$GABA_A$ Receptor Subunit mRNA Profiling: Potential and Progress

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INTRODUCTION

$GABA_A$ Receptors and Function

Historically ethanol's intoxicating effect on brain cells has been considered nonspecific. Recently specific neurotransmitter receptors have been implicated as sites for ethanol interaction. One such complex is the gamma-aminobutyric acid (GABA) receptor. GABA, the major inhibitory neurotransmitter in the vertebrate brain, binds and activates a chloride ion channel receptor complex (9). This receptor ($GABA_A$) shows allosteric potentiation by two major classes of drugs, benzodiazepines and barbituates, and is competitively inhibited by bicuculline (7). Protein purification and gene cloning have shown that multiple subunits compose the $GABA_A$ receptor (9, 10) . Expression of one or more $GABA_A$ receptor subunits in oocytes, coupled with electrophysiological studies, has revealed the complex nature of the receptor, and *in vivo* pharmacological studies have shown ethanol to increase the activity of GABA receptors (3, 12) Sixteen possible subunits make up the pentameric receptor complex, one or more of which may be essential for the expression of ethanol sensitivity. Assembly from this myriad of subunit choices varies in different cells and areas of the central nervous system (11).

Expression of whole brain mouse mRNA in frog oocytes has suggested that the $gamma_{2L}$ subunit of the $GABA_A$ receptor may be necessary for ethanol sensitivity in these receptors (12). However, the role of this subunit or others in the formation of $GABA_A$ receptors in adult mammalian brain tissue has not been established (4). It is becoming increasingly clear that $GABA_A$ receptor subunit composition and function can change

under a variety of conditions. During the embryonic and postnatal developmental periods of the brain, $GABA_A$ receptors differ dramatically in function. This results from differential expression of various receptor subunits (13). In addition, individual adult neurons likely differ in the specific receptor subunit genes expressed as compared to exogenous mRNA expression in oocytes. Clearly the stage of development and cellular environment play an important role in $GABA_A$ receptor function and subunit composition; therefore primary cultures of immature CNS neurons may not be the best model for $GABA_A$ receptor studies. It is therefore unclear whether the enhancement of $GABA_A$ receptor function by ethanol in the adult mammalian brain shows the same subunit specificity seen in oocytes or cultured neurons.

aRNA Amplification Theory

Receptor subunit composition can be examined at the mRNA (gene expression) level. Profiling gene expression in acutely isolated neurons from adult animals offers a potentially powerful approach to determining $GABA_A$ receptor subunit composition and sensitivity. Recently antisense RNA (aRNA) has been amplified from total cellular mRNA present in single cells (2). First, single live neurons are isolated from the adult rat brains. High-resistance (giga Ohm) patch-clamp recordings of whole cell currents may be obtained from the cells analyzed for mRNA expression. Following electrophysiology, the cell is aspirated into the patch pipette which contains avian myeloblastoma virus reverse transcriptase, nucleotides, oligo (dT)-T7 promoter containing primer, and buffer for cDNA synthesis. The resulting cDNA's are then processed to double stranded blunt ended molecules; at this stage the cDNA population should represent the relative quantity

of distinct mRNA species present in the cell at the time of aspiration. Linear amplification is accomplished using T7 RNA polymerase to produce single stranded antisense RNA. A second round of amplification can yield a million fold increase of the original message (2). Reverse Northern hybridizations are then used to assay the amplification product for expression of specific genes.

Potential of aRNA Amplification

The polymerase chain reaction is becoming an increasingly common method for expression analysis in single cells (6). Amplification of antisense RNA, however, offers advantages over PCR. Oligonucleotide primers used in PCR often prime DNA synthesis with differing efficiency (8). Differential primer efficiency is then exponentially amplified throughout thermal cycling. Accordingly, reactions in which multiple genes are assayed are difficult to quantify. Furthermore, total primer sets in one reaction rarely exceeds three, limiting the number of genes which can be assayed in a single cell. Antisense RNA alleviates these difficulties by linearly amplifying total cellular mRNA. This yields a large product from which expression of multiple genes may be quantitated.

EQUIPPING THE LABORATORY

The laboratory of Gerald Frye has primarily pursued electrophysiology as a method to study $GABA_A$ receptors. Therefore molecular biology equipment and reagents were

required to develop aRNA amplification, as well as technical support in this area. Much of the Fall semester was spent acquiring equipment and reagents for the following: growth and maintenance of bacteria, transformation of bacteria, DNA purification and storage, agarose gel electrophoresis, synthesis and processing of DNA, and restriction endonuclease digestion of plasmid DNA. Additional technical support was obtained from Gibco BRL, Promega, Scott Vacha of the Richard Finnell Laboratory, Tuan Pham of the University of Texas Medical Branch, and other laboratories at Texas A&M Health Science Center.

MATERIALS AND METHODS

Development of Receptor Subunit cDNA Probes

Before attempting aRNA amplification in single cells, receptor subunit cDNA clones must be obtained in sufficient quantity for Reverse Northern hybridization analysis. Dr. Frye requested small quantities of the various $GABA_A$ receptor subunit cDNA clones from several laboratories around the country late in the summer of 1994. Many labs did not respond, but mouse $gamma_{2L}$ and $gamma_{2S}$ clones were received from the David R. Burt laboratory at the University of Maryland in October 1994. Each came ligated at the EcoRI sites of pGEM7Z+ (Promega) in microgram quantities as ethanol precipitates. Each was dissolved and stored in 100ul TBE buffer.

Precompetent DH5alpha *E.coli* cells (Gibco BRL) were transformed with 5ul of the $gamma_{2L}$ and $gamma_{2S}$ containing plasmids, along with 5ul of control pUC19 vector. Following addition of the plasmid DNA, cells were incubated on ice for 30 minutes then heat shocked for 30 seconds at 37°C. Another 2 minutes on ice was followed by addition of 0.95ml of S.O.C. medium (Gibco BRL), and shaking at 150 rpm at 37°C for one hour. Cells were then diluted ten fold and plated on Luria Bertani agar media with and without 50ug/ml ampicillin (Boehringer Mannheim). Because the exact concentration of the $gamma_{2L}$ and $gamma_{2S}$ plasmids was unknown the remaining cells were pelleted and resuspended in 100ul of medium, then plated. In addition, cells to which no DNA was added were plated as a negative control. After overnight incubation at 37°C plates were examined (See figure 1). Both negative and positive controls grew as expected, and recombinant colonies appeared on the ampicillin containing plates. One colony from each ($gamma_{2S}$ and $gamma_{2L}$) was taken and stabbed into 2ml of 30% glycerol in LB broth and placed at -80°C as a freezer stocks.

Promega Wizard Miniprep kits were used to isolate plasmid from the transformed *E.coli* strains. Overnight cultures grown in 3ml ampicillin (50ug/ml) containing LB broth were used in the minipreps. Eluant from the miniprep was digested with EcoRI to determine the identity of the plasmid. Each digestion included 20ul miniprep eluant, 5 units EcoRI (Gibco BRL), 2.1ul reaction buffer (Gibco BRL), and was incubated at 37°C. After one hour the reaction was stopped by addition of 2ul 0.5M EDTA. Dilutions of the digestion product were run in parallel with a molecular weight standard (1-4.4 kb Ladder, Bio-Rad) on a 0.7% agarose gel stained with ethidium bromide (See Figure 2a). Based

FIGURE 1

E.coli DH5alpha Transformation with $GABA_A$ receptor $gamma_{2L}$ and $gamma_{2S}$ subunit cDNA

PLATE NUMBER	DNA ADDED	ANTIBIOTIC	GROWTH
1	none	amp	none
2	none	none	lawn
3	pUC19	amp	70 colonies
4	pUC19	none	lawn
5	$gamma_{2L}$	amp	140 colonies
6	$gamma_{2L}$	none	lawn
7	$gamma_{2S}$	amp	100 colonies
8	$gamma_{2S}$	none	lawn

LEGEND:

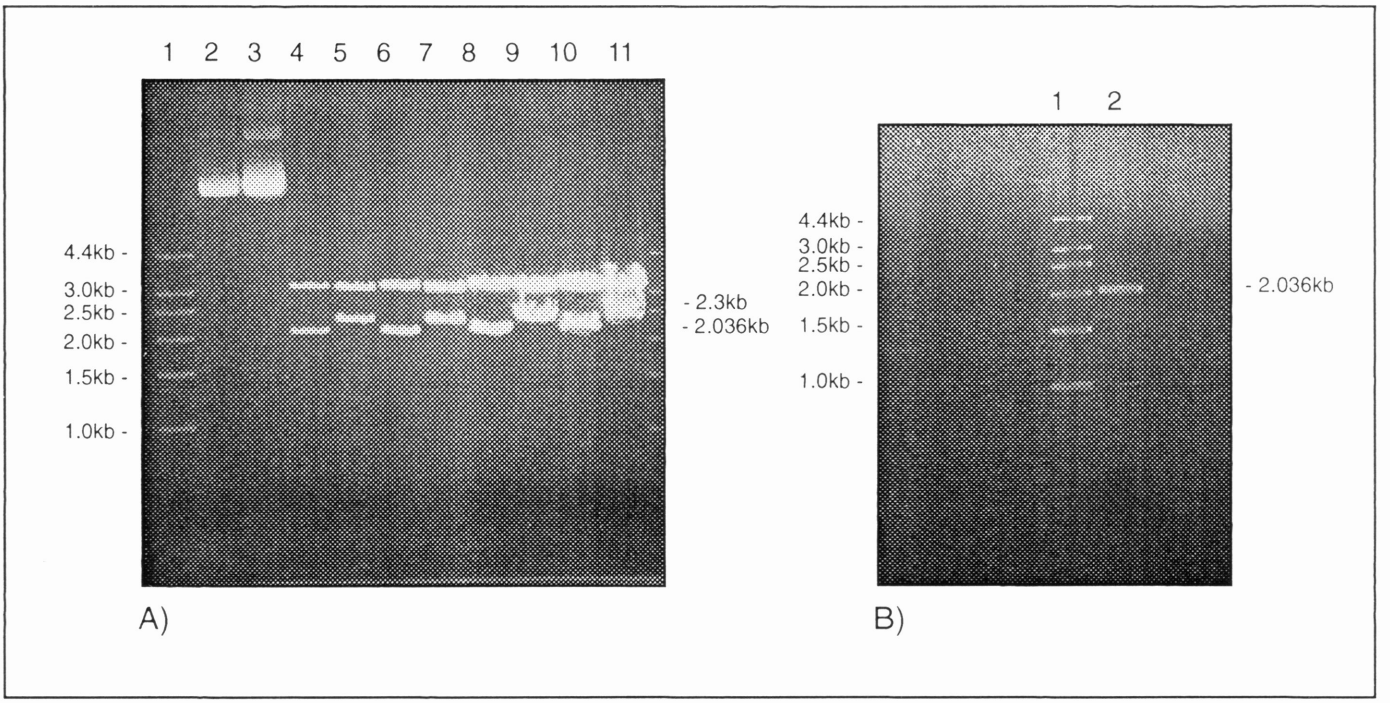
E.coli DH5alpha cells (50 ul) were transformed with 5 ul of DNA from pUC19 plasmid, $GABA_A$ receptor $gamma_{2L}$,, and $gamma_{2S}$ subunit cDNA containing plasmid according to standard protocol (see text). Following transformation, cells were plated on LB plates with and without ampicillin. Plates were incubated overnight at 37°C and examined for growth. Selected recombinant colonies were used to create freezer stocks.

upon the migration and size of the fragments, it was concluded that the transformation was successful and that the frozen *E.coli* strains carried the correct $GABA_A$ receptor subunit containing plasmids.

Plasmid isolation (Promega Wizard Maxiprep) was performed from 500 ml cultures of bacteria inoculated with 5ml from a previous overnight culture. Upon running an agarose gel of EcoRI digested product, however, the bands appeared irregular, smeared, and distorted. At the time it was thought that this was the result of using centrifuge bottles which were too small to hold the entire solutions; this resulted in extremely sloppy adherence to procedure. At the beginning of the Spring semester the procedure was repeated with proper equipment and 300 ml overnight cultures of bacteria. The $GABA_A$ $gamma_{2S}$ subunit appeared free of the contaminant, yet the $gamma_{2L}$ subunit remained contaminated. Several possibilities exist for the presence of contaminant. First, RNA could have precipitated out along with the plasmid in the isopropanol precipitation. Alternatively, chromosomal DNA may have eluted if the DNA binding resin was not allowed to dry completely. An updated protocol was obtained from Promega and used with success to isolate the $gamma_{2L}$ containing plasmid from the bacteria.

Twenty microliters from an EcoRI digest of $gamma_{2S}$ plasmid was diluted to 50ul and run on a 0.7% agarose gel to obtain a stock of the excised subunit for use in Reverse Northern analysis. The lane containing the $gamma_{2S}$ subunit was cut from the remainder of the gel and sliced into seven pieces to allow extraction. A gel extraction kit (QiaGen) was used for each of the seven pieces and the eluants were pooled. Five microliters of the pooled eluant was run on a 0.7% agarose gel with 1-4.4 kb ladder molecular weight

FIGURE 2
ISOLATION AND PURIFICATION OF GAMMA$_{2s}$ SUBUNIT cDNA



LEGEND:

A) Isolation of GABA$_A$ gamma$_{2S}$ and gamma$_{2L}$ receptor subunit cDNA from plasmid carrying *E.coli* strains. Lane one contains 1-4.4 kb ladder molecular weight standards. Lanes 2 and 3 contain uncut plasmid DNA carrying the 2S (lane 2) and 2L (lane 3) subunits. Lane 4 was loaded with 1 ul of EcoRI digested plasmid carrying the 2S subunit, and migration is consistent with the published 2.036 kb size of the cDNA. Lane 5 contains 1 ul of EcoRI digested 2L subunit containing plasmid, and migration matches the published 2.3 kb size. Lanes 6-11 duplicate lanes 4 and 5 with increasing volumes of digest which show characteristic overloading of the gel.

B) Purification of the gamma$_{2s}$ receptor subunit cDNA for use as a probe in reverse Northern analysis. Lane one was loaded with 1-4.4 kb molecular weight standards. Lane 2 contains 20 ul of eluant from a preparatory gel extraction (QiaGen) of the gamma$_{2s}$ cDNA.

markers to verify the purity of the $GABA_A$ $gamma_{2S}$ subunit cDNA (See figure 2b). Possessing a purified $GABA_A$ receptor subunit cDNA probe allowed us to concentrate on adapting patch-clamp electrophysiology for cell harvest.

Modification of Eberwine *et al* Protocol

The original technique and protocol were obtained from Eberwine *et al* with assistance from the Richard Finnell Laboratory at Texas A&M, a collaborator in the Eberwine group. These protocols provided a starting point, however specific details have been modified appropriately. The original protocol used a patch-clamp pipette filling solution that included reverse transcriptase, deoxynucleotide tripohsphates, and oligonucleotide primer. This allows access by dialysis of enzyme and primer to the intact cell therefore initiating cDNA synthesis after breaking into the cell using the whole cell recording paradigm. However, addition of the reverse transcriptase mixture alters the properties of the pipette solution making the necessary high resistance membrane seal extremely difficult to obtain. Upon removal of the reverse transcriptase mixture from the pipette filling solution patching was achieved with relative ease. Additionally, the pH of the pipette filling solution (7.2) is not optimal for AMV reverse transcriptase efficiency (pH optimum, 8.3). Length of cDNA is greatly reduced when reaction pH differs as little as 0.2 units from the optimum (8). AMV reverse transcription, therefore, is greatly reduced in the conditions of the pipette filling solution. This activity is especially critical when attempting cDNA production from single cells. Removal of the reverse transcriptase mix from the pipette filling solution therefore increases ease of cell patching.

In addition reverse transcriptase efficiency is increased by keeping the reaction pH closer to the enzyme optimum.

As mentioned above, reverse transcriptase activity is crucial for cDNA production in single cells. AMV reverse transcriptase carries a powerful RNAase H activity in addition to the polymerase activity. At the beginning of the reaction, RNAase H also binds the hybrids formed between the primer and mRNA. Therefore a competition exists between degradation of the template mRNA and synthesis of complementary cDNA. This results in lower yield and reduced length of cDNA (8). Another form of reverse transcriptase, Moloney murine leukemia virus (Mo-MLV), which exhibits a reduced RNAase H activity, is also comercially available. Mo-MLV reverse transcriptase commonly achieves greater yields than the avian enzyme, and is preferentially used in cloning (8). We therefore chose to replace the avian enzyme with the Moloney enzyme. Also, the Moloney reverse transcriptase achieves maximum efficiency at a pH of 7.6, much closer to the pH of the pipette filling solution. This should increase the yield of the reaction, allowing maximum cDNA production from the original message in the cell at the time of aspiration.

Patch-Clamp Electrophysiology

In the interest of time, we felt emphasis should be placed on achieving amplification from single cells before obtaining complete drug response data. Amplification had not been attempted in the Frye lab, and drug response data is routinely obtained. Therefore the major technical hurdle was amplification, and we felt it should be

overcome before continuing work involving pharmacologically characterizing cells. Standard patch-clamp technique was used to isolate and harvest cells used in the amplification (4, 5).

Following removal, the rat brain was cooled on iced cutting solution containing (mM): NaCl 118, KCl 3 $MgCl_2$ 6, $CaCl_2$ 0.5, HEPES 5, dextrose, 11, $NaHCO_3$ 25. This solution was continousily bubbled with 95% O_2/5% CO_2 during cutting. Coronal slices from the forebrain were obtained using a Vibratome 1000 (Polysciences Inc.). Medial septum and the nucleus of the diagonal band were microdissected from each slice and incubated in trypsin (0.7mg/ml, Sigma) for 60 minutes at 35°C. Gentle trituration through fire polished pipettes in Dulbecco's Modified Eagle Medium (Gibco BRL) isolated single cells. Cells were then dispersed on a glass cover slip coated with Alcian blue (placed in a recording chamber on the stage of a Zeiss Aviovert 35) and allowed to attach for 2.5 minutes. The recording chamber was then continually perfused with extracellular bath solution prepared from diethylpyrocarbonate (DEPC) treated water to eliminate ribonuclease (RNAse) activity. Extracellular bath solution contained (mM): NaCl 120, Kcl 3, $MgCl_2$ 2, $BaCl_2$ 2, TEA-Cl 10, HEPES 10, dextrose 10, pH 7.4, 310 mOsm (4).

Baked pipettes (200°C, 2 hr.) were pulled from 1.5 o.d. glass capillary tubing (no. 7052, Garner Glass) and filled with (mM): CsCl 110-130, EGTA 10, $MgCl_2$ 2, HEPES 10, Mg-ATP 4, GTP 0.1, pH 7.2, 290 mOsm prepared in DEPC treated water (4). Pipettes are commonly polished by heating the extreme tip until it begins to close. This smoothes the surface, allowing an easier and higher resistance seal with the cell's plasma

membrane. However, the aggressive polidhing leading to significantly decreased tip size makes cell aspiration difficult to impossible. The baking process itself achieves minimal polishing of the tip, and in several cases this was sufficient for patching. In many cases, however, it was necessary to polish the tips in order to effectively seal the cells. Once a seal had been established with a minimal leak (1-300 pA) a negative pressure was applied to the pipette through an attachable mouthpiece, and the cell contents were aspirated. Seals were maintained through the majority of cell harvest, as seen visually and electrically (Axopatch 200 amplifier, and pCLAMP software, Axon Instr.).

Cell Harvest and cDNA Synthesis

Following cell aspiration, the pipette was removed from the Axopatch amplifier head stage adapter. The tip was then broken into a microfuge tube containing 3.5 ul reverse transcriptase mixture. After addition of the harvested cell (6.5 ul usually obtained) final concentrations of the resulting reverse transcriptase mix were: oligo(dT)-T7 primer 10ng/ul (Midland Reagent), dithioreitol 10mM (Gibco BRL), the four dNTP's 0.5mM each (Gibco BRL), 20U RNAsin (Promega), and 100U Mo-MLV reverse transcriptase (Gibco BRL) (6). Negative controls of 8.0ul pipette filling solution were added to the same volume of RT mix. In addition a single tissue slice was sonicated thoroughly in 20 ul sterile water. Eight microliters of the resulting solution were added to 3.5 ul of RT mix as a positive control. Tubes were then incubated for one hour at 37°C. Following incubation, the mixture was phenol: chloroform: isoamyl extracted and precipitated in ethanol and sodium acetate (0.3M). Drying the pellets proved extremely difficult for this

and all subsequent precipitations. Liquid and visible dried precipitate remained after air drying and lyophilization. Pellets were resuspended in 20ul of freshly made 0.5N NaOH/ 0.1%SDS and 20ul 1M Tris-HCl (pH 8.0). This solution was again phenol: chloroform: isoamyl extracted and precipitated in ethanol/sodium acetate; the resulting pellet was resuspended in 10ul sterile water.

Second strand synthesis was accomplished using T4 DNA polymerase and Klenow enzyme mix containing (final concentration): Tris-HCl 5.9 mM, $MgCl_2$ 1.2 mM, NaCl 5.9mM, the four dNTP's 0.6 mM each (Gibco BRL), T4 DNA Polymerase 0.06 U/ul (Boehringer Mannheim), and Klenow 0.12 U/ul (Boehringer Mannheim). Following overnight incubation at room temperature, the solution was heated at 65°C for 3 minutes, then placed on ice. S1 Nuclease was then used to nick the single-stranded loop formed during self-priming. The reaction mixture contained: NaCl 0.2 M, sodium acetate 50 mM, $ZnSO_4$ 1 mM, and 1 unit S1 Nuclease. After 3 minutes incubation at 37°C, the solution was phenol: chloroform: isoamyl extracted and ethanol precipitated (as above). The resulting pellet was resuspended in 4.5 ul sterile water, to which dNTP's (approx. 0.6 mM each), T4 DNA polymerase (1 unit), and T4 reaction buffer (Boehringer Mannheim) were added for blunt ending. This was incubated at 37°C for 20 minutes. A final phenol: chloroform: isoamyl extraction and ethanol precipitation was performed. The pellet was resuspended in 5 ul sterile water, and placed on floating 0.025 um filters (Millipore, VS type). Samples were dialized for three hours and placed into microfuge tubes to be taken to the Richard Finnell laboratory for aRNA amplification.

Antisense RNA Amplification

Five microliters of each drop-dialyzed solution obtained from the above reactions was added to 5 ul 5X T7 buffer, 1 ul CTP (100 um), 1 ul UTP, GTP, and ATP (10 mM each), 1 ul 0.1 M DTT, 7 ul sterile water, 1 ul RNAsin (20 U/ul) and 2 ul ^{32}P labeled (3000 Ci/mM) CTP. One half microliter of the reaction mixture was then blotted onto the 'before' position of a strip of Whatman paper representing background label incorporation. Two microliters of T7 RNA polymerase (50U/ul) were added to the reaction mixture and incubated four hours at 37°C. Following incubation, 0.5 ul of the reaction was blotted onto the 'after' position of the Whatman paper. This was then washed twice (10 minutes each) in 10% TCA. Radioactivity, and therefore amplification, was measured using a BioScan counter. (See figure 3). Results suggested that little or no amplification occured in single cells.

DISCUSSION

Problems with aRNA Amplification

Successful amplification in sonicated tissue controls containing larger amounts of mRNA suggests that our difficulty is a matter of quantity. Numerous complications can prevent aRNA amplification from single cells. Finding the exact cause, therefore, can be problematic. However, several distinct possibilities for loss of mRNA substrate or cDNA

FIGURE 3

aRNA Amplification of Single Cell Extracts

A) 8.3.95

TUBE NUMBER	CONTENTS	CPM BEFORE	CPM AFTER
1	single cell	2728	2560
2	single cell	2668	2982
3	single cell	2082	2865
4	single cell	2483	3976
5	single cell	2978	4148
6	single cell	3955	4419
7	single cell	3074	4752
8	single cell	2795	2873
9	single cell	2817	2576
10	pipette solution	3865	4819

B) 3.4.95

TUBE NUMBER	CONTENTS	CPM BEFORE	CPM AFTER
1	sonicated tissue	1300	24,067
2	sonicated tissue	1506	24,000
3	sonicated tissue	1410	70,426
4	single cell	1364	1000
5	single cell	1522	1086
6	single cell	1134	1300
7	single cell	1340	1098

LEGEND:

Detection of aRNA amplification in single cells and controls. A) shows data from reactions performed on March 8, 1995. Cell extracts were processed to double stranded cDNA molecules, and amplified using T7 RNA polymerase (see text). Solution (0.5 ul) was blotted onto Whatman paper before amplification and following four hours incubation with T7 RNA polymerase ('After'). Detection was based upon incorporation of ^{32}P labeled CTP into antisense transcripts, and measured using a BioScan 2000 counter. Tube 10 contains 8 ul pipette filling solution without cell extract as a negative control. B) shows similar data from April 3, 1995, but tubes 1-3 contain 8 ul sonicated medial septum tissue as a positive control.

exist and merit discussion. First, phenol: chloroform extraction and ethanol precipitation of minute quantities of DNA require additional considerations (8). Following extraction with phenol: chloroform: isoamyl, another extraction with chloroform is suggested to remove all of the phenol (8). Also, some salts and ions will coprecipitate with the DNA, as was seen in our precipitations. These salts may affect the activity of the enzymes used in the processing reactions (above). Difficulty in drying pellets from ethanol precipitation likely resulted from phenol which remained in the pellet, which can also affect enzyme activity. Additionally, a back-extraction of the organic phase and interface with TE buffer (pH 7.8) achieves greater recovery of DNA (8). Complete recovery of DNA is critical in every step for amplification from single cells.

Another potential point of DNA loss is ethanol precipitation. First, when precipitating extremely small amounts of DNA, magnesium chloride (0.01M) should be included to improve yield. The precipitation should also be stored at -70°C for at least one hour, as opposed to 15 minutes. Also, centrifugation should be undertaken in an ultracentrifuge at 27,000 rpm for 1-2 hours, followed by microfuge centrifugation at 12,000g for 10 minutes. Only the latter was suggested in our protocol. Drying samples should be accomplished by removing the ethanol with a pipettor, and allowing the remaining ethanol to evaporate. Lyophilization can greatly reduce the yield of DNA, and is therefore not recommended with small quantities of DNA (8). Close adherence to all of the above suggestions will likely increase the yield of DNA, and possibly allow make amplification from single cell extracts possible.

As in any reverse transcription reaction, removal of ribonuclease activity is critical. Care was taken to use DEPC treated water to make all solutions, and patch-clamp pipettes were baked after pulling the glass. RNAsin (Promega), was also included in the reverse transcriptase mixture to inhibit ribonuclease activity. However, greater precaution can be taken. First, solutions should be remade periodically to prevent contamination with RNAse over time. DEPC treated water should also be made periodically for the same reason. It is especially crucial that the pipette tip be RNAse free since this first contacts the mRNA of the cell. Pipettes should be prewashed for 2 days in acetone, followed by one day in 99% ethanol. The pipettes should be washed in purified water, then baked at 200 Celsius. Following baking the pipettes should be pulled and stored at 55 Celsius. Tips should be polished right before the experiment. Gloves should be worn at all times when handling the pipettes!

Another possibility for loss of product is the number of extractions and precipitations required during processing. At each extraction and precipitation, loss of product may occur from pipetting error and adherence to plastic. Though little adherence should occur, the use of silinized plastic should further reduce loss (8).

In addition to product loss, enzyme efficiency is important for aRNA amplification. Amplification in tissue controls is possible in non optimal reaction conditions because of the large amount of mRNA substrate. At the single cell level, maximum enzyme activity is crucial. However, likely causes of non optimal reaction conditions are similar to the causes product loss, namely proper extraction and precipitation technique. Correcting our

technique should increase the likelihood of successful amplification by increasing enzyme activity and reducing product loss.

Future Direction of mRNA Profiling

Though numerous technical difficulties exist for aRNA amplification, it is feasible and has been accomplished (2). Attention to detail and closely monitoring each step should increase the possibility of success. Successful amplification in positive tissue controls suggest that the major conern is the miniscule amount of mRNA in a single cell. As such, the yield of cDNA from reverse transcription must be near optimum. A similar first step is involved in RT-PCR analysis in single cells (1, 2). However, the unmatched sensitivity of PCR may amplify from a lower cDNA yield than aRNA amplification. In addition, RT-PCR eliminates the numerous extractions and precipitations involved in aRNA amplification processing reactions. Numerous extractions and precipitations may result in product loss, and therefore lower yield of double stranded cDNA.

The Richard Finnell laboratory has acquired the oligo (dT)-T7 primer linked to polystyrene-coated magnetic micro beads (Dynal Corp.). First strand cDNA synthesis incorporates the microbeads into the DNA strand. Following this and subsequent processing reactions, Eppendorf tubes are placed into a magnetic chamber which concentrates the microbead containing DNA on one side of the tube. Remaining liquid may then be pipetted out, leaving the concentrated DNA in the tube. This may eliminate much of the product loss associated with phenol: chloroform extractions and ethanol

precipitations. We are currently attempting to amplify cell extracts using the magnetic microbeads.

Although not so powerful as aRNA amplification, PCR is a viable alternative (see introduction). We are currently having 21bp oligonucleotide probes synthesized comercially for each of the $GABA_A$ receptor subunits Although we feel that aRNA amplification can be accomplished from single cells, we must pursue other alternatives while attempting aRNA amplification. Profiling gene expression offers a powerful approach to discerning the relationship between receptor subunit composition and phenotype, in $GABA_A$ and other receptor complexes, whether using PCR or aRNA amplification.

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