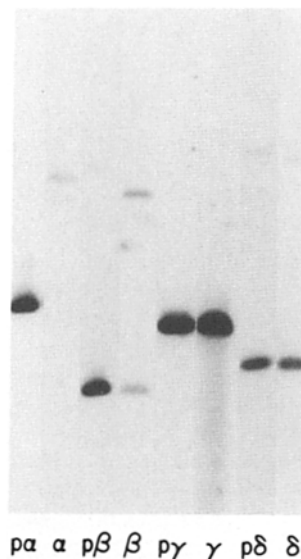
The percentage of  $\alpha$  subunits capable of binding BuTx was determined as described in the Materials and Methods section. 3T3-DOJ- $\alpha 3$  cells were solubilized, labeled with  $^{125}\text{I}$ -BuTx, incubated with anti- $\alpha$  antiserum, and precipitated with protein A-Sepharose. The precipitate was counted in a gamma counter to determine the number of moles of toxin-binding sites. The sample was then electrophoresed on an SDS gel, immunoblotted, labeled with  $^{125}\text{I}$ -protein A, treated for fluorography, and exposed to film. The total amount of  $\alpha$  polypeptide was calculated by densitometric scanning of the autoradiogram or by cutting out the gel band and counting it in a gamma counter (as described in Fig. 5). We determined that  $\sim 100\%$  of the  $\alpha$  polypeptides were capable of binding BuTx.

When 3T3-DOJ- $\alpha 3$  cells were solubilized in nonionic detergent buffer solutions and incubated with different concentrations of  $^{125}\text{I}$ -BuTx, we found that the binding was saturable and that isolated  $\alpha$  subunits bound BuTx with a  $K_d$  value of  $\sim 4 \times 10^{-7}$  M. Similar weak toxin binding was observed when *Torpedo*  $\alpha$  subunits were expressed in yeast ( $K_d$ ,  $2 \times 10^{-7}$  M; Sweet et al., 1988). This weak binding to in-

dividual  $\alpha$  subunits that both our laboratory and Hess's observed was similar to the weak binding that others had observed when attempting to bind toxin to *Torpedo* AChR after it had been denatured with SDS (Haggerty and Froehner, 1981). Once denatured in SDS, high affinity toxin-binding activity is not regained even after removal of the SDS. It was not known whether the  $\alpha$  subunits could not refold to their original conformation after denaturation in SDS, or whether other subunits were required for the correct conformation. The fact that neither our laboratory nor Hess's observed any high affinity binding to individual  $\alpha$  subunits, even though they had never been exposed to strong denaturing conditions, suggested that the toxin-binding conformation of the  $\alpha$  subunit was either unstable or unable to form in the absence of the other subunits.

### All-6 Cells

Ltk<sup>-</sup>aprt<sup>-</sup> cells were cotransfected with pSV2- $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  plasmids and a tk gene, cells were put into selective medium, eleven tk<sup>+</sup> transformants were isolated, and grown into stable cell lines as previously described (Claudio et al., 1987). DNA from one of those cell lines, all-6, was subjected to Southern (1975) blot analysis to determine the integrity and copy number of each of the integrated cDNAs. By comparing the sizes of the integrated cDNAs with the starting plasmid DNAs (Fig. 10), it appeared that the majority of the subunit cDNAs integrated intact. A comparison of the intensities of the bands with that of a single copy cDNA (an  $\alpha$  cDNA introduced into 3T3 cells using retrovirus infection) indicated that the copy number for each of the cDNAs was  $\sim 0, 1, 8,$  and  $4$  for  $\alpha, \beta, \gamma,$  and  $\delta$ , respectively. The observations that most of the DNAs integrated correctly and that the



**Figure 10.** DNA blots of the all-6 cell line. Genomic DNAs probed with  $\alpha, \beta, \gamma,$  and  $\delta$  sequences are marked  $\alpha, \beta, \gamma,$  and  $\delta$ ; the plasmid preparations digested with the same restriction enzymes as the genomic DNA are preceded by *p*. Genomic DNA was prepared from a confluent 10-cm dish of cells as described (Maniatis et al., 1982). DNA that would be probed for  $\alpha, \beta,$  and  $\delta$  sequences was digested with Sty I. DNA that would be probed for  $\gamma$  sequences was digested with Pvu II and Eco RI. 5  $\mu\text{g}$  of digested DNA was loaded onto 1% agarose gels and blotted according to Southern (1975). Probes for each of the cDNAs were as follows: a

1,090-bp PvuII-Pst I fragment for  $\alpha$ ; a 700-bp Bgl II fragment for  $\beta$ ; a 1,290-bp Bgl II-Eco RI fragment for  $\gamma$ , and a 450-bp Hind III fragment for  $\delta$ . Each was radiolabeled with  $^{32}\text{P}$  using the multiprime labeling system (Feinberg and Vogelstein, 1983) from Amersham Chemical Co., and had specific activities of  $\sim 10^9$  cpm/ $\mu\text{g}$  of DNA. The film was exposed at  $-70^\circ\text{C}$  with an intensifying screen for 18 h.

copy numbers varied, are consistent with an earlier study where the four *Torpedo* cDNAs and the *aprt* gene were cotransfected into Ltk<sup>-aprt</sup> cells (Claudio, 1987). The aberrantly migrating  $\alpha$  band seen in Fig. 10, lane  $\alpha$ , does not represent a full-length  $\alpha$  cDNA. A comparison of the intensity of this band with single copy  $\alpha$  cDNA indicated that the band was represented at less than one copy per cell. This observation was corroborated by our findings that  $\alpha$  polypeptide was not produced in these cells (Fig. 3, lane 1) and that no <sup>125</sup>I-BuTx binding material was detected either internally or on the surface of all-6 cells (data not shown). We have found that the presence of sodium butyrate (Gorman et al., 1983) in culture medium greatly increased the level of transcription of our integrated pSV2 constructs (Claudio et al., 1987). To analyze for expressed subunit polypeptides in all-6 cells, we grew a 10-cm dish of cells to confluency, and then incubated the cells in medium supplemented with 10 mM sodium butyrate for 2 d. Cells were metabolically labeled with [<sup>3</sup>H]leucine, solubilized, immunoprecipitated with a mixture of anti-*Torpedo*  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  antisera, and electrophoresed on SDS-polyacrylamide gels. As shown in Fig. 3, lane 1, only  $\beta$ ,  $\gamma$ , and  $\delta$  polypeptides were observed in all-6 cells, which is consistent with the Southern blot analysis of this line in which no  $\alpha$  cDNAs appeared to have integrated correctly. It can also be seen that there is at least fivefold more  $\delta$  subunit produced than  $\gamma$ , even though there were twice as many copies of the  $\gamma$  cDNA integrated. The discrepancy between copy number and protein expression is due to a lower-than-expected level of  $\gamma$  protein expression, rather than to a higher-than-expected level of  $\delta$  expression (compare Fig. 1 with Fig. 3 b in Claudio et al., 1987). The reason for the poor  $\gamma$  expression has not been entirely determined, but appears to be caused, at least in part, by splicing problems. Consistent with this hypothesis is the observation that we have encountered repeated difficulty with the stable expression of this subunit in mammalian cells, yet have no difficulty expressing functional AChRs after microinjection into oocytes of the in vitro-transcribed mRNA (Claudio, 1987).

### Functional AChRs

The all-6 cell line only expressed three of the four subunits and did not produce functional AChRs. We wanted to introduce the missing subunit into this line to see if the deficiency could be corrected, and if functional AChRs could be formed. If successful, then this approach could be an effective strategy for introducing a series of mutations into a single subunit of a multisubunit complex. Several methods could have been used to introduce the missing subunit into all-6 cells. Because we had integrated three of the cDNAs into Ltk<sup>-aprt</sup> cells using tk as the selectable marker, the fourth subunit could have been introduced by cotransfection using *aprt* as the selectable marker. Alternatively, cotransfection using a dominant selectable marker such as neo could have been used. We chose instead to use one of our retroviral  $\alpha$  recombinants and viral infection as the method of gene transfer because of the efficiency with which DNA and RNA can be introduced into cells using viruses.

When we attempted to infect all-6 cells with supernatant collected from a stable pDOJ- $\alpha$ - $\phi$ -2 producer cell line (titer  $\sim 2 \times 10^5$  cfu/ml), no G418-resistant colonies were formed. Several cell lines are productively infected by retroviruses

that render them resistant to superinfection (Weiss et al., 1984); however, pretreatment with tunicamycin may allow them to become transiently susceptible to viral infection (Rein et al., 1982; Steck and Rubin, 1966). All-6 cells were treated for 18 h with concentrations of tunicamycin ranging from 0 to 0.3  $\mu$ g/ml and then incubated with pDOJ- $\alpha$ - $\phi$ -2 culture supernatants. The greatest number of colonies (giving an effective virus titer of  $\sim 10^3$  cfu/ml) was obtained using 0.3  $\mu$ g/ml tunicamycin. We found that our mouse muscle BC<sub>3</sub>H-1 cells were also highly resistant to infection by retroviruses. To determine whether these cells were already productively infected by a retrovirus or whether they lacked a virus receptor, we treated the cells for 18 h with concentrations of tunicamycin ranging from 0 to 0.3  $\mu$ g/ml, and then incubated them with pDOJ- $\alpha$ - $\phi$ -2 culture supernatants. We determined that an 18-h pretreatment with only 0.05  $\mu$ g/ml tunicamycin was sufficient to allow virus entry. Thus, both Ltk<sup>-aprt</sup> and BC<sub>3</sub>H-1 cells appear to contain an endogenous retrovirus that renders them extremely resistant to superinfection by our packaged retroviral recombinants. This resistance can be overcome, however, in both cell types by pretreatment with the glycosylation inhibitor, tunicamycin.

All-6 cells were treated with tunicamycin, incubated with pDOJ- $\alpha$ - $\phi$ -2 culture supernatants, and put into G418 selection. Seven colonies were isolated, grown into separate cell lines, metabolically labeled with [<sup>3</sup>H]leucine, and immunoprecipitated with anti- $\alpha$  antiserum to determine which lines were producing the highest level of  $\alpha$  polypeptide. We determined that each of the seven lines (all-6- $\alpha$ 1, all-6- $\alpha$ 2, etc.) produced  $\alpha$  polypeptides that were recognized by subunit-specific antisera and comigrated on SDS gels with  $\alpha$  subunits isolated from electroplaque tissue (two lines are shown in Fig. 3, lanes 2 and 3).

As mentioned earlier, the expression of functional *Torpedo* AChRs in mammalian cells is acutely temperature sensitive. At 37°C, we can neither form all-*Torpedo* AChRs in fibroblasts nor can we form hybrid *Torpedo*-rat AChRs in our L6- $\alpha$  lines. At 28°C, however, both types of receptors can

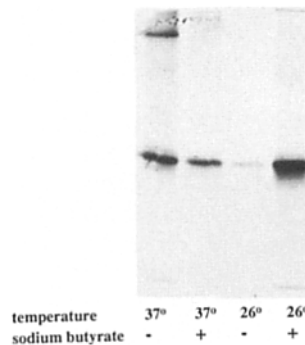


Figure 11. The effect of sodium butyrate and temperature on protein expressed from an MLV LTR. Four 10-cm dishes of 3T3-DOJ- $\alpha$ 3 cells were grown at 37°C until confluent. Two dishes were then supplemented with sodium butyrate and two were not. One dish with normal medium and one containing supplemented medium were grown at 28°C for 2 d while the other two dishes (treated and untreated)

remained at 37°C for 2 d. Each dish of cells was then starved for leucine for 10 min, labeled for 20 min with 400  $\mu$ Ci of [<sup>3</sup>H]leucine, solubilized, immunoprecipitated with anti- $\alpha$  antiserum, electrophoresed on SDS-polyacrylamide gels, and treated for fluorography. The film was exposed at -70°C with an intensifying screen for 30 h. (Lane 1) Cells grown for 2 d at 37°C; (lane 2) cells grown for 2 d at 37°C in the presence of 10 mM sodium butyrate; (lane 3) cells grown for 2 d at 28°C; and (lane 4) cells grown for 2 d at 28°C in the presence of 10 mM sodium butyrate.

be expressed. Temperature is therefore critical for expression of *Torpedo* AChRs in mammalian cells. Also critical to the expression of *Torpedo* AChRs in cell lines with our pSV2 constructs, is the inclusion in the medium of 10 mM sodium butyrate which significantly increases the level of transcription (Claudio et al., 1987). Therefore, to test for expression of functional AChRs in the all-6- $\alpha$  cell lines (containing  $\beta$ ,  $\gamma$ , and  $\delta$  cDNAs under the control of an SV-40 promoter, and an  $\alpha$  cDNA under the control of the MLV LTR) we grew the cells at 28°C in the presence of sodium butyrate for 5–7 d before assaying for cell surface AChRs. To determine the effect of sodium butyrate on the MLV LTR, we incubated dishes of 3T3-DOJ- $\alpha$ 3 cells at 37 and 28°C in the presence or absence of 10 mM sodium butyrate for 2 d. The cells were metabolically labeled for 20 min;  $\alpha$  was immunoprecipitated, electrophoresed on SDS gels, and autoradiographed; and the bands were scanned with a densitometer. At 37°C, the amount of  $\alpha$  polypeptide synthesized in 20 min in the presence of sodium butyrate was actually less (25–50%) than the amount synthesized in untreated cells (Fig. 11, lanes 1 and 2). In contrast, at 28°C, there was  $\sim$ 10–20-fold more polypeptide synthesized in sodium butyrate-treated cells compared with untreated cells (Fig. 11, lanes 3 and 4). Thus, for both types of expression vectors, sodium butyrate had a pronounced effect when the cells were grown at 28°C.

Cell surface expression was tested with  $^{125}\text{I}$ -BuTx. Although BuTx binds specifically to the  $\alpha$  subunits of the AChR, the binding affinity for isolated  $\alpha$  subunits is weak ( $K_d = 4 \times 10^{-7}$  M) whereas the affinity for  $\alpha$  subunits that are part of the  $\alpha_2\beta\gamma\delta$  pentameric complex is very strong ( $K_d = 7.8 \times 10^{-11}$  M). We assayed for cell surface AChRs by first incubating cells with  $^{125}\text{I}$ -BuTx for 140 min, removing unbound toxin by washing, and then counting the cells in a gamma counter. Because the assays were performed using nanomolar concentrations of toxin, such that low affinity toxin binding would not be detected, and because individual  $\alpha$  subunits are not expressed on the surface in this system and do not bind toxin with high affinity, any toxin-binding activity that we observed on the surface of all-6- $\alpha$  cells could be attributed to the presence of AChR complexes. We assayed the cell lines for the ability to bind  $^{125}\text{I}$ -BuTx and determined that all seven cell lines were capable of binding toxin. The number of cell surface receptors expressed per cell was  $\sim$ 550, 360, 120, 75, 95, 80, and 150 for all-6- $\alpha$ 1 through all-6- $\alpha$ 7 cells, respectively.

The best AChR-expressing line (all-6- $\alpha$ 1) was further tested to determine if the expressed AChRs were also functional; i.e., responded to their natural ligand ACh by opening channels permeable to cations. If the AChRs were evenly distributed over the surface of the fibroblast, we would expect a surface density of  $\sim$ 1 AChR per  $\mu\text{m}^2$  and  $\sim$ 10 AChRs per outside-out patch (Hamill et al., 1981) of membrane. Although this is a low surface density of channels, we were able to detect single-channel activity, and recordings from all-6- $\alpha$ 1 cells showed ACh-induced single-channel currents having properties similar to single *Torpedo* AChR currents previously observed. Currents were recorded before and after the application of ACh to an outside-out patch of membranes. Channel activity increased and then desensitized in response to "puffs" of ACh (Fig. 12 A). Amplitude histograms (Fig. 12 B) showed a single class of events with an inward current amplitude of  $5.3 \pm 0.2$  pA and a mean channel open time

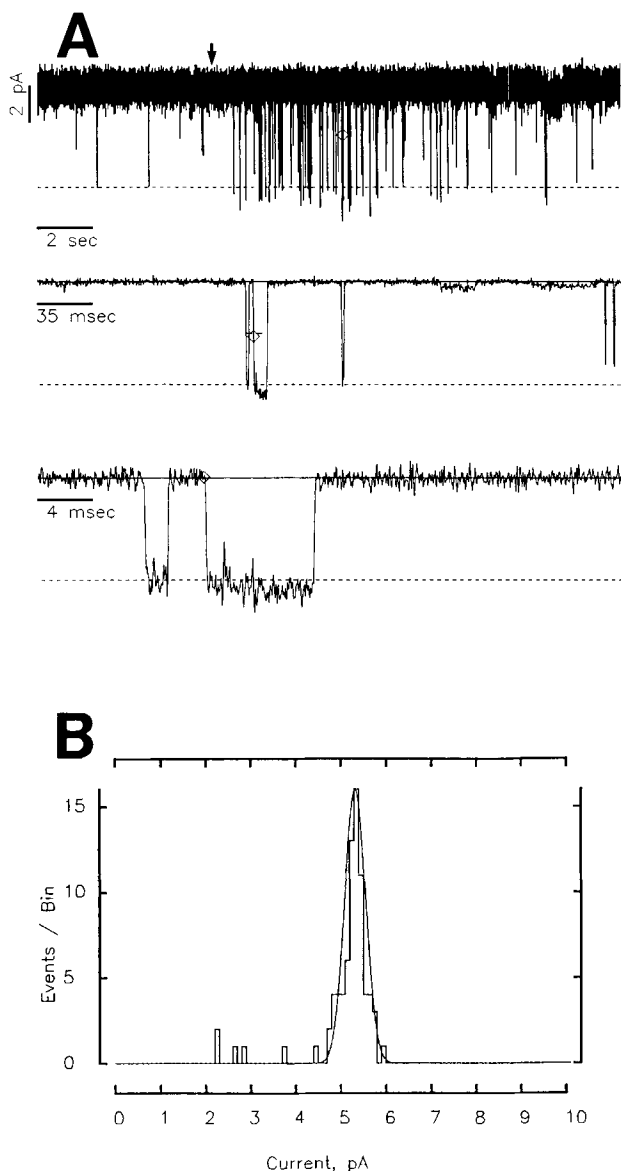
of  $\sim$ 0.5 ms at a membrane potential of  $-60$  mV and 17°C. The single-channel properties of the *Torpedo* AChRs expressed in all-6- $\alpha$ 1 cells were similar to those of this same AChR expressed in fibroblasts when the cDNAs were introduced simultaneously into the same cell (Claudio et al., 1987) and to those expressed in *Xenopus* oocytes (Claudio, 1987; Claudio et al., 1988).

## Discussion

Two gene transfer methods were used to introduce AChR subunit cDNAs stably into cultured cell lines: DNA-mediated gene transfer using the calcium phosphate precipitation method and viral infection using packaged retroviral recombinants. Transfection was most useful for quickly introducing multiple cDNA constructions into the same cell and for introducing material into some cell types that were refractory to viral infection. Viral infection was most useful for introducing a single cDNA construction into many cell types and for introducing material into some cell types that did not take up DNA well by transfection. Using one or both of these gene transfer methods, we have introduced combinations of subunit cDNAs or each subunit cDNA individually into mouse fibroblast NIH3T3 cells, mouse fibroblast Ltk<sup>-</sup>aprt<sup>-</sup> cells, mouse C2 muscle cells, rat L6 muscle cells, and mouse BC<sub>3</sub>H-1 muscle cells.

Each of the expressed subunits was recognized by subunit-specific antisera, and all, except  $\gamma$ , migrated on SDS-polyacrylamide gels with the proper electrophoretic mobility. This subunit was recognized by polyclonal antisera but its mobility was  $\sim$ 3 kD smaller than expected. The altered mobility is probably due to small differences in posttranslational modifications between *Torpedo* electrocytes and other expression systems. Experiments with tunicamycin and endoglycosidase H indicated that  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  had incorporated one, one, two, and three units of oligosaccharide, respectively, consistent with values predicted from the carbohydrate mass of each subunit (Nomoto et al., 1986). The level of expression of the different subunits varied among the cell lines whether the lines were established by transfection or infection, with the highest level of expression being  $\sim$ 270,000 molecules per cell. In general, we observed higher levels of expression when the cDNA was driven by an LTR rather than by an SV-40 promoter. Half-life studies were conducted on the cell lines expressing individual AChR subunits. We found that  $\alpha$ ,  $\gamma$ , and  $\delta$  each had the same short half-life of  $\sim$ 43 min at 37°C whether expressed in fibroblasts, undifferentiated muscle cells, or differentiated muscle cells. In contrast, the  $\beta$  subunit had an extremely short half-life of only  $\sim$ 12 min at 37°C. When the cell lines were grown at 28°C, we found that although all the subunit half-lives increased, the  $\beta$  subunit was the most profoundly affected. Further investigation of the short  $\beta$  half-life and acute temperature sensitivity is continuing. It is intriguing to postulate that  $\beta$  might play some critical regulatory role in the process of subunit assembly and that these observed unique properties of  $\beta$  might be related to this role.

In cell lines expressing only one subunit type, we were unable to detect any subunits on the surface of fibroblasts at 37 or 28°C. When all four subunits were present, however, and the cells were grown at temperatures lower than 37°C, we could detect functional *Torpedo* AChRs on the surface of



**Figure 12.** ACh-induced single-channel currents from all-6- $\alpha$ 1 cells. (A) Recordings from an outside-out patch at  $-60$  mV and  $17^\circ\text{C}$ . In the top trace the frequency of the single-channel current events increased in response to a "puff" (arrow) of  $30\ \mu\text{M}$  ACh pressure delivered from a pipette ( $\sim 1\text{-}\mu\text{m}$  tip)  $\sim 40\ \mu\text{m}$  away from the patch. In the middle of the top trace (diamond), the record (downward spikes) is expanded and shown in the middle trace. The record in the middle trace (diamond) is further expanded and shown in the bottom trace to better display the single-channel current events. The bath solution was  $145\ \text{mM}$  KCl,  $1\ \text{mM}$   $\text{CaCl}_2$ ,  $25\ \text{mM}$  Hepes, pH 7.4; the pipette solution (in contact with the inside of the membrane) was  $80\ \text{mM}$  KF,  $20\ \text{mM}$  KCl,  $2\ \text{mM}$   $\text{MgCl}_2$ ,  $40\ \text{mM}$  KAspartate,  $1\ \text{mM}$  EGTA,  $10\ \text{mM}$  Hepes, pH 7.4. (B) An amplitude histogram obtained from events longer than  $0.3\ \text{ms}$  at  $-60\ \text{mV}$ . The amplitudes cluster into a single peak at  $5.3 \pm 0.2\ \text{pA}$  (SD) fit by a single Gaussian curve represented by the continuous line.

fibroblasts. These cell surface AChRs have been analyzed in terms of subunit stoichiometry and found to be assembled into proper  $\alpha_2\beta\gamma\delta$  pentamers (Hartman et al., 1989).  $\alpha$  subunits assembled into  $\alpha_2\beta\gamma\delta$  pentamers have a  $K_d$  for BuTx of  $7.8 \times 10^{-11}\ \text{M}$  (Claudio et al., 1987). In contrast, al-

though essentially 100% of unassembled  $\alpha$  subunits are capable of binding BuTx, they have a  $K_d$  for BuTx of only  $\sim 4 \times 10^{-7}\ \text{M}$ . It would appear that for *Torpedo*  $\alpha$  subunits, high affinity BuTx binding cannot be achieved with isolated  $\alpha$  subunits. Whether subunit combinations with  $\alpha$  other than proper pentamers can also produce high affinity BuTx binding has not yet been determined.

One of the advantages of working with cDNAs is that specific mutations can be made in the molecules fairly easily, and the gene products can then be expressed in a system where the effects of those mutations on structure or function can be monitored. Because of the time involved for establishing stable cell lines ( $\sim 2$  months) compared with several transient expression systems ( $\sim 2$  days), the stable system would not be a practical method for screening potentially interesting mutations. Using a two-step transfection or a transfection-infection protocol, one might more readily combine stable expression of heterologous multisubunit protein complexes with site-directed mutagenesis. We recently demonstrated that the cotransformation efficiency of introducing all four subunit cDNAs plus a selectable marker gene was 80% (Claudio, 1987). Although this method is efficient, the copy number of the integrated subunit cDNAs varies as does the level of expression of each of the subunits, necessitating the analysis of several cell lines to obtain one with the desired level of expression. A significant amount of screening would thus be necessary when studying the effects of various mutations in any one of the subunits. One method for simplifying this problem would be to first establish and characterize a cell line that expressed just three of the four subunits. The characterized cell line would then serve as the recipient cell for introducing the fourth wild-type (or mutant) cDNA. This approach alleviates the necessity of characterizing four gene products with each transduction and establishes a more uniform system (three of the four subunits are identical in all cell lines) for interpreting the effects of a mutant gene product. We have demonstrated in this paper that when the  $\alpha$  subunit cDNA is introduced into one of our cell lines that only expresses  $\beta$ ,  $\gamma$ , and  $\delta$  subunits, fully functional AChR complexes are subsequently produced.

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