CLONING AND EXPRESSION OF THE GENES ENCODING THE CHICK MUSCLE NICOTINIC ACETYLCHOLINE RECEPTOR

by

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ABSTRACT

The nicotinic acetylcholine receptor (AChR) within Torpedo electric organ and vertebrate skeletal muscle has been shown to be an oligomeric integral membrane glycoprotein composed of four homologous subunits in a stoichiometry of $\alpha_2\beta\ \gamma\ \delta$. This thesis describes the isolation and application of cDNA clones encoding in part the α, β and δ subunits of the chicken muscle AChR. Maximum levels of the AChR protein were found in E12 - E14 pectoral muscle. Messenger RNA from this source was therefore used to construct a cDNA library in λ gt10. Restriction fragments of clones encoding Torpedo AChR genes previously shown by Northern blot analysis to cross hybridise with the chicken AChR genes were used to screen this library. Positive clones were plaque purified through several rounds of rescreening, and the inserts were sequenced. The deduced amino-acid sequence identified these clones as coding for the α, β and δ subunits of the chicken AChR, by their homology with the respective subunits of the Torpedo and bovine AChR. These clones were then used in conjunction with a γ subunit probe (M. Ballivet, University of Geneva) in experiments designed to examine the control of AChR expression in innervated, denervated and embryonic muscle. The change in the ratio of the embryonic 4 subunit mRNAs was measured by Northern blotting during the course of chick pectoral muscle development and in innervated adult and

chronically-denervated muscle. Using appropriate standards the absolute steady-state levels of each of the 4 subunit mRNAs were also determined.

In a collaborative project with Dr. A. Mudge (University College London), the effects of calcitonin-gene-related-peptide on the level of AChR protein and its encoding mRNAs were examined by similar methods in embryonic chicken pectoral muscle. The results provide information on the coordination of the transcripts of four genes in regulating the production of this receptor in the muscle cell.

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ABBREVIATIONS

ACh	Acetylcholine
AChR	Nicotinic acetylcholine receptor
ARIA	AChR inducing agent
ATP	Adenosine 5' triphosphate
α Butx	α Bungarotoxin
BSA	Bovine serum albumin
cAMP	Adenosine 3' : 5' cyclic monophosphate
cDNA	Complementary DNA
CEE	Chick embryo extract
CGRP	Calcitonin gene related peptide
CT	Cholera Toxin
DMSO	Di-methyl-sulphoxide
DEPC	Diethylphrocarbonate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethyleneglycol-bis-(β -amino ethyl ether)-N,N,N',N'-tetraacetic acid

HEPES	N-2-hydroxyethyl piperazine-N'-ethane sulphonic acid
IPTG	Isopropyl-β -D-Thio galactopyrnoside
Kb	Kilo base
Kd	Kilo dalton
PEG	Polyethylene glycol
PBS	Phosphate buffered saline
PIPES	Piperazine N'-N'-bis (Z-ethane sulphonic acid) 1,4 piperazine diethane sulphonic acid
PMSF	Phenyl methyl sulphonyl flouride
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TRIS	Tri-(hydroxymethyl) amino methane
TCA	Tri-chloroacetic acid
X-gal	5-bromo-4-chloro-3-indoyl- β -D galactopyranoside

CHAPTER 1

GENERAL INTRODUCTION

Theories of nervous system function are dependent on the existence and properties of the synapse, the communicative junction between neurons. For this reason the synapse has been the focal point of neuroscience research for many decades. The transfer of information between neurons at synapses is achieved by neurotransmitters. The best characterised synapse is the neuromuscular junction, where synaptic transmission is mediated by the neurotransmitter acetylcholine (ACh). At this synapse ACh is released from the presynaptic nerve terminal on depolarisation. Once released ACh diffuses across the synaptic cleft and interacts with specific receptor sites on the postsynaptic membrane. The interaction of ACh with its receptor leads to a rapid transitory increase in the permeability of the postsynaptic membrane to Na+ ions (Katz 1960). The physiological effect of this transitory depolarisation is to initiate muscle contraction. Postsynaptic stimulation is terminated by the action of acetylcholine esterase, which specifically hydrolyzes ACh molecules. A specific receptor for ACh was identified by the classical pharmacological observations of Dale et al (1936). Subsequent research on the AChR is seen to address two significant questions:-

1) How does the interaction of ACh with its receptor lead to a transitory production of a specific ion channel? In this context the AChR may serve as a general model of signal

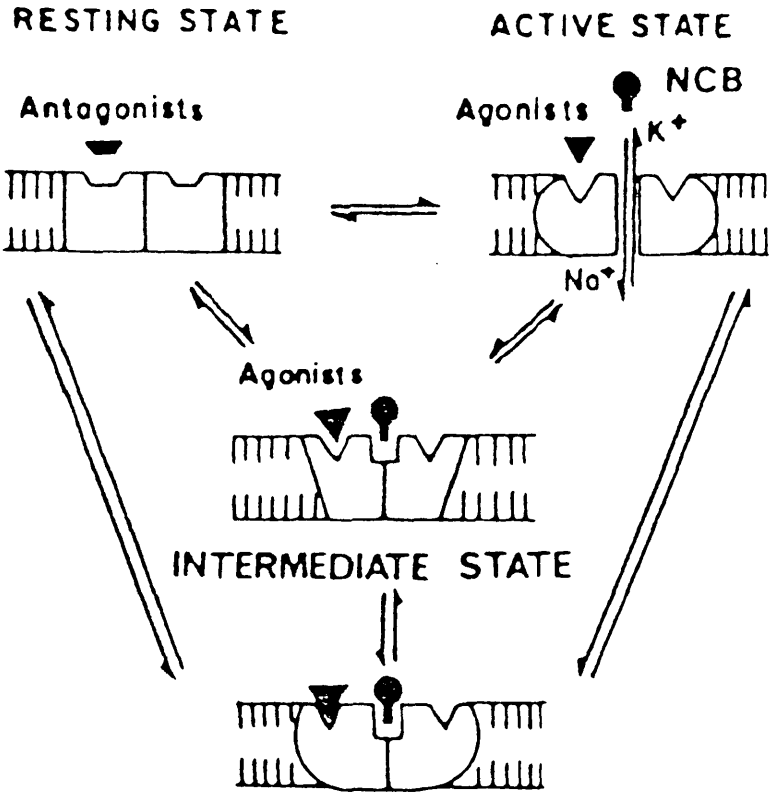
transduction for other ligand gated ion channels within the central nervous system, which are less accessible to electrophysiological and biochemical analysis.

2) The mechanisms underlying the developmental control of AChR biosynthesis, which will increase the understanding of the process of synaptogenesis.

Primary research on the AChR was dependent on, and limited by, the only assay available; the ability of the AChR to depolarise the postsynaptic membrane. This research effort established a complex pharmacological and physiological profile of the receptor (see Figure 1.1) as an allosteric membrane spanning protein (reviewed by Changeux et al. 1983, Popot and Changeux 1984). Four distinct functional states of the AChR have been identified: resting, active (channel open), fast onset desensitized, and slow onset desensitized. Specific agonists and antagonists are thought to bind close to the ACh binding site as determined by competitive ligand binding assays (reviewed by Popot and Changeux 1984). Non-competitive blockers are thought to stabilise the resting or desensitized states binding within the ion channel (Changeux et al 1987). The observations that the AChR is phosphorylated and that phosphorylation enhances the rate of rapid desensitization (Huganir et al 1986, Huganir and Greengard 1987), suggests the existence of

FIGURE (1.1)

The functional states of the AChR (after Changeux et al 1983).



Minimum four-state model for the allosteric transitions of *Torpedo* AChR. The channel is open only in the active state. (NCB) Noncompetitive blocker.

additional modulators of receptor activity. Specific phosphorylation sites have been identified in the intracellular portion of the AChR (Huganir and Greengard 1983). However, while the physiological role of the open and resting state of the AChR are well documented, the actual physiological relevance of the two desensitized states remains to be established.

Progress in understanding the structure of the AChR has been hampered because of the difficulties involved in isolating integral membrane proteins. The properties of the snake α toxins and the high density of AChR within the electric organs of the electric eel, Electrophorous electricus and of the electric rays Torpedo marmorata and californica, have been exploited to provide a detailed picture of AChR structure. Snakes from the Elapidae family produce a group of toxins that bind with high affinity to the AChR blocking synaptic function. Alpha toxins are classified according to size and potency for the AChR (Lee 1972). Type II toxins (70 - 74 amino acids) show essentially irreversible binding to the AChR (Kd ≈ 10^{-14}M). Alpha Bungarotoxin (α Butx) isolated from the venom of Bungarus multicinctus, when labelled to high specific activity, is used as a means of quantifying the levels of AChR in a given preparation. Alpha cobra toxin, a Type I toxin (60 - 62 amino acids) isolated from Naja naja siamensis has a lower affinity for the AChR (Kd ≈ 10^{-9}M) and has been widely used for the affinity purification of the AChR (Changeux et al 1983).

The electric organs of the electric eel or electric ray Torpedo are embryologically derived from skeletal muscle. Both tissues are extremely rich in AChR protein and contain approximately 1000pmols/g of tissue (O'Brien et al 1972) as compared to innervated muscle where the density of receptor is in the order of 1pmol/g (Sumikawa et al 1982b). In addition, the pharmacology of the Torpedo AChR and muscle AChR have been demonstrated to be Similar (Changeux and Sealock 1975).

This combination of α toxin and electric organ has revealed that the AChR of the Torpedo electric organ is a pent meric glycoprotein composed of four different polypeptide chains with a stoichiometry α_2, β, γ and δ (Raftery et al 1980, Popot and Changeux 1984). The apparent molecular weights determined by SDS-PAGE are 40, 50, 60 and 65Kd respectively, giving the AChR complex a molecular mass of 268000Kd, in agreement with values determined by biophysical methods (Popot and Changeux 1984). The stoichiometry and number of subunits found in skeletal muscle AChRs appear to be the same as found in Torpedo, although skeletal muscle AChRs have a sedimentation coefficient of 9S and are not found as the 13S dimmers characteristic of the Torpedo receptor (Chang and Bock 1977).

Preparations containing high densities of AChR or membranes derived from Torpedo electric organ can be shadowed or negatively stained and the receptors can then

be visualized under the electron microscope. The AChRs appear as discs of about 90A^{o} in diameter with a low density pit in the centre (Brisson and Unwin 1984). The experiments of Kistler et al (1982) indicate that each of the peptides spans the membrane extending about 15A on the intracellular side and 55Å on the extracellular side. Analysis of electronmicrographs of biotinylated α Butx and subunit specific monoclonal antibody fragments avidin labelled bound to the AChR, suggested that the arrangement of the subunits around the central pit is with the γ subunit between the two α subunits (Karlin 1983).

A more detailed investigation of AChR structure, and structure/function relationships, requires the determination of the structure of its polypeptides; the easiest way of achieving this utilises gene cloning techniques. Cloning of the AChR presented a formidable problem in molecular biology because the protein consists of four different polypeptides, each encoded by a separate mRNA (Mendez et al 1980, Anderson and Blobel 1981). The complexity of the AChR eliminated approaches utilizing DNA mediated gene transfer, which have been successfully used in cloning other cell surface receptors (Littman et al 1985). The ability to clone the Torpedo receptor was dependent on the relatively high abundance of the four mRNAs in electroplaque tissue (ca 0.5%), the availability of limited sequence data (Raftery et al 1980) and also the availability of subunit specific

antibodies (Lindstrom et al 1979).

Two basic approaches were independently employed in obtaining the Torpedo genes. Both of these strategies were aimed at isolating the clones from a complementary DNA (cDNA) library representing the mature electroplaque mRNA population. In the first of the two approaches, the protein sequence information (Raftery et al 1980) was used to construct synthetic oligonucleotides corresponding to all possible triplet representations of a five to six amino acid segment in the N-terminal region (Numa et al 1983, Barnard et al 1983). These oligonucleotides were then radiolabelled and used to screen a Torpedo cDNA library. This approach yielded an α subunit clone to one group (Sumikawa et al 1982c) and all four subunits to another (Noda et al 1982, 1983a,b).

In the second approach, clones containing genes expressed uniquely in the electroplaque tissue were first selected by screening a Torpedo library with probes made by reverse transcription of electroplaque and brain mRNA, and picking clones hybridising to the former probe but not to the latter. Clones identified by this "plus/minus" screen were then tested for their ability to select AChR subunit mRNA, by DNA-RNA hybridisation. Messenger RNAs which hybridised to the various clones were translated in vitro and their protein products analysed by immunoprecipitation with anti-AChR subunit antisera and followed by gel electrophoresis. Thus,

identification of AChR clones depended on the existence of antibodies that recognised the primary translation products of the AChR mRNA. This approach yielded an α clone to one group (Giraudat et al 1982) and a γ clone to another (Ballivet et al 1982).

As noted by Raftery et al (1980), on examination of partial N-terminal amino acid sequence the most striking feature of the cloned polypeptides was a high degree of homology between them (Noda et al 1983b). Clones for AChR subunits from other vertebrate species have been obtained using Torpedo clones as probes, since there is the extraordinary conservation of nucleic acid sequence between species. In fact, the Torpedo α subunit is more homologous to the calf muscle or human α subunit than it is to other Torpedo subunits (Noda et al 1983c). This approach has yielded the four calf cDNAs (Noda et al 1983c, Tanabe et al 1984, Kubo et al 1984, Kubo et al 1985), all four murine cDNAs (Heinemann et al 1986), and chick genomic γ and δ clones (Nef et al 1984). This extraordinary conservation of structure suggests that the primitive AChR was a homoligomer, and that this structure evolved by gene duplication and divergence in two steps to produce four kinds of chains. The fact that the basic pentameric structure of AChR has been conserved from Torpedo to mammals (Raftery et al 1980), argues that duplication and modification of the subunit genes was an early evolutionary event that afforded significant selective advantage over the homoligomeric structure,

probably in allowing the molecule more subtle and complex modes of allosteric regulation (Changeux et al 1987).

Analysis of genomic clones (Noda et al 1983c, Ballivet et al 1983, Nef et al 1984) suggests that the protein segments encoded by distinct exons correspond to different structural domains. Transmembrane spanning domains (discussed below) are each encoded by distinct exons. In the human α subunit gene there are 8 distinct introns dividing the gene into 9 exons. If the exons do represent functional domains, then this allows for the evolution of the AChR by exon shuffling. The α, γ and δ subunit genes appear to be present as a single copy within the genome of the human and the chicken (Noda et al 1983c, Nef et al 1984).

Based on hydropathy analysis of the primary amino acid sequence, a model for the transmembrane topology common to each subunit was proposed (Figure 1.2) (Noda et al 1982, Claudio et al 1983, Devillers-Thiery et al 1983) which displays the following features :

1) A large amino terminal hydrophilic domain orientated towards the synaptic cleft, which contains the ACh binding site, and a potential site for N-glycosylation.

2) Three transmembrane, uncharged α helical segments (M1, M2 and M3) separated by short

FIGURE (1.2)

The structural models of the AChR (after Huganir and Greengard 1987).

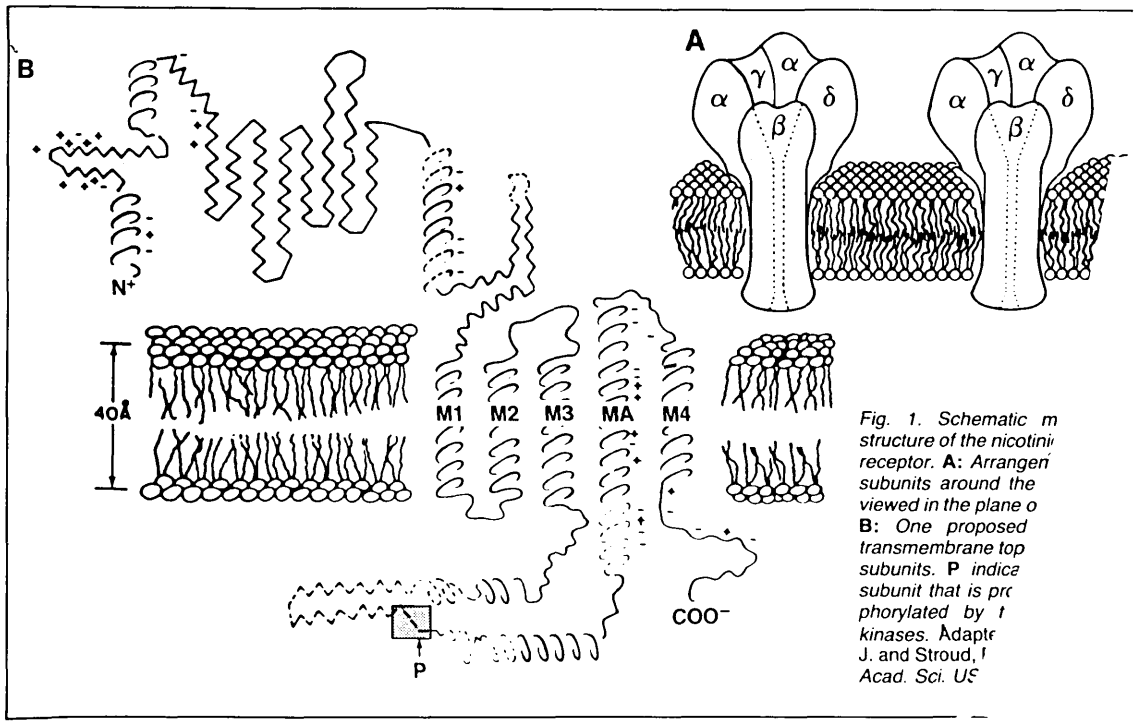


Fig. 1. Schematic m structure of the nicotini receptor. **A:** *Arrangen subunits around the viewed in the plane o* **B:** *One proposed transmembrane top subunits.* **P** *indica subunit that is pr phorylated by t kinases.* Adapt J. and Stroud, *Acad. Sci. US*

hydrophilic stretches and bound by charged residues.

3) A second hydrophilic domain facing the cytoplasm.

4) A fourth membrane spanning α helix (M4) orientated with the carboxy-terminus on the synaptic side of the membrane.

The ion channel is assumed to lie in the axis of quasisymmetry of the putative transmembrane helices (M1 - M4). An alternative model postulates that the ion channel would be lined by charged amino acid side chains from a fifth membrane spanning amphipathic helix (termed helix A), which would be formed at the expense of the cytoplasmic domain. As a consequence, the carboxy-terminus would face the cytoplasm (Guy 1984, Finer-Moore and Stroud 1984).

The cloning of the AChR genes and the elucidation of the primary protein structure of the receptor allows one to examine structure/function relationships. Relevant studies are dependent upon the reconstitution of the receptor within a membrane environment. Sumikawa et al (1981) demonstrated that when mRNA from Torpedo electric organ was microinjected into Xenopus oocytes, AChR subunits were synthesised and inserted into the oocyte membrane. The newly synthesised receptors gave α Butx

binding and subsequent electrophysiological experiments showed that fully functional ion channels were formed in the oocyte membrane with characteristics of those found in Torpedo electric organ (Barnard et al 1982). This system has since been used to identify a wide range of other receptor mRNAs (Barnard and Bilbe 1987). Using mRNA encoding the α, β, γ, and δ subunits of the Torpedo AChR produced from the cloned genes, it was possible to demonstrate that all four subunits were required to produce a functional ligand gated channel (Mishina et al 1984). The use of the bacteriophage Sp6 and T7 promoters (Melton 1984), allowing the production of large quantities of subunit specific mRNA, has made the oocyte the system of choice in determining the effects of genetic manipulation on AChR function.

The first attempts to use mutagenesis as a means to locate functional regions of the α subunit of the Torpedo AChR (Mishina et al 1985) were inconclusive, in that the measurement of functional activity did not indicate whether it was a functionally important or structurally important region of the α subunit that had been identified. This question can only be resolved by determining the tertiary structure of the AChR, which has been hampered by the problems of crystallizing membrane proteins. The use of hybrid AChRs produced from different species has proved more productive because in these cases a change in a particular receptor property is desired on injection of a different subunit, and so one

is always dealing with a functional AChR. Using this technique, a region of the δ subunit between M2 and M3 has been shown to be of importance in determining ion channel conductance (White et al 1985, Imoto et al 1986).

Research into receptor structure/function relationships is also being pursued by other experimental approaches e.g. immunological studies using antibodies directed against synthetic peptides, to define specific sites on the AChR (Neumann et al 1985) and labelling of specific sites on the AChR using radiolabelled photoactivatable ligands (Changeux et al 1987). A combination of these approaches suggests that the toxin binding site and the ACh binding site are within the vicinity of amino acids 192 and 193 of the α subunit. 4-N-Maleimidolbenzyl-tri[^{3}H]methylammonium iodide, (MBTA), is known to react with cysteine groups located in the vicinity of the ACh binding site after reaction of the AChR with DTT (Damle and Karlin 1978). Analysis of digests of the α subunit after reaction with MBTA, or p-N,N-dimethylamino-benzenediazonium fluoroborate, (DDF), by high performance liquid chromatography showed that MBTA labelled cysteine residues 192 and 193 (Kao et al 1984) and DDF labelled amino acids surrounding 192 and 193. Synthetic peptides of amino acids 185 - 196 bind specifically to α Butx and this binding is competitive with the binding of d-tubocurare (Neumann et al 1985). The contribution to the toxin binding site of the other regions of the AChR has yet to be determined. Time-resolved photo-affinity

labelling is yielding information on the binding sites for non-competitive blockers (Changeux et al 1987). The results suggest that these compounds act within, or just within, the ion channel. Serine residues within M2 of the β and δ subunits have been identified as comprising part of these binding sites (Giraudat et al 1986, Hucho et al 1986).

The one respect in which the Torpedo AChR falls short as a model for skeletal muscle AChR concerns developmental regulation. Muscle AChRs undergo distinct changes in their distribution and also in certain biochemical and electrophysiological properties during the process of synaptogenesis. Muscle fibres are formed by the fusion of myoblasts. AChRs are first detected by α Butx binding at this stage in muscle development. Initially, these receptors are incorporated throughout the membrane at about $200\mu m^{-2}$ (Fambrough 1979). As motor neurons make contact with muscle and functional synapses form, AChRs are found to cluster at the synapse. The AChRs at the synapse are termed junctional and those spread diffusely on the membrane extra-junctional. As synaptogenesis proceeds the density of extra-junctional receptors declines with a parallel decrease in AChR biosynthesis, while the density of junctional receptors increases to reach densities of between 1 - 10 x $10^3/\mu m^{-2}$ (Dennis 1981). This process is reversed by muscle denervation, as extra-junctional receptors once again appear and the rate of AChR synthesis is greatly enhanced (Miledi 1960,

Brockes and Hall 1975).

Junctional and extra-junctional receptors are distinct in certain properties. For example, in chick muscle the turnover rate of the AChR increases from 4 hrs in embryonic muscle, to greater than 120 hrs in innervated muscle (Burden 1977a). In the rat, extra-junctional and junctional receptors have different isoelectric focusing points (Brockes and Hall 1975). Also in both rat and mouse muscles extra-junctional and junctional receptors have distinct antigenic determinants (Hall et al 1983). The physiology of junctional and extra-junctional receptors are also distinct in that junctional receptors have shorter channel open times, and increased channel conductance (Sakman and Brenner 1978). This change in channel properties is universal to all species studied with the exception of the chick (Schuetze 1980). The characteristics of extra-junctional AChRs in embryonic and denervated muscle are essentially the same (Fambrough 1979). This suggests that the innervating neuron is responsible for the maintenance of AChR distribution and also certain receptor properties. The observation that direct electrical stimulation of denervated muscle suppresses AChR synthesis (Hall and Reiness 1977), suggests that in some way the electrical activity of muscle regulates the synthesis of the AChR. However, the maintenance of, and the late increase in, AChR number at the level of the endplate, while extra-junctional AChR levels decrease during development (Betz and Changeux

1980), requires the intervention of an anterograde signal to override the effects of electrical activity on AChR biosynthesis in junctional regions. Numerous factors of neuronal origin have been shown to increase AChR levels in muscle cultures, but these have yet to be fully characterised (Salpeter 1987).

The differences in channel properties between junctional and extra-junctional AChRs have been elegantly explained by Mishina et al (1986). A novel subunit, the ε subunit, was cloned from calf muscle (Takai et al 1985). Microinjection in Xenopus oocytes of cloned mRNAs encoding the α, β, γ, and δ subunits of the calf AChR, gave AChRs in the oocyte membrane of those found in fetal calf muscle; whereas injection of mRNAs for the α, β, ε, and δ gave AChRs in the oocyte membrane that had the characteristics of mature bovine muscle. Thus the change in channel properties can be related to the substitution of the γ subunit in fetal muscle for the ε subunit in adult muscle. These findings were supported by Northern blot analysis. Witzemann et al (1987) have demonstrated further that muscle denervation results in renewed synthesis of γ subunit mRNA, strengthening the observations of Mishina et al (1986). These experiments explain certain differences observed between junctional and extra-junctional receptors, but are clearly insufficient to account for all developmental changes in AChR properties. The interactions of the AChR with the components of the basal lamina such as the 43Kd protein

(Burden 1987) and Agrin (Wallace 1987) are of importance in localisation, clustering and stabilisation of the AChR at the neuromuscular junction. Post-translational modification of the AChR, such as changes in glycosylation and phosphorylation, have been implicated in developmental changes in certain properties of the AChR (Hall et al 1983, Schuetze et al 1986).

Central to understanding the developmental changes in AChR density and functional properties, is an understanding of the mechanisms controlling receptor biosynthesis. Each AChR molecule is the product of expression of four genes (Anderson and Blobel 1981, Noda et al 1982, 1983c). Thus, the control of AChR mRNA transcription presents a highly complex problem in coordinating gene regulation. The only comparable system studied in detail to date is that of the globin genes (α and β). Recent data have shown that the transcription of the α and β globin genes is controlled in very different ways during erythroid differentiation (Charnay et al 1984). Thus, one may anticipate that the transcriptional control of AChR will be extremely complex. However, it may be a long time before the AChR can be effectively studied in the same detail as globin. For example, while AChR has been successfully transiently expressed in heterologous cell types by mRNA injection (Mishina et al 1984), its expression directly from cloned DNAs introduced into cell lines by gene transfer has only recently been achieved for the α subunit (Claudio 1987).

The observation of Nef et al (1984) of the close linkage of the δ and γ genes in the chick provides an interesting possibility to study their control, especially since the γ subunit gene is not expressed in adult muscle, yet the δ subunit gene is. Several groups have recently begun to investigate the control of AChR gene transcription by examining upstream regions of these genes. In the case of both the α and γ subunit genes, both tissue and developmental specificity is controlled by DNA motifs 1 - 2Kb upstream of the start of transcription (Fontaine et al 1987).

Thesis Goals

Developmental changes in AChR levels and biochemical properties have been discussed. A key point in understanding the control of expression of the AChR is the level of expression of the 4 mRNAs encoding this protein. The ease with which embryonic tissues may be obtained from Avian species makes the chick an ideal system to study the control of AChR expression. The aim of this thesis is the isolation of α, β, γ, and δ subunit specific cDNAs encoding the chick muscle AChR. These clones can then be used to study the relationship of expression of the AChR protein, and the level of its four encoding mRNAs during muscle development. These clones can be further utilised to study the relationship between the level of AChR and its encoding mRNAs in response to muscle denervation, and on exposure to candidate neuronally derived anterograde factors.

CHAPTER 2

THE CLONING OF cDNA s ENCODING THE α, β AND δ SUBUNITS OF THE CHICK MUSCLE AChR

INTRODUCTION

An essential prerequisite for the cloning of a gene using cDNA methodology is the identification of a suitable enriched source of the respective mRNA. This is of particular importance when the cloning of low abundance mRNAs is attempted. Assay of the levels of receptor mRNA is dependent on its translation and the assembly of the respective subunits in such a form that the receptor protein can be assayed by either biochemical or electrophysiological methods.

Cell free systems for the translation of mRNAs are incapable of faithfully synthesising intact functional AChRs. Although the translation of the subunits can be detected in cell free systems (Sumikawa *et al* 1981, Anderson and Blobel 1981), the products fail to exhibit α Butx binding. The addition of canine rough microsomal membranes to *in vitro* translation systems still fails to produce translation products that exhibit α Butx binding (Anderson and Blobel 1983, Merlie *et al* 1983).

Xenopus oocytes have been shown to be highly efficient systems for the translation of microinjected exogenous mRNA and to faithfully carry out post-translational processing such as glycosylation, sequestration, pre-peptide cleavage and secretion (Gurdon 1974, Lane *et al* 1979). When *Torpedo* poly(A)+ RNA isolated from electric organ was microinjected into *Xenopus* oocytes, α

Butx binding activity was detected in the translation products, and subsequent analysis showed the synthesised AChR to be glycosylated and have the biochemical characteristics of the native Torpedo AChR (Sumikawa et al 1981). Further studies (Barnard et al 1982) showed AChRs synthesised in this system to be correctly orientated in the oocyte membrane and to have the identical electrophysiological properties of the AChR found within the Torpedo electroplaque. The Xenopus system has since been widely used for the translation of mRNAs encoding for various neurotransmitter receptors (reviewed by Barnard and Bilbe 1987). It has also been used to establish that the four mRNAs encoding the α, β, γ, and δ subunits of the AChR from the Torpedo contain all the information necessary for the correct translation and assembly in the oocyte membrane (Mishina et al 1984). Once a suitably enriched source of receptor mRNA has been identified, cDNA libraries can then be constructed which are enriched for receptor mRNAs. The first AChR cDNAs to be isolated were those encoding the α subunit of the Torpedo receptor (Sumikawa et al 1982c, Noda et al 1982). The cloning of these cDNAs was dependent on the high abundance of receptor mRNA within the electroplaque, and on partial N-terminal amino acid sequence. The much lower abundance of muscle AChR and its susceptibility to degradation has made the cloning of muscle AChR cDNAs more difficult using the methodology outlined for the Torpedo receptor (Barnard et al 1984). The cloning of muscle AChR cDNAs has been dependent upon the ability of

the Torpedo cDNAs to cross hybridise with their counterparts encoding muscle AChR subunits. Cross hybridisation has subsequently been used to isolate cDNAs for the calf muscle α, β, γ, δ and ε subunits (Noda et al 1983c, Tanabe et al 1984, Mishina et al 1985), and the α, β, γ, and δ subunits of the murine AChR (Patrick et al 1986). The regions used from the Torpedo genes as probes to obtain the above clones were those encoding the putative transmembrane regions M_1, M_2 and M_3. In addition, Buonanno et al (1987) have shown that an oligonucleotide based on a consensus sequence of 9 AChR subunits encoding from nucleotide 402 - 426, a region that is identical in all AChR subunits, was sufficient to isolate the murine β and γ subunit cDNAs.

In this Chapter the developmental expression of the chick pectoral muscle AChR was analysed. In a collaborative project with D. Beeson (Royal Free Hospital, London) the levels of receptor mRNA were analysed by translation in Xenopus oocytes. Once a suitable source of receptor mRNA was identified it was used to obtain chick AChR cDNAs using Torpedo AChR cDNAs as hybridisation probes.

METHODS AND MATERIALS

Maintenance of Embryonic Chickens

Fertile chicken eggs (Rhode Island Reds x Light Sussex) were obtained from Orchard Farms Limited. Fertile eggs were incubated in a force draft incubator at 37°C for periods between 10 - 21 days. Pectoral muscles were dissected at time points between E10 - E21 and stored under liquid nitrogen until used. The number of animals utilised at each time point was sufficient to give 2g of pectoral muscle. At E10, 30 chicken embryos were utilised. 1 day post hatch pectoral muscles were a gift from K. Tsim. ^{125}I - α Butx (200 - 1000ci/mmol) was obtained from Amersham International. Formalin fixed staphylococcus aureus cells were obtained from BRL. Xenopus Laevis were obtained from Xenopus Ltd. Restriction enzymes were obtained from BRL, Biolabs or BCL, and were used according to manufacturers instructions. Bulk chemicals were obtained from either BDH or Sigma.

Preparation of AChR from Chick Muscle

The preparation of crude AChR was as described in D. Beeson's Ph.D. thesis (1987). Tissue from chick pectoral muscles (E10 to 1 day post hatch) was homogenised in 10 volumes of ice cold buffer A: 50mM potassium phosphate buffer pH 7.6, EDTA 5mM, EGTA 1mM,

PMSF 1mM. The homogenate was then centrifuged at 10,000xg. for 30 mins. The supernatant was then discarded and the remaining pellets solubilised in 2 volumes of Buffer A plus 2% Triton X-100. The extract was centrifuged again at 10,000rpm for 30 mins. The supernatant was then filtered through glass wool and stored in 200μl aliquots at -70^{o}C.

Assay of Crude AChR α Butx Binding

Receptor assays were performed by the method of Dolly and Barnard (1977) with small modifications. α Butx binding was measured by incubating soluble samples (100 - 150μl) in 10nM ^{125}I - α Butx for 1 hr at 23^{o}C. Aliquots (60 - 100μl) were pipetted onto Whatman DE 81 discs (2.3cm), left for 1 - 2 mins, then washed (x3, 4 mins each) in 300ml of ice cold 25mM Na phosphate, 0.2% Triton X-100 pH 7. Discs were dried under an infra-red lamp and counted in a gamma counter with counting efficiency of 45 - 50%. All assays were performed in triplicate. Non-specific binding was determined in the presence of 5μM α Butx, which was added 15 mins before ^{125}I - α Butx. Specific binding was defined as the total minus non-specific binding.

Measurement of Protein Content

Protein was determined by the method of Lowry et al (1951).

The Preparation, Handling and Assay of RNA

It must be noted that RNA is readily degraded, and therefore great care should be taken in its preparation and subsequent storage and handling. Where suitable, solutions used in the preparation of RNA were first treated with Diethylpyrocar bonate (DEPC) at a concentration of 0.1%, for 15 mins. DEPC inactivates translational activity of mRNA. However, it has a relatively short half-life in water and was removed from solutions by autoclaving. Similarly, glassware was either treated with DEPC or baked at 160^{o}C after having been acid and alkali washed. To ensure maximum RNA recovery, glassware used in steps after the initial homogenisation was siliconised using Dimethy dichlorosilane (BDH).

Isolation of RNA using Guanidinium Thiocyanate

A modification of the method of Chirgwin et al (1979) was used. Muscle tissue was homogenised in the following buffer:

4M guanidinium thiocyanate
25mM sodium citrate
0.1M 2-mercaptoethanol

1g of muscle was homogenised in 10ml of the above solution in a glass homogeniser using a Polytron at full

speed, for 15 - 30 secs. The homogenate was transferred into two 30ml corex tubes, and spun in a Sorval HB4 swing out rotor for 10 mins at 10,000xg. The supernatant was made 2% with respect to sodium lauryl sarcosine, heated at 65°C for 2 mins, then layered over a 2.5ml cushion of 5.7M CsCl in 100mM EDTA pH 8, in polyallomer tubes and spun at 35,000 rpm in a Beckman SW41 swing out rotor for 18 hrs at 20°C. The upper layer and first 1ml of CsCl were carefully removed, the tubes inverted and the bottom 1.5cm cut off. The RNA, seen as a translucent pellet, was immediately resuspended in 5ml of:

20mM Tris-HCl pH 7.5
10mM EDTA
0.5% SDS

To facilitate solubilisation it was heated at 65°C for 2 mins. An equal volume of phenol/chloroform/ isoamylalcohol (25 : 24 : 1) was added and mixed for 2 mins and the phases separated by centrifugation at 10,000rpm for 5 mins in a Sorval HB4 rotor. After a further extraction with chloroform/isoamylalcohol the upper phase was removed and RNA precipitated by the addition of 1/20th volume 4M NaCl and 2.5 volumes of 100% Ethanol. RNA was sedimented by spinning at 10,000rpm in a Sorval HB4 rotor for 10 mins. Pellets were washed with 70% ethanol, resedimented, lyophilised and dissolved in 5mM Hepes pH 7.5 at 1 - 4mg/ml.

Oligo (d T)Cellulose Chromatography

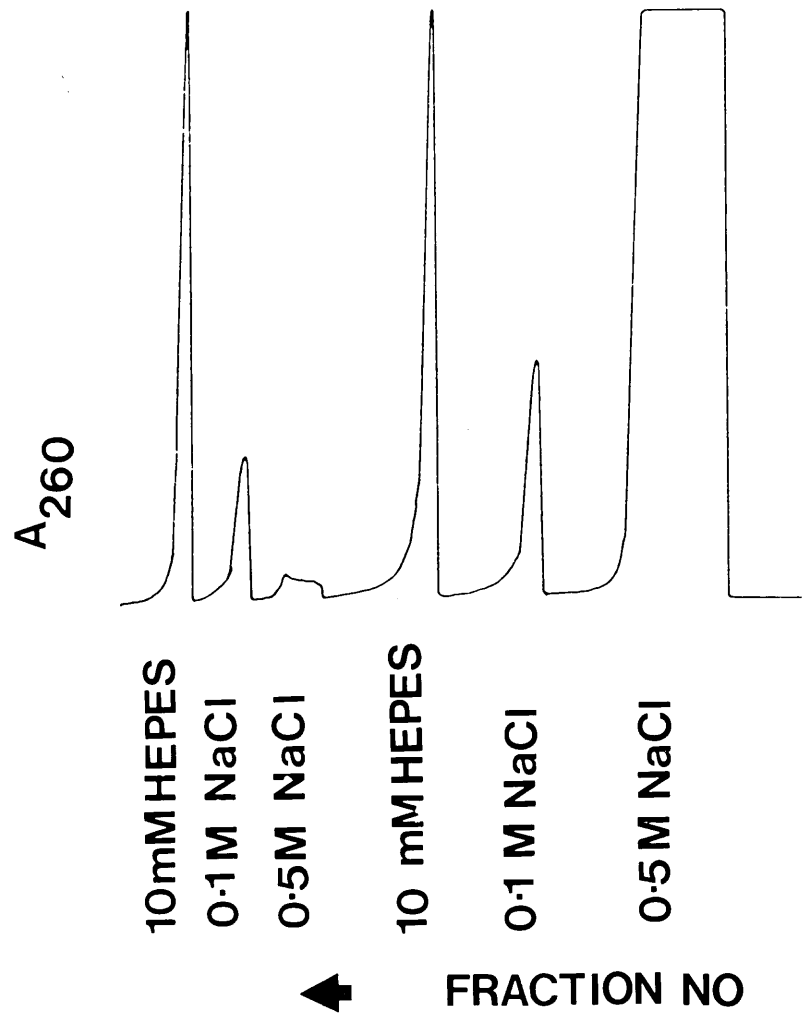
The use of oligo (d)T cellulose chromatography to separate poly(A)$^+$ RNA (mRNA) from total RNA is based on the method of Aviv and Leder (1972). RNA was applied to an oligo d(T) cellulose column, and the profile of the RNA coming off the column was monitored using an LKB Uvicord ultraviolet absorption meter at 266nm. A typical elution profile is shown in Figure (2.1). Briefly, total RNA in 0.5M NaCl/10mM Hepes pH 7.5, 0.5% SDS was applied to an oligo (d)T cellulose column equilibrated in 0.5M NaCl, 10mM Hepes pH 7.5, 0.5% SDS. The column was washed extensively with 0.5M NaCl, 10mM, Hepes pH 7.5, 0.5% SDS until the UV absorption reading returned to zero. A similar washing procedure was carried out using 0.1M NaCl/10mM Hepes pH 7.5, 0.5% SDS. Final poly(A)$^+$ RNA was eluted from the column in as small a volume as feasible of 10mM Hepes pH 7.5, between 2 - 5mls Poly(A)$^+$ RNA was subjected to two cycles of chromatography, and finally precipitated by the addition of potassium acetate pH4.2 to 0.2M and 2.5 volumes of ethanol.

Poly(A)$^+$ RNA was sedimented by centrifugation at 10,000rpm in a Sorval HB4 swing out rotor for 30 mins at 4^{o}C, washed with 70% ethanol, lyophilised, and dissolved in 100 - 200μl of 5mM Hepes pH 7.5, usually at 1 - 4mg/ml. RNA was then stored in aliquots at -70^{o}C.

Between cycles of chromatography the column was washed

FIGURE (2.1)

Isolation of chick pectoral muscle mRNA by oligo (d)T cellulose chromatography.



briefly with 0.1M NaOH, which removed non-specific binding from the column, and then washed extensively with 0.5M NaCl/5mM Hepes pH 7.5 until the pH of the buffer coming off the column had returned to 7.5.

Analysis of Total RNA Preparations by Formamide Gel Analysis

Total RNA preparations were analysed by Formamide gel electrophoresis on 1cm tube gels as described by Maniatis et al 1983. The gels were then scanned for UV absorption at 260nm using a Joyce Leobl UV scanner. A typical foramide gel profile is shown in Figure (2.2).

Translation of mRNA in Rabbit Reticulocyte Lysate Assays

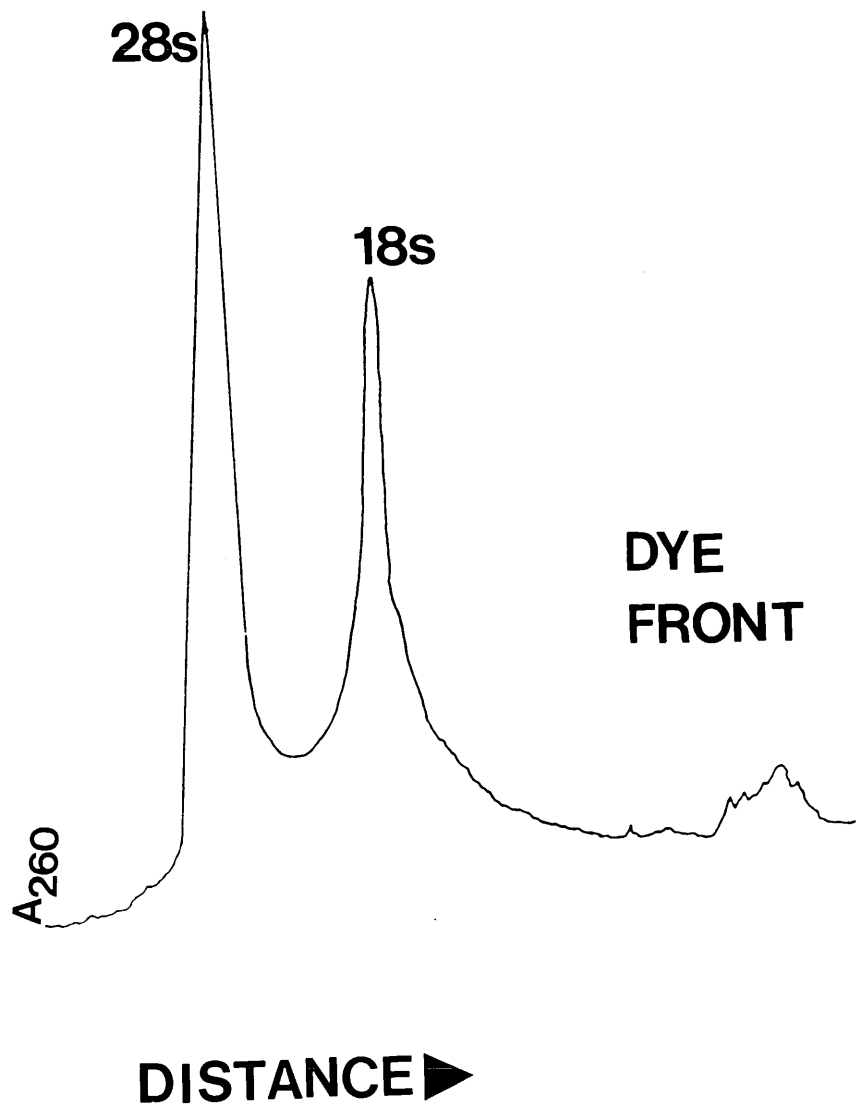
The reticulocyte lysate assay was used as a routine screen to assess the integrity of mRNA preparations by $[^{35}S]$ methionine incorporation into high molecular weight polypeptides as analysed by gel electrophoresis or TCA precipitation.

Time Course of mRNA Translation

The rabbit reticulocyte lysate used was as supplied by Amersham International. The lysate and the mRNA to be tested were thawed and placed on ice. The following reaction mix was then made up:

FIGURE (2.2)

Formamide gel analysis of E12 pectoral muscle total RNA.



Lysate 40μl

mRNA 1μg

[^{35}S] Methionine 50μCi

TOTAL Volume 50μl

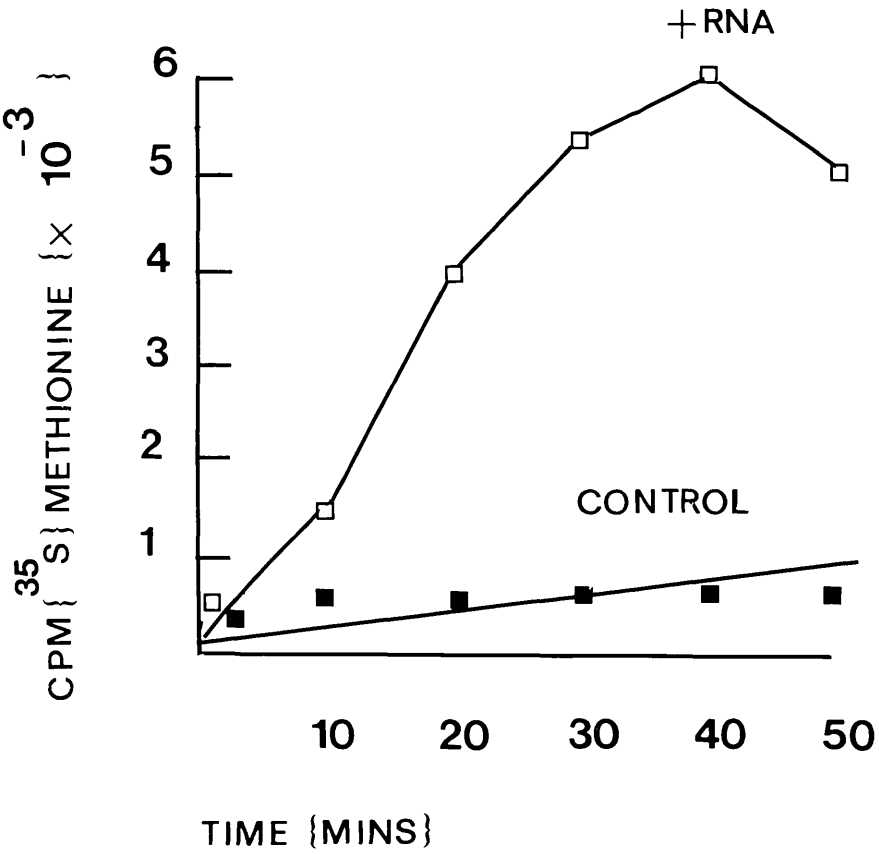
The reaction was then incubated at 30^{o}C, 1μl samples were removed in duplicate at regular time intervals and added to 0.5ml of 0.5M NaOH 6% w/v H_2O_2. Samples were then placed in a 37^{o}C water bath for 10 mins to hydrolyze amino acyl-tRNA complexes. The tubes were then removed to an ice bath and 2ml of ice cold 25% TCA solution was added. 100μg of BSA was added as a carrier. After 30 mins incubation, acid insoluble counts were isolated by filtration onto Whatman GF/C filters, and washed twice with 3mls of ice cold 6% TCA. The discs were then diced under an infra red lamp. Radioactive incorporation was determined after addition of 4mls of Biolour (New England Nuclear) by Liquid Scintillation counting. The results of such an experiment are shown in Figure (2.3).

Gel Electrophoresis of Lysate Products

Once the optimal time of translation has been established the lysate assay was performed as above, except half reaction volumes were used. The reaction was allowed to proceed for 40 mins, 5μl of the reaction mixture was removed and diluted with 100μl of distilled water. 60μl of 45% TCA was then added and the samples were allowed to precipitate on ice for 30 mins. Pellets were collected

FIGURE (2.3)

Time course of incorporation of [^{35}S]-Methionine by Rabbit Lysate assay. Each Lysate mix received 1μg of mRNA.



by centrifugation (12,000 rpm, 15 mins). The supernatant was discarded and the pellet was washed in 500μl of 12% TCA and centrifuged as above. The supernatant was removed and finally washed in 500μl of acetone. After centrifugation the pellet was then dissolved in 10μl of FSB.

FSB:

10% Glycerol

5% β-mercaptoethanol

3% SDS

50mM Tris-HCl pH 6.8

0.01% Bromophenol Blue

Samples were stored at -20^{o}C. Prior to electrophoresis samples were boiled for 5 mins before loading.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Gels were fixed in Amplify (Amersham International) before being dried under vacuum, prior to autoradiography at -70^{o}C using an intensifying screen and Fuji X-ray film. A typical autoradiograph is shown in Figure (2.4).

The Microinjection of Xenopus Oocytes

Oocyte microinjection was performed by D. Beeson (Royal

FIGURE (2.4)

Autoradiograph of chick muscle and fibroblast mRNA[^{35}S] lysates, after 12% polyacrylamide gel electrophoresis.

Track 1 lysate contained fibroblast mRNA.

Track 2 E10 pectoral muscle mRNA

Track 3 E12 pectoral muscle mRNA

Track 4 E14 pectoral muscle mRNA

Track 5 E16 pectoral muscle mRNA

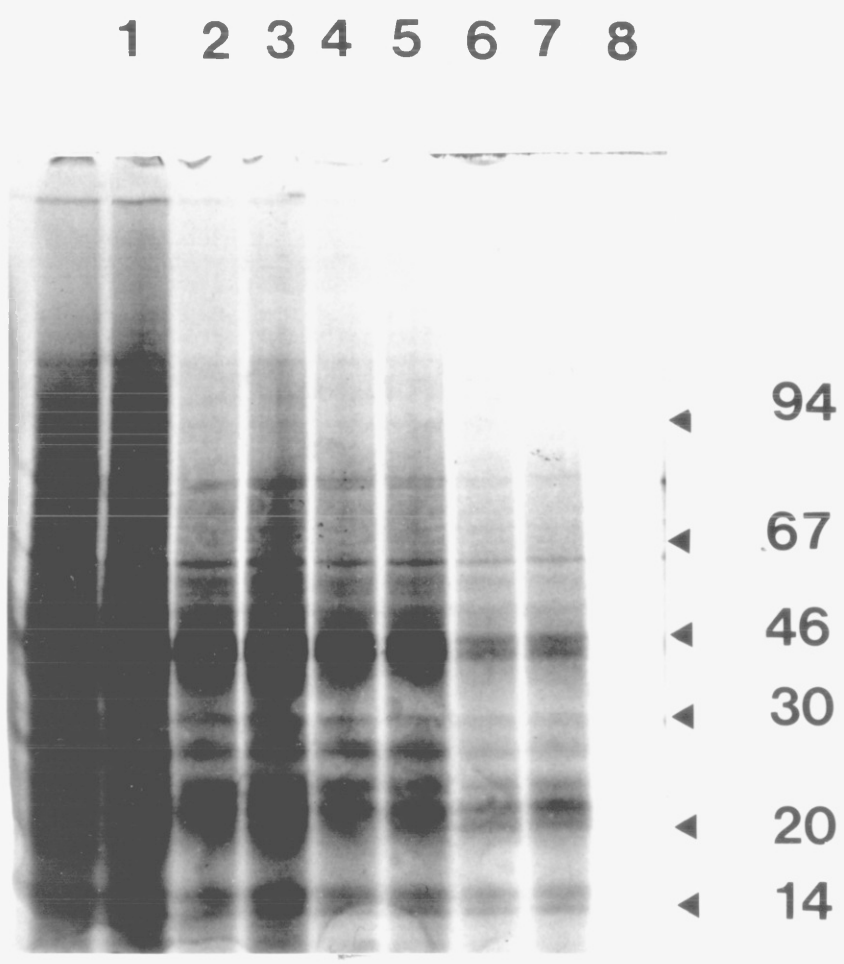
Track 6 E18 pectoral muscle mRNA

Track 7 1 day post hatch muscle mRNA

Track 8 control sample containing no RNA

The standard proteins were phosphorylase b (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), soybean trypsin inhibitor (20.1K) and alpha lactalbumin (14.4K).

FIGURE (2.4)



Free Hospital, London), oocytes were obtained from female Xenopus laevis, the African clawed toad. Toads were killed and the oocytes removed by dissection. The oocytes, usually several thousand from one female, were placed in a 90mm petri dish containing Barth's medium (Gurdon 1974). Oocyte injection and α Butx binding assays on oocyte extracts were performed in conjunction with D. Beeson.

Barth's Medium:

88mM NaCl

1mM KCl

2.4mM $NaHCO_3$

0.8mM $MgSO_4$

0.3mM $Ca(NO_3)_2 .4H_2O$

0.4mM $CaCl_2 .6H_2O$

7.5mM Tris-HCl pH 7.6

Before use gentamycin at 100μg/ml was added.

Individual oocytes were teased away from one another with forceps and left in Barth's solution overnight at 21°C. Healthy oocytes were then selected for microinjection. Microinjection was carried out using a Singer MK 1 Micromanipulator and Microsyringe outfit (Singer Instruments Co. Ltd) Microinjection needles were prepared from hard glass capillary tubes (BDH). Oocytes were usually injected with 25ng mRNA dissolved in sterile distilled water at 0.5mg/ml. After injection

they were incubated in Barth's at 21°C overnight, and then those which still appeared healthy selected and incubated for a further 12 - 36 hrs. Oocytes were then used for assay of AChR content.

α Butx Assay for AChR in Xenopus Oocytes

The method used was as described . Sumikawa et al (1981). Batches of 20 or 40 oocytes selected as healthy after microinjection were homogonised in 150μl of Phosphate buffer

Phosphate buffer:

50mM phosphate buffer pH 7.2

2% Triton X-100

0.1mM PMSF

1mM EDTA

1mM EGTA

The homogenates were shaken for 1 hr at 4°C and centrifuged at 10,000g for 30 mins. The supernatant was split into two duplicate samples and incubated with excess ^{125}I - α Butx at room temperature for 2 hrs. Subsequently 2μl of rabbit anti-Torpedo AChR serum was added, mixed and incubated at 4°C overnight. The antibody/AChR/α Butx complex formed was then precipitated either with S. aureus cells or purified goat anti-rabbit IgG as a second antibody as described previously

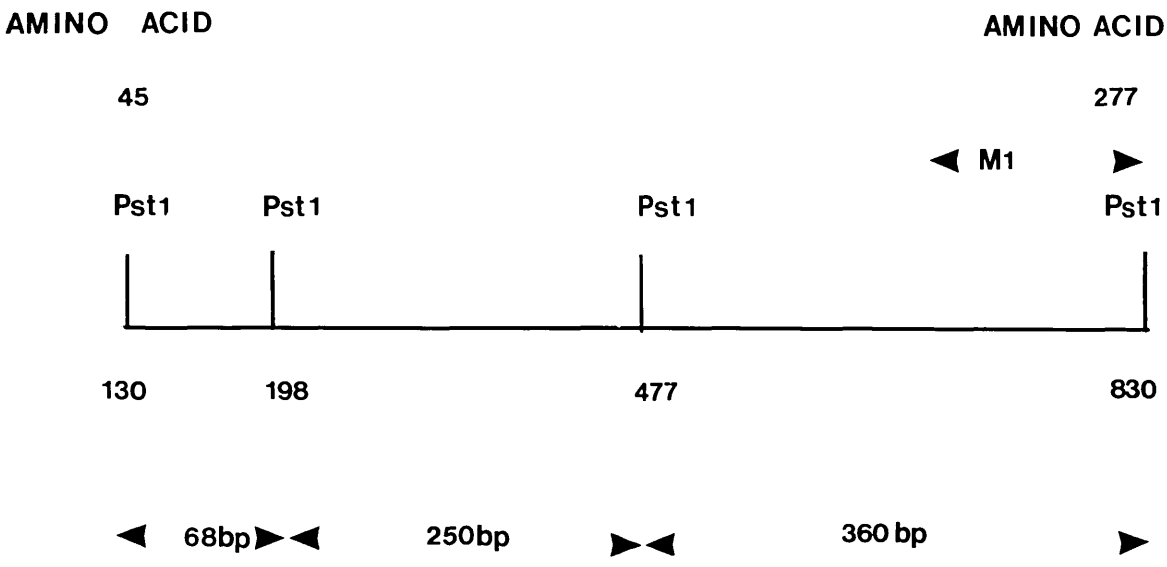
(Sumikawa et al 1981).

Preparation of Plasmid PT50

Plasmid PT50 a pBR322 derivative containing a PstI insert encoding from amino acid 45 to 277 of the mature β subunit of Torpedo californica (Hershey et al 1983) was a gift from N. Davidson (Caltech, Pasadena, U.S.A. - see Figure (2.5)). Single colonies of PT50, transformed in E.coli strain HW87 (British Bio-technology Ltd), were innoculated into 10mls of MNB Broth containing 12.5μg/ml tetracycline. After overnight growth at 37°C, 1ml of this culture was innoculated into 500mls of MNB Broth 100 μg/ml tetracycline in a 2L baffle flask and grown for 18 hrs at 37°C. Cells were then harvested by centrifugation at 10,000xg for 10 mins. Plasmid DNA was purified by alkali lysis (Birnboim and Doly 1979). Plasmid DNA was further purified free of RNA and chromosomal DNA by using Sephacryl S-300 (Pharmacia) columns. Crude plasmid DNA was prepared as described above, and resuspended in 190μTE and 10μl, 4M NaCl. To this 20μl RNase A (1mg/ml) and 5μl RNase T_1 (1mg/ml) were added and incubated at 37°C for 30 mins. The solution was extracted once with phenol (equilibrated with TE) and ethanol precipitated. The precipitate was dried and resuspended in 90μl TE, 10μl 100% glycerol and loaded onto a sephacryl column equilibrated in TNE.

FIGURE (2.5)

Restriction map of clone PT50 (after Hershey et al 1983).



TNE:	TE:
50mM Tris pH 7.5	10mM Tris pH 8.0
100mM NaCl	1mM EDTA
10mM EDTA	

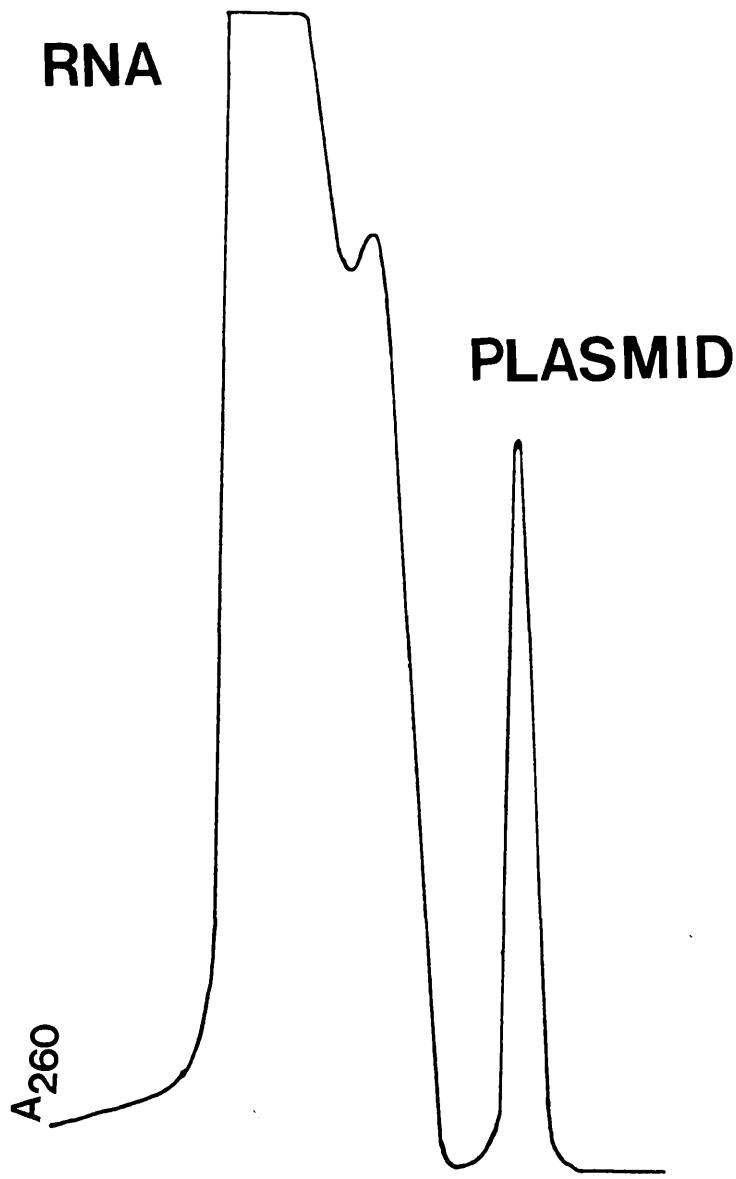
The elution profile was monitored on a UV spectrophotometer at 260nm and the first excluded fraction (Figure (2.6)) was collected. An equal volume of 7.5M ammonium acetate was added and the plasmid was precipitated with 2.5 vols of ethanol. After overnight incubation at -20°C, the precipitated DNA was collected by centrifugation at 10,000rpm for 10 mins and resuspended in 500µl TE. The yield was estimated by UV spectroscopy (50µg DNA = 1.0 OD unit at 260nm). The yield was typically between 250 - 500µg of DNA per 500ml culture.

Preparation of Insert DNA from Plasmid PT50

Purified plasmid PT50 was digested with a 10 fold excess of PstI. DNA restriction digests were then run on low melting temperature agarose gels (Maniatis et al 1982), containing 1µg/ml ethidium bromide at 70V. Bands were viewed under 366nm UV light and the 250bp and 360bp bands (Figure (2.5)) were excised and placed in eppendorf tubes. After addition of 1 volume of 10 x TE, 1 volume of 3M Na acetate pH 5.2 and 2 volumes of distilled water, the tubes were incubated at 65°C for 5 mins. The

FIGURE (2.6)

Purification of plasmid DNA by chromatography on Sephacryl-S300 resin.



solution was then extracted with an equal volume of phenol (equilibrated in TE buffer). The initial extraction was for 5 mins at 65°C. Subsequently the aqueous phase was extracted twice with phenol chloroform (1 : 1) (equilibrated in the above buffer), and finally with 1 volume of chloroform, before precipitation with 2.5 volumes of ethanol. DNA was recovered by centrifugation, washed in 70% ethanol and resuspended in a suitable volume of TE. The yield of DNA was determined by running a small aliquot on a second gel.

Labelling of PT50 Insert to High Specific Activity.

The 250 and 360bp fragments purified as above were labelled to high specific activity using the random priming method as described by Feinberg and Vogelstein (1983). Unincorporated nucleotides were removed by using G25 spun columns (Maniatis et al 1982). The specific activity of the probes produced by this method was in excess of 1 x 10^9cpm/μg, as determined by the calculation of the percentage incorporation of hot nucleotide: 2 x 1μl aliquots of the probe after G25 purification were dotted onto DE81 filters (Whatman) and washed extensively in 0.5M Na phosphate buffer. The filters were then dried under infra red light, followed by Cerenkov counting. Probes were boiled for 10 mins before hybridisation.

Northern Blots

Northern blots were performed essentially as described by Thomas (1980). Glyoxal solution 6(M) was deionised using amberlite number 1 fixed bead resin (BDH) and stored in aliquots at -20°C. RNA samples were denatured by incubation in 1M glyoxal/50% dimethyl sulphoxide (DMSO)/10mM sodium phosphate buffer at pH 7.0 at 50°C for 1 hr. Samples were then run on 1.1% agarose in 10mM sodium phosphate buffer gels at 90V for between 4 and 8 hrs. The buffer was circulated continuously in order to prevent the pH rising above 8 at which point glyoxal dissociates from RNA. Chick poly(A)- RNA and λ*Hin*dIII markers were glyoxalated as above and run in parallel with RNA samples. Marker lanes were cut off after electrophoresis and treated as follows. The gel was treated with a large volume of 50mM NaOH for 30 mins at room temperature, followed by neutralisation in 100mM Tris-HCl pH 7.4 containing 2μg/ml Ethidium bromide for a further 30 mins. The size markers were then visualised under UV illumination. The RNA was then transferred to a Gene Screen (NEN) membrane as follows. The gel was laid on 2 sheets of Whatman 3MM saturated with 20 x SSPE. A sheet of Gene Screen membrane was then placed on top of the gel and its surface rolled flat to remove any bubbles. Two sheets of Whatman 3MM paper saturated with distilled water were placed over the Gene Screen membrane, followed by three inches of paper towels, a glass plate and a weight. RNA was transferred overnight.

Blots were allowed to air dry and were then baked for 2 hrs at 80°C.

Prehybridisation and Hybridisation of Northern Blots

The exact conditions of hybridisation and prehybridisation varied according to the experiment. Prehybridisation was for 1 - 4 hrs in 50% formamide, 5 x SSPE, 1 x Denhardts, 0.5% SDS, 100μg/ml salmon sperm DNA, 100μl/ml yeast transfer RNA at 42°C.

5 x SSPE:- 0.75M NaCl, 0.04M Na_2H_2 PO_4 pH 7.4, 0.5mM EDTA

1 x Denhardts:- 0.02% bovine serum albumin
0.02% polyvinyl pyrrolidone
0.02% Ficoll 400

Hybridisation was in the same buffer at 42°C for 18 hrs with the addition of 5 x 10^5 cpm/ml of hybridisation probe.

Blots were washed according to the conditions of each experiment, as outlined in individual figure legends.

Embryonic Chick Muscle cDNA Library

An amplified E12 pectoral muscle λgt10 cDNA library was a generous gift from Dr. J. Jackson (Imperial College,

London). The initial library consisted of 8 x 10^5 independent clones.

Screening of E12 Chick Pectoral Muscle cDNA Library

E.coli strain C_{600} HFLA were grown overnight in MNB liquid including 0.2% Maltose. Cells were harvested at 4000rpm in a bench top centrifuge and resuspended in half the culture volume of 10mM $MgSO_4$.

c600 hfl_A = E.coli hsdR - $hsdm^+$ supE thr leu thi lacY1 tonA21 hflA150 (chr : Tn10)

Screening was carried out essentially as described by Huynh et al (1985). λgt10 recombinant phage were plated out either an 150mm or 90mm dishes, containing MNB agar.

MNB liquid:

Per litre
- 10g NZ amine
- 2g Casamino acids
- 10g NaCl
- 5g $MgSO_4$

MNB agar as above including 12g agar

Primary screening on large 150mm plates was at a density of 3.5 x 10^4 pfu/plate. For 150mm plates 600μl of C_{600} HFLA cells were infected, for 90mm dishes 200μl of C_{600} HFLA cells were infected. Plates were grown at 37°C overnight. Plates were chilled at 4°C for 2 hrs. Schleicher and Schuell BA85 nitrocellulose filters were used for adsorption of the plaques. Denaturation was

achieved by placing the filters in 0.5M NaOH/1.5M NaCl for 1 - 2 mins. Filters were then neutralised in 1M Tris pH 7.5/1.5M NaCl for 3 - 5 mins. After air drying filters were baked at 80°C for 2 hrs prior to prehybridisation.

Prehybridisation and Hybridisation for Library Screening

Prehybridisation and hybridisation were essentially as described for Northern blots.

Hybridisation buffer:

5 X SSPE

0.5% SDS

5 X Denhardts solution

100μg/ml heat denatured

Salmon Sperm DNA

Prehybridisation was at 55°C for 4 - 6 hrs. Hybridisation was in the above buffer overnight using 5ml per large filter and 3ml per small filter, with the addition of probe at 5 x 10^5 cpm/ml. Filters were washed as described in figure legends.

Preparation of λDNA

λDNA was purified from small scale liquid lysates, as described by Maniatis et al (1982).

Preparation of Vectors mp18 and mp19 for Cloning of Inserts Isolated from cDNA Library Screening

Vectors mp18 or mp19 (Biolabs) were digested with the appropriate restriction enzymes. Where appropriate, phosphate groups were removed by use of calf intestinal alkaline phosphatase (CIP) as described by Maniatis et al (1982).

Ligation of cDNA Fragments into EcoRI Cleaved Vector mp18

mp18 vector cleaved as above was mixed with a 3-fold molar excess of cDNA insert purified as above, ligation was in 1 x ligase buffer (Maniatis et al 1982). ATP was added to a final concentration of 1mM, 1 unit of T4 DNA ligase were added and ligation was allowed to proceed at room temperature for 3 hrs. The ligation mixtures were then used to transform E.coli strain TG1.

Production of Competent TG1 Cells

Competent TG1 cells were produced using the protocol of Maniatis et al (1982). 200μl aliquots of TG1 cells above were incubated at 4^{o}C with ligation mixes for 40 mins. The cells were then heat shocked at 42^{o}C for 5 mins and returned to ice. The transformed cells were plated onto 2TY plates, containing 20μl of 20mg/ml IPTG, and 20μl of 20mg/ml X-GAL. Plates were then incubated overnight at 37^{o}C.

Preparation of Single-stranded Template DNA

PEG/NaCl

20% (w/v) polyethylene glycol 6000

2.5M NaCl

2 x TY per litre

16g bactotryptone

10g yeast extract

5g NaCl

100ml of 2 x TY was innoculated with 1ml of an overnight culture of E.coli strain TG1, and dispensed into 1.5ml aliquots in glass 10ml culture tubes. Each tube was innoculated with a clear plaque taken from a plate of M13 transfectants. Tubes were shaken for 5 hrs vigorously at 37°C, and the cells then removed from the solution by centrifugation in an Eppendorf microfuge. The supernatant was added to 200µl PEG/NaCl, shaken and then left to stand at room temperature for 15 mins. Tubes were centrifuged for 5 mins, and all traces of PEG carefully removed before resuspension in 100µl TE. 50µl of phenol/chloroform was added to each tube and they were then left to stand for 15 mins prior to a chloroform extraction and ethanol precipitation at -20°C. Pelleted DNA was redissolved in 30µl TE and stored at -20°C.

Sequencing Reactions

The following reagents were used:

Primer Mix M13 17mer primer (Biolabs) at 2.5μg/ml
TM 100mM Tris-HCl pH 8.5
50mM $MgCl_2$
H_2O

mixed in a ratio of 1 : 1 : 1

NTP Mixes Obtained from P & L Biologicals Ltd.

Klenow Mix H_2O
0.2M DTT
Klenow (4μ/μl) obtained from BRL Ltd.
^{35}-S dATP obtained from Amersham International
SJ 304 mixed in a ratio of 14 : 2 : 2 : 1

Chase 1mM dNTP obtained from Amersham International Sequencing kit

Formamide Dyes 98% formamide
0.1% Xylene cyanol
0.1% bromophenol blue

Sequencing reactions were carried out according to the procedures used at the MRC, Cambridge (M. Hunter,

personal communication). To allow for scale up of the number of clones being sequenced at any one time, a microtitre plate (Falcon 8311) was used.

	A	T	G	C	A	T	G	C
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0

Etc.

To each well 2μl of primer mix plus 2μl of single-stranded template DNA was added. These were centrifuged for 20 secs to mix, covered with saran wrap, and then incubated at 55^{o}C in an oven for 1 hr. 2μl of dNTP mixes were dispensed into the appropriate wells using a Hamilton syringe, followed by 2μl of Klenow mix dispensed onto the side of the microtitre wells. The polymerase reaction was initiated by a quick spin and followed by incubation at 25^{o}C for 15 mins. 2μl of chase was added and the reaction continued for a further 15 mins at 25^{o}C. The chase reactions were stopped by the addition of 2μl formamide dyes and the microtitre dish then incubated at 80^{o}C in an oven for 15 mins to denature the DNA. During this procedure the microtitre dish was not covered by saran wrap so as to allow evaporation to concentrate the solutions in the microtitre wells. The denatured DNA was then run on sequencing gels.

Sequencing Gels

To maximise the number of bases that could be read from each gel buffer gradient gels were prepared as described by Biggin et al (1983).

Top Gel	6% acrylamide
	7M urea
	0.5 x TBE
Bottom Gel	6% acrylamide
	7M urea
	5 x TBE
1 x TBE	10.9g/l Tris/HCL
	5.4g/l Boric acid
	0.9g/l EDTA

60ml of top gel and 10ml of bottom gel were degassed and polymerised by the addition of 300μl 10% ammonium persulphate and 40μl TEMED, and 60μl 10% ammonium persulphate and 8μl TEMED respectively. Gradients were formed by drawing 6ml of top gel followed by 6ml of bottom gel into a syringe and introducing this to the bottom of a 33cm set of gel plates. Once an even layer had been formed top gel was added and a 'shark's tooth' comb containing 52 teeth clamped in an inverted position at the top of the gel. The plates were placed at an angle of 30^{o} to the horizontal, and left to set for 1 hr.

The plates were then clamped into a Searle 33 cm gel apparatus, the area behind the comb flushed with TBE and samples loaded behind the comb. The gels were run in 1 x TBE at 40w for 2.5 hrs, or for 15 mins after the bromophenol blue dye had run off the end of the gel. They were fixed in 10% acetic acid 10% methanol for 15 mins, transferred to Whatman 3MM paper, covered by saran wrap and dried on a Biorad A75 gel dryer at 80^{o}C for 30 mins. The saran wrap was removed and film placed in direct contact with the gel and exposed at room temperature for a period of 18 - 48 hrs.

RESULTS

Developmental Expression of Chick Pectoral Muscle AChR

The profile of AChR expression as determined by α Butx binding is shown in Figure (2.7). α Butx was found to be maximal at 14 days. Betz et al (1980) have analysed the developmental expression of the AChR within pectoral and posterior latissimum dorsi muscles and report a similar pattern of developmental expression as that shown in Figure (2.7), maximal expression being evident at E14 decling rapidly towards day 1 post hatch.

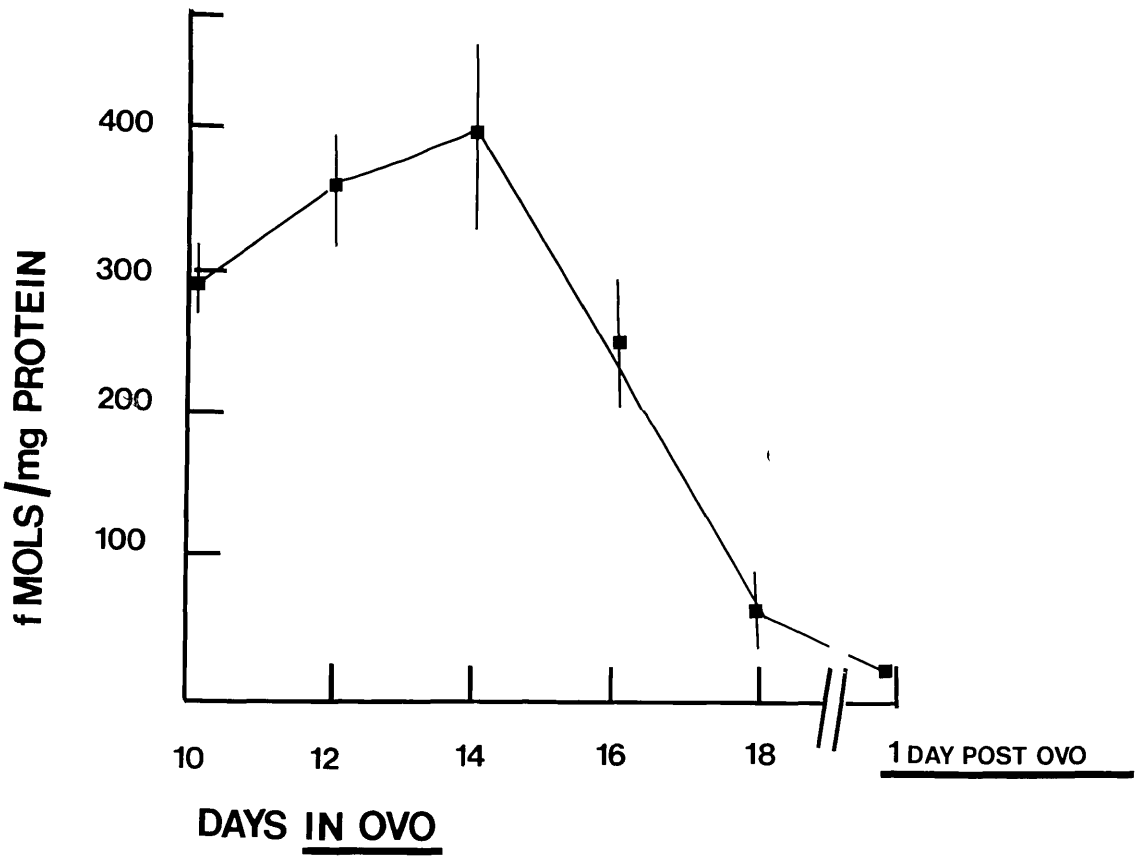
Isolation of mRNA from Chick Pectoral Muscle

Stringent precautions are required to extract undegraded biologically active mRNA from eukaryotic tissues. The methods currently available are dependent on rapid tissue disruption utilising potent chaotropic agents such as guanidinium thiocyanate (Chirgwin et al 1979), or LiCl/Urea (Auffray and Rougeon 1980). The method of choice employed in this study was that of Chirgwin et al (1979). This method was chosen primarily because of its simplicity and speed.

As a means of determining the integrity of total RNA produced above, samples of RNA were subjected to formamide gel electrophoresis followed by UV scanning. The distinct 28S and 18S RNA peaks (Figure (2.2))

FIGURE (2.7)

The developmental expression of the chick muscle AChR, as determined by α Butx binding. Crude receptor extract was labelled for 1 hr with 10nM ^{125}I α Butx, as described by Sumikawa et al (1981). Non specific binding was determined in the presence of a large excess of unlabelled toxin. The results are the mean of triplicate observations.



Error bars are +- s.e.m. of three observations.

demonstrate the integrity of the RNA isolated using this procedure.

Once reproducible total RNA preparations could be obtained , total RNA preparations were subjected to oligo (d)T cellulose chromatography to enrich for mRNA species. Messenger RNA preparations were then tested for integrity and biological viability by translation in rabbit reticulocyte lysate assays (Pelham and Jackson 1976). A time course of translation is shown in Figure (2.3) as judged by incorporation of ^{35}S L-methionine into acid insoluble material. Maximal incorporation was found after 40 mins incubation, decreasing after this period; 40 mins incubation was therefore routinely used in further assays. The products of translation were analysed by SDS-PAGE. The products of translation are shown in Figure (2.4). Thus the detection of high molecular weight proteins indicates that the mRNA prepared by the methods outlined are intact and biologically active. Several other methods are available for assaying the integrity of mRNA such as glyoxal gel analysis or conversion to cDNA. The advantage of the rabbit reticulocyte lysate assay is that it measures both the integrity and translational ability of mRNA preparations in a single assay and requires only sub microgram quantities of mRNA.

The Detection of Total AChR mRNA by Detection of α Butx Binding to Xenopus Oocytes Injected with Chick Pectoral Muscle mRNA.

It has been previously demonstrated that α Butx binding activity could be detected in Xenopus oocytes after injection of Xenopus oocytes with Torpedo mRNA (Sumikawa et al 1981). Comparison of the levels of AChR mRNA present within different tissue types have been made using the oocyte translation system (Barnard et al 1982). The different levels of AChR mRNA between Torpedo electric organ and feline denervated muscle have been shown to be directly comparable with the differences in receptor content of the two tissue types.

Changes in the levels of AChR in the developing chick pectoral muscle have been illustrated above, and also by Betz et al (1980). It is important for the purposes of cDNA cloning that the peak of AChR expression during chick pectoral muscle development corresponds with a peak in AChR mRNA levels. In collaboration with D. Beeson (Royal Free Hospital, London) mRNA preparations from different stages of pectoral muscle development were injected into Xenopus oocytes and assayed for α Butx binding. Translational activity was observed to vary considerably depending on the batch of oocytes used, and strict comparisons could only be made with oocytes from one donor. A plot of α Butx binding to extracts of oocytes microinjected with mRNA derived from chick

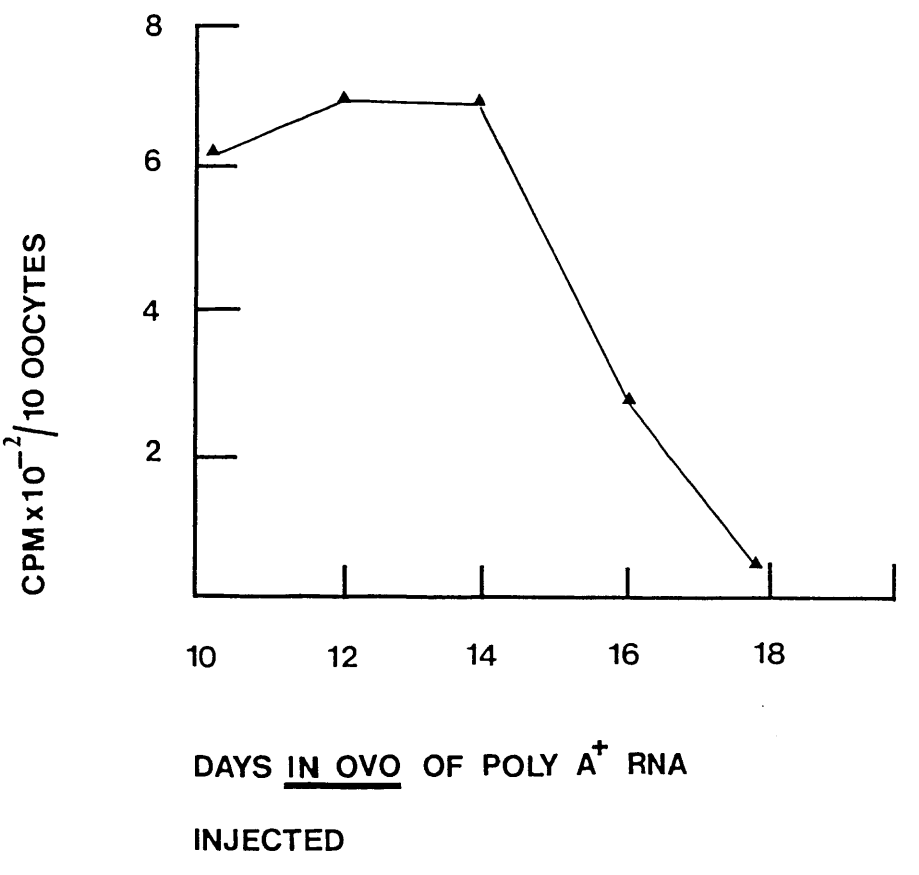
pectoral muscle is shown in Figure (2.8). The level of detectable α Butx binding was high from E12 - E14 and difficult to detect above background after E18. It is clear from these results that the highest level of AChR mRNA is present within pectoral muscle at E12 - E14. Furthermore, the level of translatable AChR mRNA directly corresponds to the level of AChR, suggesting AChR levels are controlled by transcriptional mechanisms. Messenger RNA preparations from E12 and E14 pectoral muscle were therefore used in further cloning studies.

Cross Hybridisation of Torpedo cDNAs to Chick Pectoral Muscle mRNA by Northern Blot Analysis

Cross hybridisation using Torpedo cDNA restriction fragments has proved an extremely efficient means of cloning AChR genes expressed in muscle, as reviewed earlier. Since the complete coding sequences of the α, γ and δ subunits of the chick muscle AChR have been elucidated (Nef et al 1988, Nef et al 1984), priority was given to obtaining chick β subunit cDNAs. As a result of this, the ability of a Torpedo β subunit cDNA probe to cross hybridise with chick muscle mRNA was tested. Northern blots were performed using mRNA derived from E12 and E14 pectoral muscle. Blots were hybridised with a cDNA probe; PT50 encoding from amino acid 45 to amino acid 277 of the mature β subunit from Torpedo californica (Hershey et al 1983). This probe contains the highly conserved putative transmembrane region M1, and also the

FIGURE (2.8)

Detection of α Butx binding in Xenopus oocytes injected with muscle mRNA. Batches of 20 or 40 oocytes were used per time point. After microinjection and incubation overnight in Barths media, oocytes were homogenised in phosphate buffer containing 2% TRITON X-100. After centrifugation the supernatant was assayed for α Butx binding activity, as outlined in Sumikawa et al (1981). Receptor-toxin complexes were purified by immuno-precipitation using rabbit anti-Torpedo AChR serum. The results represent the data derived from one experiment.



region 402 - 426, conserved in all AChR subunit sequences so far analysed (Buonanno et al 1987). Using low stringency hybridisation, three distinct mRNA species were detected of 3.6, 3.0 and 2.0Kb. No mRNA species were detected in the control tissues used. The probe hybridised very strongly to Torpedo electric organ mRNA (the lane in Figure (2.9) has been removed to prevent smearing) detecting a single mRNA species of 1.59Kb in accordance with Noda et al (1983b). The signal obtained from both E12 and E14 mRNA was similar suggesting the cross hybridising species identified using PT50 are of similar abundance at both days. These results suggest that the β Torpedo probe outlined would function as a suitable probe to clone AChR subunit cDNAs from chick pectoral muscle. From this data it is difficult to determine whether the PT50 probe is hybridising to one or more subunit mRNAs or that the different mRNA species are the product of a single gene. The most likely based on homology (Shibahara et al 1985) would be the β chick subunit gene product.

Screening of Embryonic Pectoral Muscle Library using β Torpedo cDNA Restriction Fragments

The library was screened essentially as for Northern blots. Initially 400,000 recombinant phage were screened. After washing at 0.1 x SSPE at room temperature for half an hour, filters were monitored with a hand held counter. If background counts persisted,

FIGURE (2.9)

Cross hybridisation of probe PT50 to chick pectoral muscle mRNA. RNA was denatured using 1M glyoxal, 50% DMSO, electrophoresed on a 1.0% agarose gel and transferred to a Gene creen membrane. The membrane was then hybridised in 50% Formamide, 5 X SSPE, 1 x Denhardts, 0.5% SDS, 100μg/ml salmon sperm DNA, 100μg/ml yeast tRNA. The blot was then hybridised with insert derived from plasmid PT50. Insert DNA was labelled to a specific activity of 8.5 x 10^8cpm/μg by random hexamer priming. Hybridisation was for 24 hrs at 42^{o}C. Prehybridisation was in the same buffer for 2 hrs. The blot was then washed in 0.1 x SSPE at room temperature for 1 hr, followed by 10 mins at 50^{o}C in 6 x SSPE. The blot was then exposed to Fuji film at -70^{o}C using an intensifying screen.

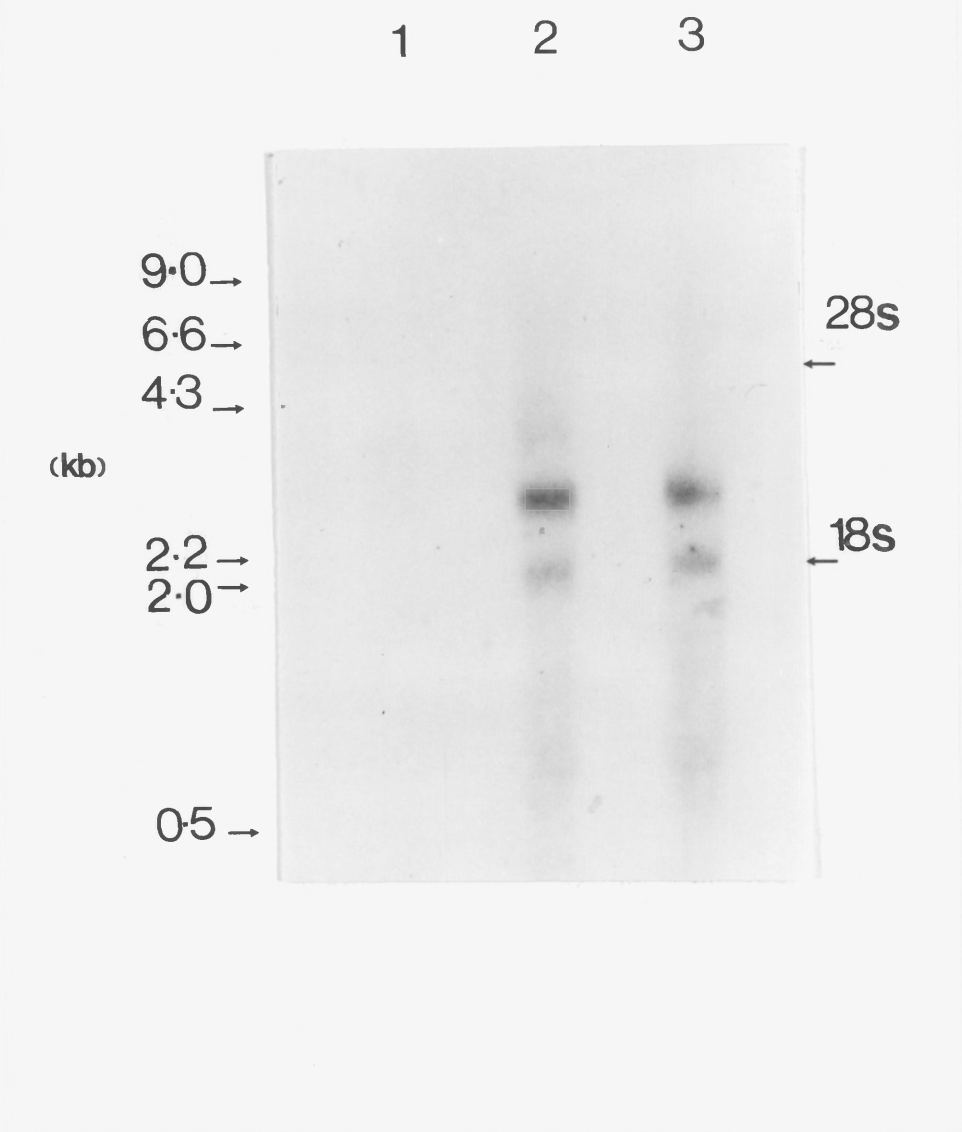
Track 1 fibroblast mRNA

Track 2 E12 pectoral muscle mRNA

Track 3 E14 pectoral muscle mRNA

The positions of chick ribosomal 28S and 18S are shown and also the positions of λHindIII DNA markers.

FIGURE (2.9)



filters were then washed at 6 x SSC at 50°C for 10 mins before exposure to film. Using these conditions 15 duplicating positives were detected. Phage corresponding to these hybridisation signals were picked using the large end of a Pasteur pipette. Phage were then rescreened at a density of between 500 - 1000pfu per 90mm dish. Phage DNA was then transferred to nitrocellulose and screened as before. All phage picked from the first round screen showed positive on rescreening. These positive clones were then classified according to hybridisation signal as strong, medium or weak intensity. Typical members of each of these hybridisation classes are shown in Figures (2.10) - (2.12).

Analysis of Insert Sizes of Positive Clones

Final fourth round screening (Figure (2.13)) resulted in all plaques on the plates showing hybridisation positive. Single plaques were then picked and used as a phage stock to produce small scale plate lysates from which phage DNA was isolated. Phage DNA was then digested with EcoRI restriction enzyme and the insert sizes were analysed on 1.0% agarose gels. The results of this analysis are shown for largest clones of each of the respective hybridisation classes in Table (2.1).

In order to determine the relationship of the clones to the Torpedo probe, and also to other cloned chick AChR genes, the largest clones from each of the hybridisation

FIGURE (2.10)

Second round positive clone λSM_1 (strong hybridisation intensity). Phage DNA was transferred to nitrocellulose, denatured and baked. Hybridisation was for 24 hrs at $55^{o}C$ in 5 x SSPE, 1 x Denhardts, 0.5% SDS, 100μg/ml SSDNA and 10^{6}cpm of labelled probe PT50 (specific activity approximately 8 x 10^{8}cpm/μg). Nitrocellulose filters were then washed at 0.1 x SSPE at room temperature for 1 hr followed by 10 mins at $50^{o}C$ 5 x SSPE. Filters were then exposed to Fuji film at $-70^{o}C$ for 24 hrs using an intensifying screen.

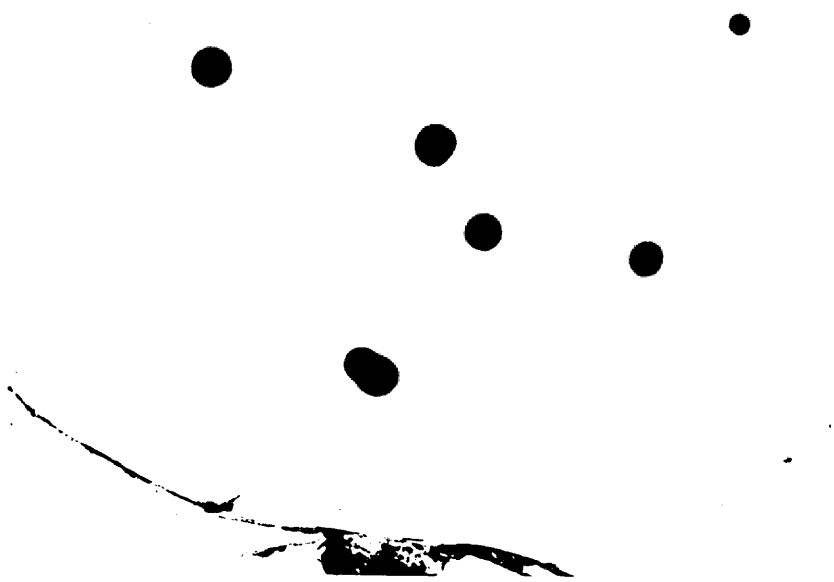


FIGURE (2.11)

Second round positive clone λSM_8 (medium hybridisation intensity). Hybridisation conditions were as described in Figure (2.10).

FIGURE (2.12)

Second round positive clone λSM_{14} (weak hybridisation intensity). Hybridisation conditions were as described in Figure (2.10).

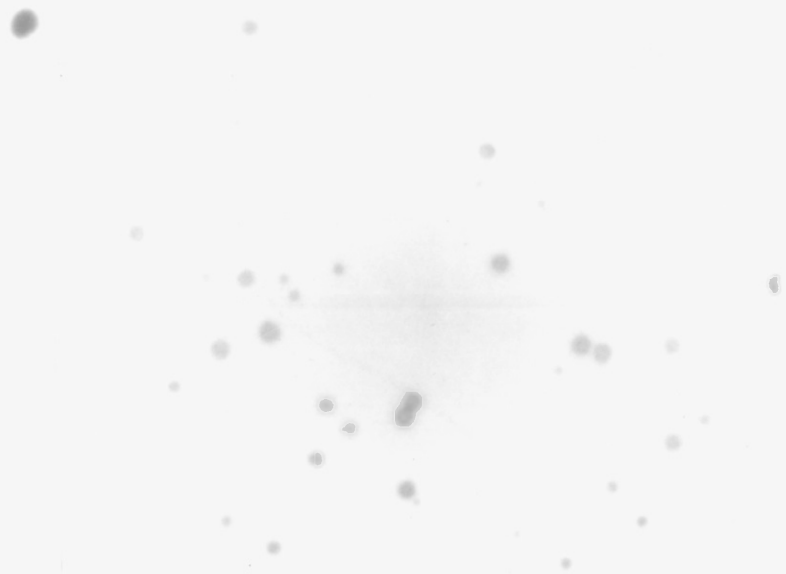


FIGURE (2.13)

Final round positive clone λSM_8 (medium intensity). Hybridisation conditions were as described in Figure (2.10).

TABLE (2.1)

CLONE	INSERT SIZE	HYBRIDISATION INTENSITY
λSM_1	1.9Kb	Strong
λSM_4	0.8Kb	Strong
λSM_7	1.1Kb	Strong
λSM_8	0.2Kb	Medium
λSM_9	0.4Kb	Medium
λSM_{13}	0.3Kb	Weak
λSM_{14}	0.5Kb	Weak

classes was cloned into vector mp18 or mp19. Colourless recombinant plaques were then used to produce single stranded template for DNA sequence analysis. Clones were sequenced by the dideoxy method of Sanger (1977), utilising gradient gels to maximise the amount of DNA sequence information that could be obtained. A typical sequencing gel is shown in Figure (2.14), both orientations of clone λSM15 can be seen, up to 325bp of sequence could be obtained routinely from high quality gels. The time of growth of M13 single strand cultures was found to be critical. Templates used for DNA sequence analysis produced after periods of greater than 5 hrs incubation resulted in high background, and therefore difficulty in interpreting DNA sequence.

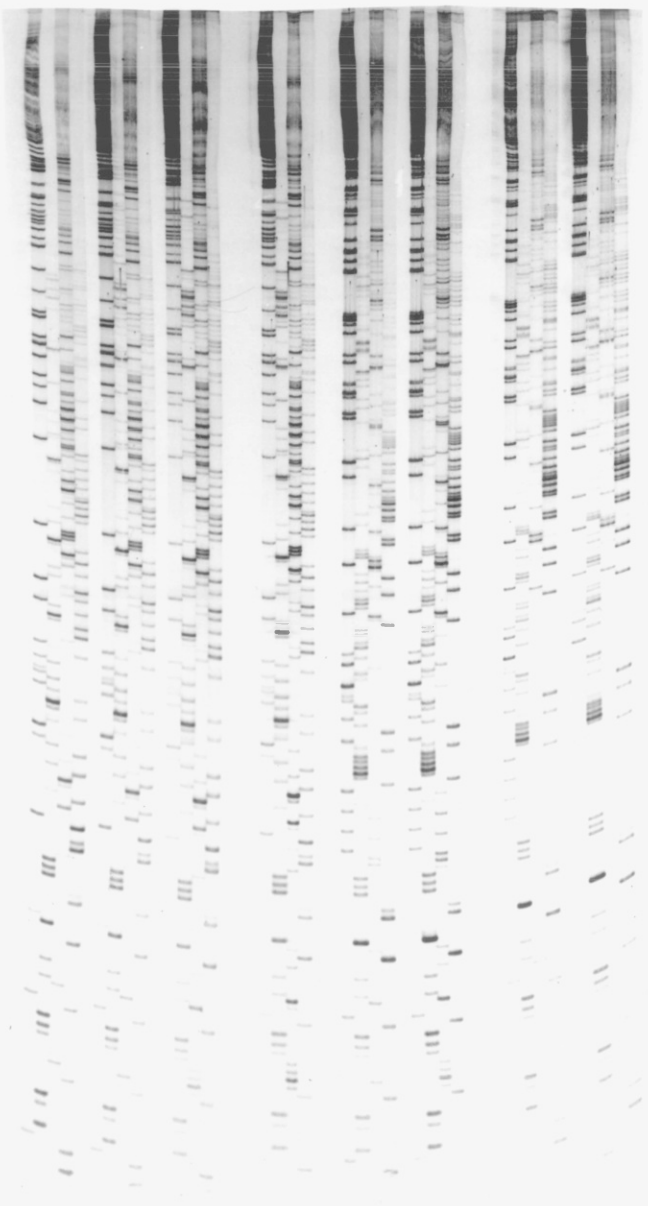
DNA Sequence Analysis

DNA sequence was analysed by the in-house Staden programmes at the MRC Centre, Cambridge.

The sequence information from both ends of clone λSM_1 showed that this clone encoded the α subunit of the chicken muscle AChR. 300bp of DNA sequence was read from each end of the respective clones. When DNA sequence was translated, the deduced amino acid sequence of the 5′ end of the clone was found to be identical with that reported by Beeson et al (1988) from amino acid -19 to amino acid 81. Presumably clone λSM_1 encodes the

FIGURE (2.14)

DNA sequence analysis of clone λSM_{14} cloned as an EcoRI fragment in vector mp18.



entire coding sequence of the α subunit mRNA. For further use of clone λSM_1 it was first necessary to determine its full sequence, this required extensive DNA sequencing. A full length α subunit cDNA clone was obtained from D. Beeson (Royal Free Hospital, London), to use in further experiments as a hybridisation probe. A restriction map of this clone is shown in Figure (2.15).

Clone λSM14 representing the largest clone from the weakest hybridising group of clones was subcloned into vector mp18 and sequence data was analysed as for the α subunit clone λSM_1. Due to the small size of this insert the entire DNA sequence was obtained on different strands (see Figure (2.14)). The actual size of this clone from DNA sequence analysis was found to be 498bp. The deduced amino acid sequence was found to be identical to that described by Nef et al (1984) encoding the δ subunit of the chick muscle AChR from amino acid 22 to amino acid 188 (results not shown). The homology of the α and δ clones isolated with the Torpedo probe was determined after alignment of the amino acid sequences (Figure (2.16)) over the entire length of the β Torpedo probe (Hershey et al 1983). The sequences were 42% and 46% identical to the β Torpedo sequence respectively for the α and δ subunits (See Figure (2.16)).

Both inserts from the medium intensity hybridisation group were cloned into vector mp18 and the entire sequence of both clones was determined on both strands.

FIGURE (2.15)

Restriction map of α subunit chick muscle cDNA (Beeson et al 1988).

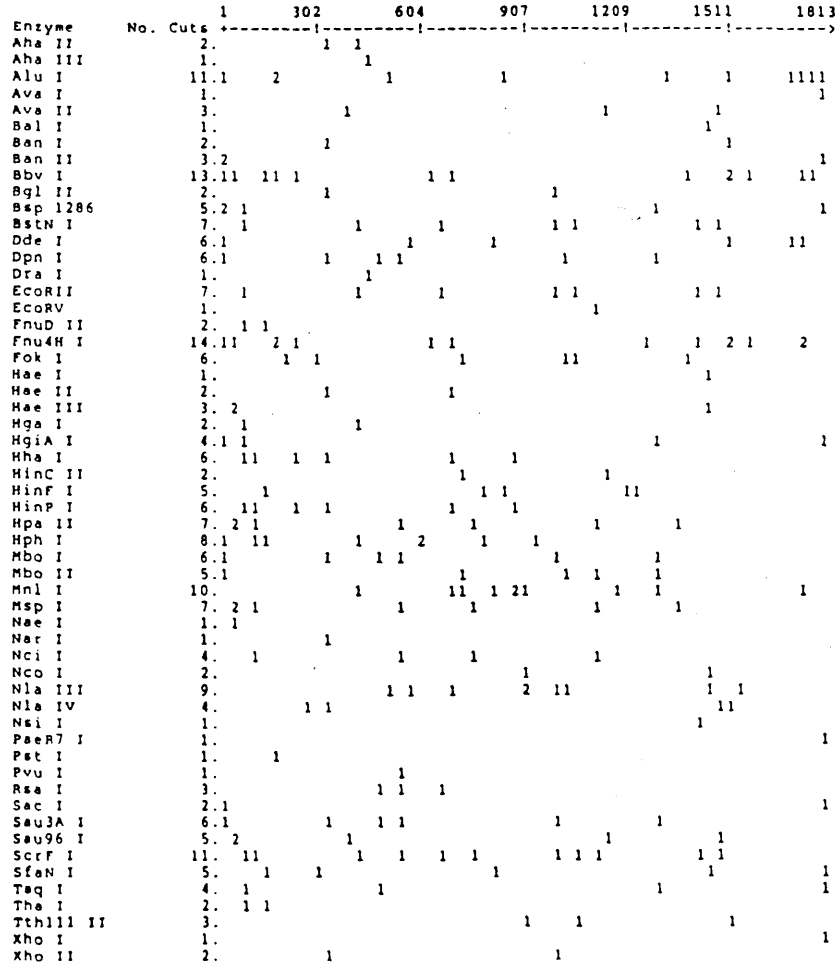
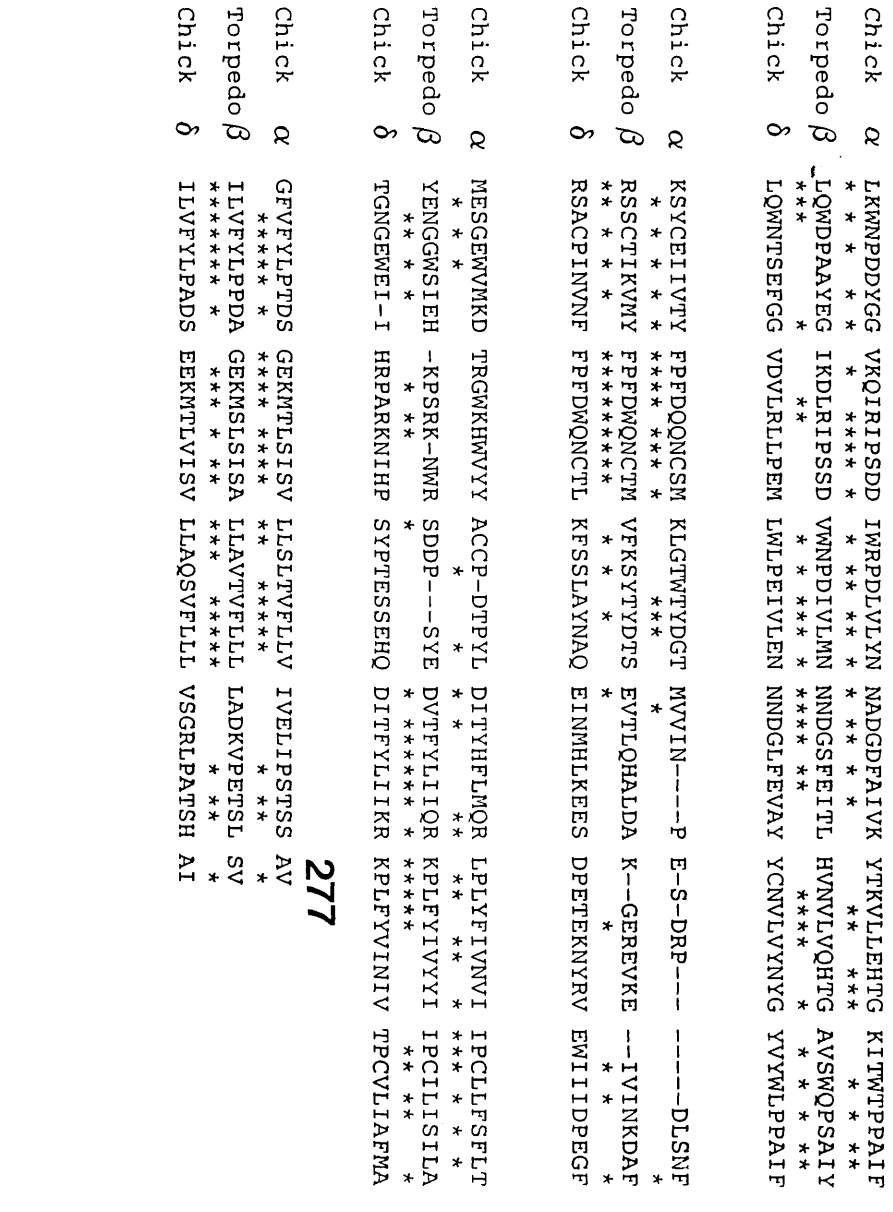


FIGURE (2.16)

Alignment of the protein coding sequence of chick muscle α subunit (Beeson et al 1988), Torpedo β subunit (Noda et al 1983a), and chick δ subunit (Nef et al 1984) from amino acids 44 - 277. * = identical residue. Gaps have been inserted to achieve maximal homology.



The sizes of these 2 clones were found to be 198bp for λSM_8 and 354bp for λSM_9 respectively. Significantly, linker sequence was found only at the 5' end of each of these clones. These two clones were found to overlap, the consensus sequence being shown in Figure (2.17), the smaller clone encoding amino acids 39 - 100. The first 14 amino acids of this sequence have the characteristics of a signal peptide. Unfortunately, the larger clone does not extend as far as the initiating methionine. However, the signal peptide has a characteristic central hydrophobic segment, and a good cleavage point at the alanine residue-1 in accordance with the rules of Heijne (1983). This makes the first amino acid of the protein encoded by this clone an alanine. In order to identify the protein encoded by the clones λSM_8 and λSM_9, the consensus protein sequence was aligned with the published sequence of AChR subunits from a number of species (Figures (2.19)). It is clear from this analysis that clones λSM_8 and λSM_9 encode the β subunit of the chick AChR since they show greatest homology to the β subunit from the bovine, i.e. 80% considering conserved amino acid substitutions. A further comparison was then made between the β chick subunit sequence and all other published β subunit sequences. The result of this analysis is shown in Figure (2.20). At the level of identity the β chick sequence shows highest homology to the murine and bovine sequences than to the Torpedo sequence as might be expected from an evolutionary standpoint. Clone λSM_9 was used as a probe to isolate

FIGURE (2.17)

Nucleotide and deduced amino acid sequence of clones λSM_8 and λSM_9.

```
  A  P  T  V  A  L  L  L  L  C  A  L  C  S  A  A  D  A  P  P
AGCTCCCACCGTTGCTCTCCTCCTCCTCTGTGCCCTCTGCAGCGCTGCCGACGCCCCCCC
        10        20        30        40        50        60

  P  P  P  S  A  A  A  S  A  D  G  A  Y  H  A  G  V  R  P  A
CCCCCCGCCGAGCGCTGCTGCAAGCGCTGATGGAGCGTACCACGCGGGGGTCCGCCCCGC
        70        80        90       100       110       120

  P  T  P  R  D  R  V  E  V  R  V  G  L  S  L  A  Q  L  V  S
CCCCACCCCACGGGACCGCGTGGAAGTGAGAGTGGGGCTGAGCCTGGCACAGCTCGTCAG
       130       140       150       160       170       180

  L  D  E  K  N  E  E  L  T  T  K  V  Y  L  D  L  S  W  W  D
TCTGGATGAGAAGAACGAGGAGCTGACCACCAAAGTGTACCTCGATCTGAGCTGGTGGGA
       190       200       210       220       230       240

  P  R  L  Q  W  D  P  H  D  Y  G  G  L  G  G  L  R  V  A  A
CCCCCGGCTGCAGTGGGACCCCCACGATTATGGGGGTTTGGGGGGGCTGCGCGTGGCTGC
       250       260       270       280       290       300

  S  R  L  W  L  P  D  I  G  L  D  N  N  N  D  G  E  F
ATCGCGACTGTGGCTGCCCGACATTGGGCTGGATAACAATAACGACGGTGAATTC
       310       320       330       340       350
```

FIGURE (2.18)

Alignment of nucleotide sequence of clones λSM_8 and λSM_9 and β Torpedo nucleotide sequence (Noda et al 1983).

```
          1          11          21          31          41          51
Chick  β  AGCTCCCACC GTTGCTCTCC TCCTCCTCTG TGCCCTCTGC AGCGCTGCCG ACGCCCCCCC
             *     * *    *   *  * *           *  * **** *  *   **  *
Torpedoβ  GATGGCGGTG GGTCTGGTGG TGATGATGGC GCTGGCCCTC AGCGGCGTGG GGGCGTCGGT
        1          11          21          31          41          51

         61          71          81          91         101         111
Chick  β  CCCCCCGCCG AGCGCTGCTG CAAGCGCTGA TGGAGCGTAC CACGCGGGGG TCCGCCCCGC
            *    *                          *   * ***  ** **   * * ** ** **
Torpedoβ  GATGGAGGAC ACACTCCTGA GCGTGCTGTT CGAGACCTAC AACCCGAAAG TGCGGCCGGC
         61          71          81          91         101         111

        121         131         141         151         161         171
Chick  β  CCCCACCCCA CGGGACCGCG TGGAAGTGAG AGTGGGGCTG AGCCTGGCAC AGCTCGTCAG
           *  **      * **    * *    *****  ********* * **** **  * *** ***
Torpedoβ  GCAGACGGTG GGCGATAAAG TCACGGTGAG GGTGGGGCTG ACCCTGACAA ACCTCCTCAT
        121         131         141         151         161         171

        181         191         201         211         221         231
Chick  β  TCTGGATGAG AAGAACGAGG AGCTGACCAC CAAAGTGTAC CTCGATCTGA GCTGGTGGGA
           *** ****  ** *  ** * ** *** *** ***  *** *  *  * ***    ***   **
Torpedoβ  CCTGAATGAA AAAATTGAAG AGATGAGCAC CAACCTGTTC TTGAACCTGG CGTGGACAGA
        181         191         201         211         221         231

        241         251         261         271         281         291
Chick  β  CCCCCGGCTG CAGTGGGACC CCCACGATTA TGGGGGTTTG GGGGGGCTGC GCGTGGCTGC
           *  *** *** ********** *    *  **  *  **  *     *  ** * ** *  *  *
Torpedoβ  CTACCGCCTG CAGTGGGACC CTGCTGCCTA CGAAGGCATC AAAGATCTCC GCATTCCCTC
        241         251         261         271         281         291

        301         311         321         331         341         351
Chick  β  ATCGCGACTG TGGCTGCCCG ACATTGGGCT GGATAACAAT AACGACGGTG AATTC
           **     *  **** ***** **** *  ** *   ****** ********     **
Torpedoβ  CTCCGACGTC TGGCAGCCCG ACATCGTCCT GATGAACAAT AACGACGGGT CCTTT
        301         311         321         331         341         351
```

FIGURE (2.19)

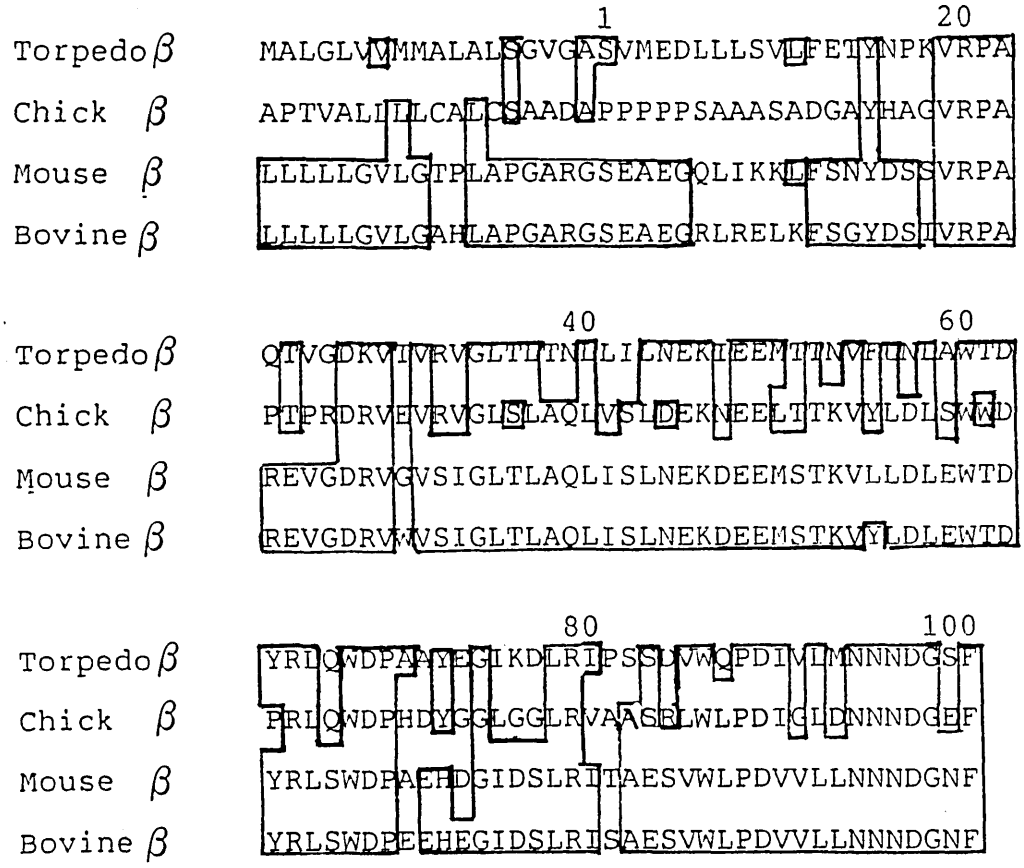
Alignment of the deduced amino acid sequence derived from the clone λSM_8, in comparison with that of AChR subunit cDNAS: α chick muscle (Beeson et al 1988), calf β (Tanabe et al 1984), chick γ and δ (Nef et al 1984) calf ε (Mishina et al 1985) and Torpedo β (Noda et al 1983).

	39	100	%I	%I+C
CH α	QLINVDEVNQIVTTNVRLKQQWTDINLKWNPDDYGGVKQIRIPSDDIWRPDLVLYNNADGDF		45	66
	39	100		
CA β	QLISLNEKDEEMSTKVYLDLEWTDYRLSWDPEEHEGIDSLRISAESVWLPDVVLLNNNDGNF		59	80
	39	100		
CH β	QLVSLDEKNEELTTKVYLDLSWWDPRLQWDPHDYGGLGGLRVAASRLWLPDIGLDNNNDGEF		56	74
	39	100		
CH γ	NLISLNEREETLTTNVWIEMQWSDYRLRWDPDKYDDIQQLRVPSAMVWLPDIVLENNIDGTF		50	74
	41	102		
CH δ	NLISLKEVDETLTTNVWVEQSWTDYRLQWNTSEFGGVDVLRLLPEMLWLPEIVLENNNDGLF		51	75
	39	100		
CA ε	NLISLNEKEETLTTSVWIGIDWQDYRLNYSKGDFGGVETLRVPSELVWLPEIVLENNIDGQF		48	77
	39	100		
TOR β	NLLILNEKIEEMTTNVFLNLAWTDYRLQWDPAAYEGIKDLRIPSSDVWQPDIVLMNNNDGSF		100	100

All sequences were compared to that of beta Torpedo. I = identity, C = conserved.

FIGURE (2.20)

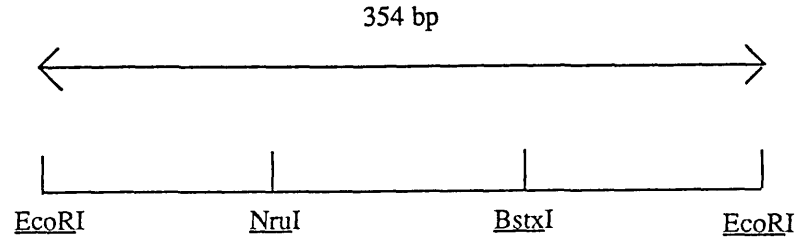
Alignment of the deduced amino acid sequence derived from the clone λSM_9 in comparison with that of AChR subunit cDNAs: β Torpedo (Noda et al 1985), calf β (Tanabe et al 1984), β mouse (Buonanno et al 1987) deduced amino acid sequences.



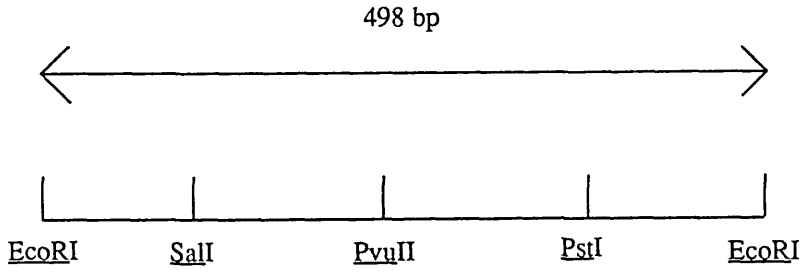
the larger clones encoding the remainder of the chick β subunit. After subcloning this insert into plasmid p-GEM1, and screening 400,000pfu of the cDNA library described previously, no larger clones were identified. The reason for this could possibly be due to an internal EcoRI site as indicated by the lack of linker sequence at the 3′ end of both clones λSM_8 and λSM_9. If the methylation step during library synthesis was not successful, then the 3′ end of the β subunit cDNA would be lost and not detected using λSM_9 as a probe. The cDNAs isolated while not all being complete, were sufficient for use as hybridisation probes in the following experiments. The partial sequence was used to generate restriction maps of the β and δ cDNAs and these are shown in Figure (2.21).

FIGURE (2.21)

Restriction maps of clones λSM9 (β subunit cDNA) and λSM14 (δ subunit cDNA).



λSM 9



λSM14

DISCUSSION

The isolation of cDNAs is dependent on the abundance of the particular mRNA of interest. The high abundance of AChR mRNA within the Torpedo electroplaque facilitated the cloning of the four cDNAs encoding the Torpedo AChR (Sumikawa et al 1982c, Noda et al 1983a,b). The density of AChR within innervated skeletal muscle is 1000 times less than that found within the Torpedo electroplaque (Sumikawa et al 1982a). The ability to clone muscle AChR cDNAs has therefore been dependent on finding muscle tissues enriched with receptor RNA. Fetal or denervated muscle has been demonstrated to be highly enriched in both AChR and receptor mRNA (Barnard et al 1982, Mishina et al 1985). Denervated or embryonic muscle has therefore been used by numerous groups to clone cDNAs encoding the four subunits of the muscle AChR.

The developmental expression of the chick pectoral muscle AChR was examined in this Chapter, and the results demonstrate that embryonic pectoral muscle is highly enriched in AChR in accordance with the earlier observations of Betz et al (1980). Measurements of receptor mRNA utilising Xenopus oocytes demonstrated further that embryonic pectoral muscle also contains high levels of the receptor mRNAs. The correlation of receptor mRNA levels and AChR protein levels suggests that receptor expression is controlled at least in part by transcriptional mechanisms. This observation is

discussed at length in later Chapters.

Cross hybridisation has proven an extremely successful method of obtaining cDNAs and genomic clones encoding muscle AChR subunits using Torpedo cDNAs as hybridisation probes, as reviewed earlier. Using a Torpedo β subunit cDNA partially encoding from within the extracellular domain to within the putative transmembrane domain M_1 (Hershey et al 1983) under conditions of low stringency, it was possible to isolate cDNAs encoding the α, β and δ subunits of the chick muscle AChR. The degree of homology between the Torpedo β subunit cDNA and the chick cDNA clones was 56% for the β subunit cDNA and between 41% - 46% for the α and δ subunit cDNAs, at the level of identical amino acids. Using similar hybridisation conditions Nef et al (1988) have isolated cDNAs encoding neuronal AChRs with up to 70% mismatch with the probe.

The degree of homology between AChR subunits is greatest within the putative transmembrane regions M_1, M_2, M_3 and M_4 (Shibahara et al 1985). As demonstrated by the results in this Chapter, AChR subunits also exhibit very high homology between amino acids 30 - 150. Buonanno et al (1987) have used the region between the cysteine residues at 128 and 142 to isolate β and γ subunits encoding the murine AChR. As yet, however, no functional significance has been assigned to these regions, although the asparagine residue at position 141 is the only site available for N-glycosylation within each AChR subunit

sequence. It is perhaps of great significance that the cysteine "bridge" region is conserved in other ligand-gated integral ion channel receptor subunits. It is found within both subunits of the $GABA_A$ receptor and also within the 48K subunit of the strychnine sensitive glycine receptor (Schofield et al 1987, Grenningloh et al 1987)

CHAPTER 3

DEVELOPMENTAL EXPRESSION OF THE 4 SUBUNIT mRNA S ENCODING THE CHICK PECTORAL MUSCLE AChR

INTRODUCTION

The level of AChR expressed within muscle changes during myogenesis upon innervation and in response to muscle denervation (Fambrough 1979). Early during myogenesis, as myoblasts fuse to form multinucleated myotubes, AChRs appear over the entire surface of the newly formed myotubes. The receptors are incorporated throughout the extent of the membrane and reach a density of about two hundred per square micron. As muscle/nerve contacts are first made, AChRs begin to form distinct clusters around the site of innervation. In part this is due to lateral migration from extra-junctional regions, and in part due to synthesis of new AChR molecules (Burden 1977a,b, Salpeter 1987). After the establishment of receptor clusters first detected in the chick embryo at day E10 (Burden 1977a), the density of extra-junctional receptors starts to decline. In the chick this occurs from day E16 and continues to decline after hatching. In mature innervated chicken muscle (3 weeks post hatch), AChRs are almost exclusively localised to the neuromuscular junction at levels 1,000 - 10,000 times of that in extra-junctional regions (Fertuck and Salpeter 1976, Salpeter and Loring 1985). While these events are taking place, certain properties of the receptors biochemistry and physiology also change, as reviewed in the Introduction.

To some extent these developmental changes are reversible

if synaptic activity is blocked by either muscle denervation (reviewed by Fambrough (1979), see Chapter 4) or by a pharmacological blockade of the AChR (Betz et al 1980). Both of these results demonstrate the importance of electrical activity in regulating the level of AChR in developing muscle.

Comprehension of the mechanisms regulating the genes encoding the AChR oligomer in relation to myogenesis and innervation is a prerequisite to the effective study of AChR expression. With cDNA or genomic clones that encode the various subunit mRNAs, it is possible to measure the amount of mRNA in a given tissue. Thus, the natural development of chick embryo muscles, where the amount of AChR synthesis is stimulated upon innervation and subsequently declines as synaptogenesis proceeds, provides an ideal system to examine the regulation of AChR expression.

METHODS AND MATERIALS

Clones p8 and p9 were generous gifts from M. Ballivet (University of Geneva, Switzerland). Clone PCDB δ was a gift from D. Beeson (Royal Free Hospital, London). Enzymes and plasmids were from either Promega Biotec Limited or Biolabs Limited. All other reagents were of the highest grade commercially available.

Maintenance of Embryonic Chickens

Chick embryos were maintained as described in Chapter 2. Pectoral muscle were dissected and stored at -70°C before use. The number of embryos per time point was sufficient to give 2g of tissue. At E10 25 embryos were used, while at E18 5 embryos were sufficient.

Isolation of mRNA

Total RNA was isolated by the method of Chirgwin et al (1979). Messenger RNA was isolated by 2 rounds of oligo (d)T cellulose chromatography (Aviv and Le der 1972). Both of these methods are described in Chapter 2.

Standardisation of mRNA by ^{3}H Poly(U) Assay

Messenger RNA preparations were first quantified by UV spectroscopy, the comparative levels of poly(A)$^{+}$RNA were measured by poly(U) assay (Bishop et al 1974). ^{3}H

poly(U) was from New England Nuclear (50mCi/mmol) and was between 47 - 147 ribonucleotides in length. Poly(A) was from Pharmacia and was 100 ribonucleotides in length. Poly(A) samples between 0 - 15ngs were used as standards, in triplicate. Samples of mRNA between 0.01 - 0.1μg and of total RNA between 0.1 - 1.0μg were used for calibration.

Each sample was added to 2ml of 2 x SSPE containing 5μl of ^{3}Hpoly(U) and vortexed briefly. Samples were placed at 45^{o}C for 10 mins and then on ice for 10 mins. 20μl of boiled RNase A (1mg/ml) was added and the incubation period was extended for a further 15 mins. 100μl of 1mg/ml BSA was added and the poly(A) - poly(U) hybrids were precipitated by addition of 220μl of 100% w/v T.C.A. followed by incubation on ice for 5 mins. This last step was carried out in batches of no more than 10 samples. The acid insoluble material was then collected by filtration onto GF/C Filters, washed in 15mls of ice cold 10% T.C.A. (w/v), dried and counted in a toluene-based scintillant.

Northern Blots

Northern blots were performed as described in Chapter 2. Conditions of hybridisation varied according to the probe type used. Prehybridisation for both RNA and DNA probes was at 42^{o}C, in 50% Formamide, 5 x Denhardts, 0.5% SDS, 100 μg/ml denatured sonicated salmon sperm DNA, 100μg/ml

tRNA for 6 hrs. Hybridisation using DNA probes was in the same buffer with the inclusion of 1 x 10^6cpm/ml of radiolabelled probe. Hybridisation was for 24 hrs at 42°C. Blots using RNA probes were prehybridised as above. Radiolabelled RNA was then added to 1 x 10^6cpm/ml and the temperature of hybridisation was increased to 60°C. Blots were then hybridised for 18 hrs. Washing of blots was as described in individual figure legends.

Hybridisation Probes

Subunit specific hybridisation probes for the α, β and δ subunits were derived from the cDNA clones described in Chapter 2. The γ subunit probe utilised was the clone p9. This genomic clone extends from within exon 2 to beyond exon 6 of the chicken γ subunit gene (Nef et al 1984). p9 was provided as a PstI fragment cloned in the polylinker of plasmid pUC18.

The β and δ cDNAs described in Chapter 2 were cloned as EcoRI fragments (isolated from M13 RF DNA) into EcoRI cleaved and phosphatased vector p-GEM2. Clones were then orientated by restriction analysis. The β clone was orientated via an asymmetric NruI site. The δ clone was orientated via an asymmetric SalI site. For convenience all constructs were orientated to allow "sense" transcription from the SP6 promoter. The structure of plasmids PCSMβs and PCSMδs is shown in Figure (3.1).

The γ probe p9 was released from pUC18 as a 639bp ClaI/PstI fragment encoding from exon 2 to within exon 5 of the chick muscle γ subunit (Nef et al 1984), and cloned into plasmid p-GEM1 digested with AccI and PstI to yield plasmid PCSMγs. The 1817bp, α subunit cDNA, was digested with EcoRI/HincII to yield a 725bp fragment coding from within the 5'untranslated region to amino acid 217 of the mature α subunit and was cloned into EcoRI/HincII cut p-GEM2 to yield plasmid PCSMαs. The structure of plasmids PCSMαs, PCSMβs, PCSMγs and PCSMδs is shown in Figure (3.1).

Subcloning Procedures

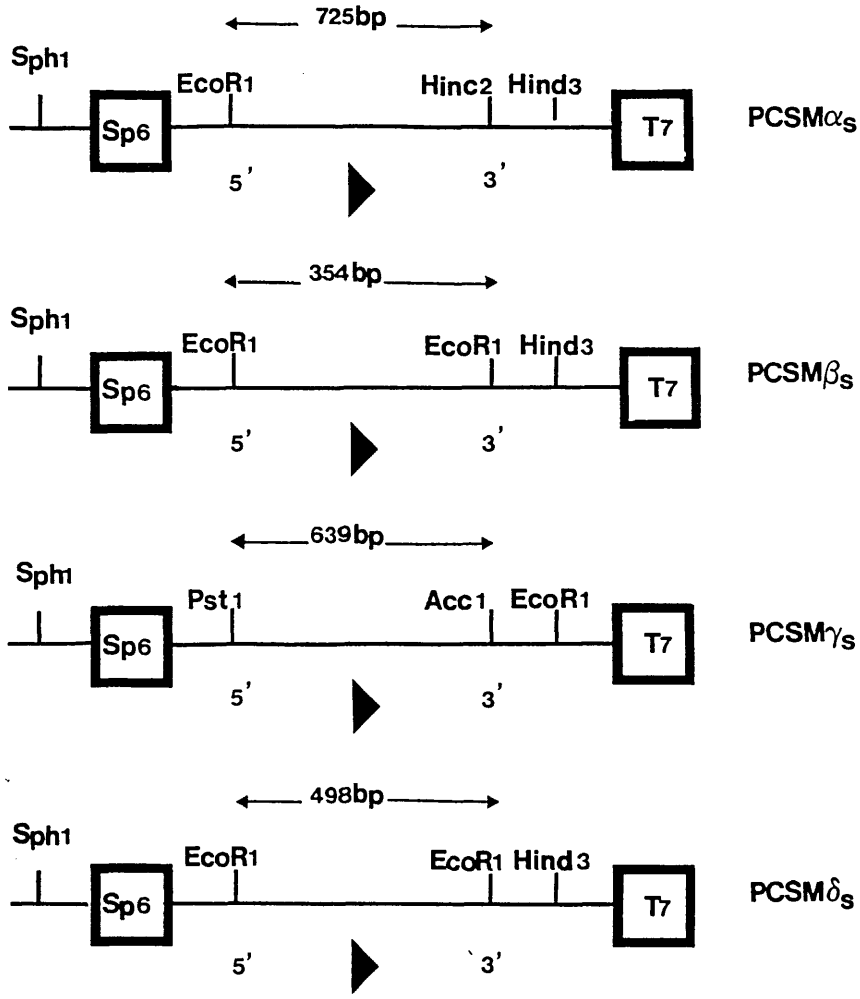
Standard manipulations such as restriction digests, transformations, ligations, growth, maintenance and preservation of bacterial strains and the preparation of media and standard buffers were all described in Chapter 2 or as by Maniatis et al (1982). All plasmids were transformed into bacterial strain HW87 (British Bio-technology Ltd).

Isolation of RF DNA of β and δ cDNA Clones

10ml small scale M13 RF cultures were grown as described by M. Goedert (Laboratory of Molecular Biology Cambridge, personal communication), and RF DNA was isolated by the alkali lysis method of Birnboim and Doly (1979). Purified RF DNA (the yield was typically between 5 - 10μg

FIGURE (3.1)

Restriction maps of clones PCSMαs, PCSMβs, PCSMγs and PCSMδs.



per 10ml culture) was resuspended in TE and digested with EcoRI. The respective inserts were then purified on 1.2% low melting point agarose gels, as described in Chapter 2. Ligations of inserts into plasmids p-GEM1 or p-GEM2, transformation and production of competent HW87 cells was described in Chapter 2.

Screening of Transformants

Ampicillin resistant transformants were screened by restriction analysis. Plasmid DNA was purified by alkali lysis (Birnboim and Doly 1979). DNA was resuspended in TE and digested with the appropriate restriction enzyme. Digests were analysed on 1.2% agarose gels. Clones were orientated such that "sense" transcription was always from the SP6 promoter (see Figure (3.1)).

Preparation of Template DNA for In Vitro Transcription

10μg of plasmids PCSMαs, PCSMβs and PCSMδs were linearised by digestion with HindIII. PCSMγs was linearised by digestion with EcoRI. The digests were then extracted with an equal volume of phenol (equilibrated with TE), the aqueous phase was then ethanol precipitated. Linearised plasmids were then resuspended in 100μl of DEPC treated water (Chapter 2) and stored in 20μl aliquots until used.

Production of Sense RNA Standards

5 X TB: 200mM Tris pH7.5
30mM $MgCl_2$
10mM Spermidine
100mM NaCl

2μg of the appropriate linearised template was incubated in 1 x TB buffer, 20 units RNAsin, 20 units S 6 (Promega Biotec) 500μM of rATP, rGTP, rCTP, rUTP, 10mM DTT. The final reaction volume was 100μl, and the reaction was allowed to proceed for 2 hrs at 40°C . 1 Unit of DNase1 (Promega Biotec) was added and the reaction was allowed to proceed for a further 15 mins. The sample was then extracted with an equal volume of phenol (equilibrated with TE) and ethanol precipitated. Unincorporated ribonucleotides were removed using G25 mini columns as described by Maniatis et al (1982). The yield of RNA was determined by UV spectroscopy and was typically between 7 - 10μg of RNA.

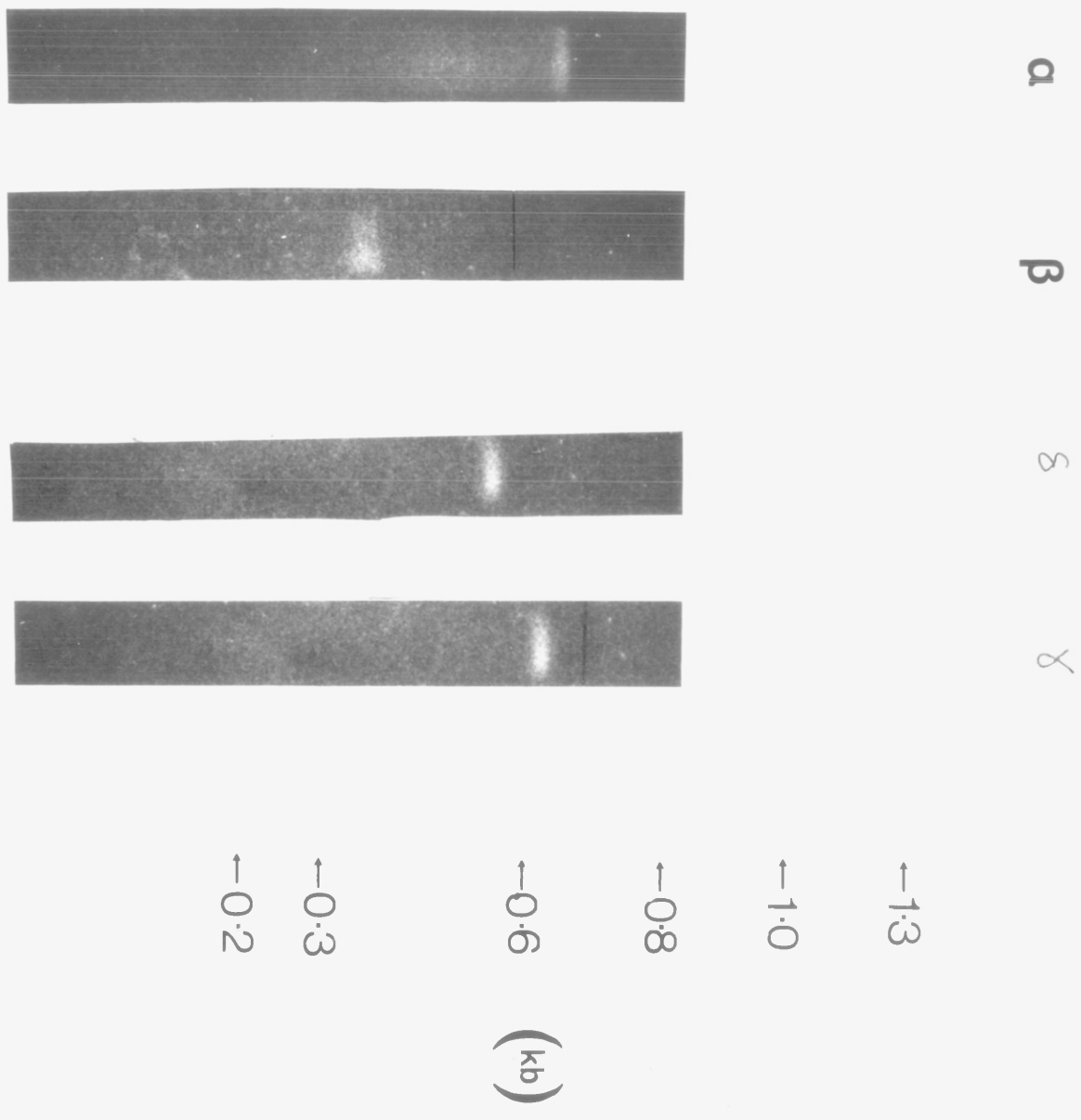
Determination of Sense Standard Integrity

The size of the RNA standards produced was determined by glyoxal gel electrophoresis. 1μg of each template was glyoxalated as for Northern blots (see Chapter 2), and then electrophoresed on 1.1% agarose gel. The gel was then stained with ethidium bromide (see Chapter 2). A typical result is shown in Figure (3.2).

FIGURE (3.2)

Denaturing gel analysis of sense standards. 1μg of RNA was denatured in 1M glyoxal/50% DMSO and electrophoresed on a 1% Agarose gel, and stained with Ethidium bromide.
Track 1 RNA produced by in vitro transcription of plasmid PCSMαs
Track 2 RNA produced by in vitro transcription of plasmid PCSMβs
Track 3 RNA produced by in vitro transcription of plasmid PCSMγs
Track 4 RNA produced by in vitro transcription of plasmid PCSMδs
Molecular weight markers were derived from a HaeIII digest of ϕ x 174 DNA.

FIGURE (3.2)



Production of Labelled Antisense Probes by In Vitro Transcription

Labelled antisense RNA was produced by transcription using the T7 promoter using SphI linearised PCSMαs, PCSMβs, PCSMγs and PCSMδs. Before being used as templates, the SphI site was converted to a blunt end to prevent transcription anomalies (Melton 1984). This was achieved using the Klenow fragment of E.coli DNA polymerase I, essentially as described by Maniatis et al (1982). The transcription mixture (10μl final volume) had the following composition:

2μl of 5 x TB
1μl of 100mM DTT
0.5μl of 10mM rGTP, rATP and rGTP
10 units RNasin
10 units of T7 polymerase
5μl of ^{32}P-UTP 400 Ci/mmol

After incubation at 40°C for 1 hr the reaction mixture was phenolextracted and ethanol precipitated. Unincorporated nucleotides were removed using G25 mini columns. Specific activity was determined by dotting a small sample of the probe before purification on to a DE81 filter. Unincorporated counts were removed by washes with 0.5M $Na_2 HPO_4$. Specific activities in excess of 8 x 10^8cpm/μg were achieved.

Production of Labelled Double Stranded Probes

Plasmids PCSMβs and PCSMδs were digested with EcoRI. PCSMαs was digested with HincII/EcoRI, PCSMγs was digested with BamHI/PstI. Insert DNA was purified on low melting point agarose gels followed by phenol extraction (as described in Chapter 2). Purified inserts were then labelled to high specific activity by the random hexamer priming (Feinberg and Vogelstein 1983), as described in Chapter 2.

SP6/T7 Quantitative Dot Blots

10 - 1·25 μg of mRNA and 2ngs - 1pg of the relevant sense standard were denatured as for Northern blots, tRNA was included to ensure that the RNA concentration remained constant. After 1 hr at 50°C an equal volume of ice cold 20 x SSPE was added and the samples were chilled on ice for 10 mins. The samples were then applied to a Gene Screen membrane equilibrated with 10 x SSPE using a Bio-rad Bio-dot apparatus. Samples were then allowed to absorb at room temperature for 1 hr. After binding the blot was washed under vacuum with an excess of 10 x SSPE, followed by 1M ammonium acetate. The filter was then baked for 2 hrs under vacuum. Prehybridisation was as for Northern blots. Hybridisation was with the relevant antisense probe or DNA probe and was as described for Northern blots. Filter washing and autoradiography was as described in individual figure legends.

Quantitation of Hybridisation Signals

Hybridisation signals were quantified by densitometric scanning using a Joyce lobel gel scanner, as described in Chapter 2.

Values were compared statistically by Student's t-test.

RESULTS

Determination of the AChR Subunit mRNA Sizes in E13 Chicken Pectoral Muscle

The sizes of the respective mRNAs were determined by Northern blotting. Messenger RNA from E13 pectoral muscle was denatured and transferred to Gene Screen membranes and probed with the respective subunit probe, labelled by random hexamer priming.

Using an α subunit specific probe encoding from within the prepeptide to amino acid 217, a major mRNA species of 3.2Kb was detected (Figure (3.3)). A size of 2.8Kb for the α subunit mRNA has been reported previously by Klarsfeld and Changeux (1985). Additional mRNA species of 4.2 and 5.9Kb were also observed on further exposure of autoradiograms. The level of these larger mRNA species was less than 15% of that of the more abundant 3.2Kb species as determined by scanning densitometry. These larger mRNA species do not result from cross hybridisation to other subunit mRNAs (discussed below). Multiple RNA species encoding AChR subunits have been observed in other species. Murine α (Boulter *et al* 1985), Bovine γ (Takai *et al* 1984), *Torpedo*, Murine and Bovine δ (Hershey *et al* 1983, LaPolla *et al* 1984, Kubo *et al* 1985). In the case of the Bovine γ subunit two mRNAs of 2.0 and 3.7Kb were observed. Takai *et al* (1984) were able to clearly demonstrate that the larger species

FIGURE (3.3)

Determination of size of mRNA encoding the α subunit in E13 pectoral muscle.

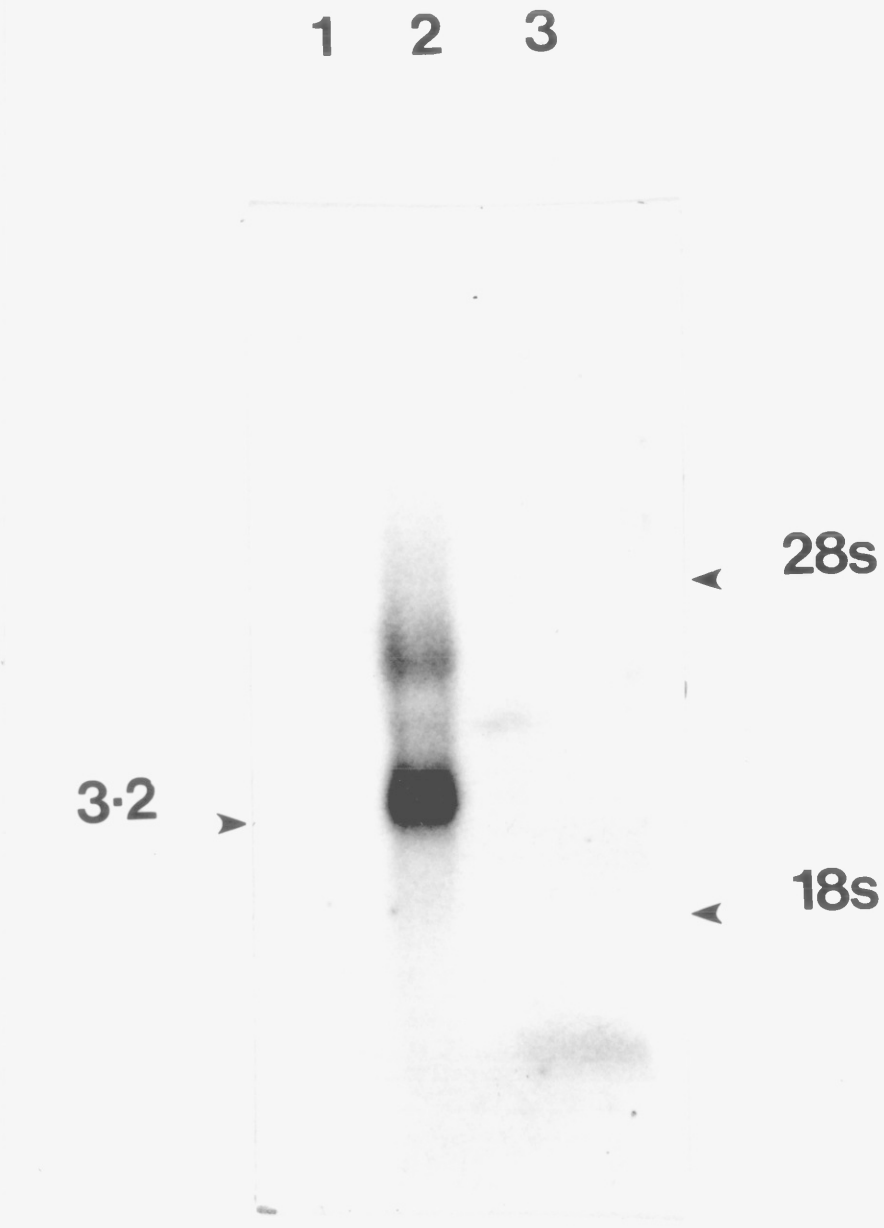
Track 1 10μg of heart mRNA

Track 2 10μg of E13 pectoral muscle mRNA

Track 3 10μg of fibroblast mRNA

were denatured in 1M glyoxal/50% DMSO electrophoresed on a 1.1% Agarose gel and transferred to a Gene Screen membrane. The blot was then hybridised for 24 hrs at 42°C in 50% Formamide 5 x SSPE, 0.5% SDS, 100μg/ml salmon sperm DNA and radiolabelled probe derived from plasmid PCSMαs, labelled by random hexamer priming to 1.1 x 10^9 cpm/μg at 10^6 cpm/ml. Prehybridisation was in the same buffer without probe for 2 hrs. The blot was then washed at 65°C in 0.1 x SSPE for 1 hr before exposure to Fuji X-ray film at -70°C for 24 hrs. The migration of 28S and 18S chick ribosomal RNA is also shown.

FIGURE (3.3)



resulted from incomplete processing as it hybridised specifically to an intron probe. In the case of the Torpedo δ mRNAs it is most likely that the pattern arises from the occurrence of multiple poly adenylation signals, since the larger mRNA species is the most abundant. By analogy to these examples it is most probable that the larger mRNA species detected by Northern blotting represent unprocessed forms of the 3.2Kb species detected. More conclusive evidence of this is provided in Chapter 5. Using RNase protection analysis it was possible to detect the presence of intron sequences specific to the α subunit gene in pectoral muscle RNA.

Single mRNA species of 2.8 and 1.8Kb were observed respectively for the β and δ subunit mRNAs, using a β subunit cDNA probe encoding from within the signal sequence to amino acid 100, and the modified γ subunit specific probe P9 (Figures (3.4) and (3.5)). Using a cDNA probe encoding from amino acids 22 - 182 of the mature δ subunit 2 mRNA species of 2.3 and 1.9Kb (Figure (3.6)) were detected. The significance of this observation is discussed below with respect to the developmental expression of the δ subunit mRNA.

These differences in mRNA sizes permit the unambiguous identification of each subunit mRNA encoding the pectoral muscle AChR and exclude cross-hybridisation, which would complicate interpretation of data in the following experiments. In addition, the failure to detect any

FIGURE (3.4)

Determination of size of mRNA encoding the β subunit in E13 pectoral muscle.

Track 1 10μg of heart mRNA

Track 2 10μg of E13 pectoral muscle mRNA

Track 3 10μg of fibroblast mRNA

Conditions were as described in Figure (3.3), except that the blot was hybridised with insert derived from plasmid PCSMβs, labelled by random hexamer priming to 7.5×10^8 cpm/μg.

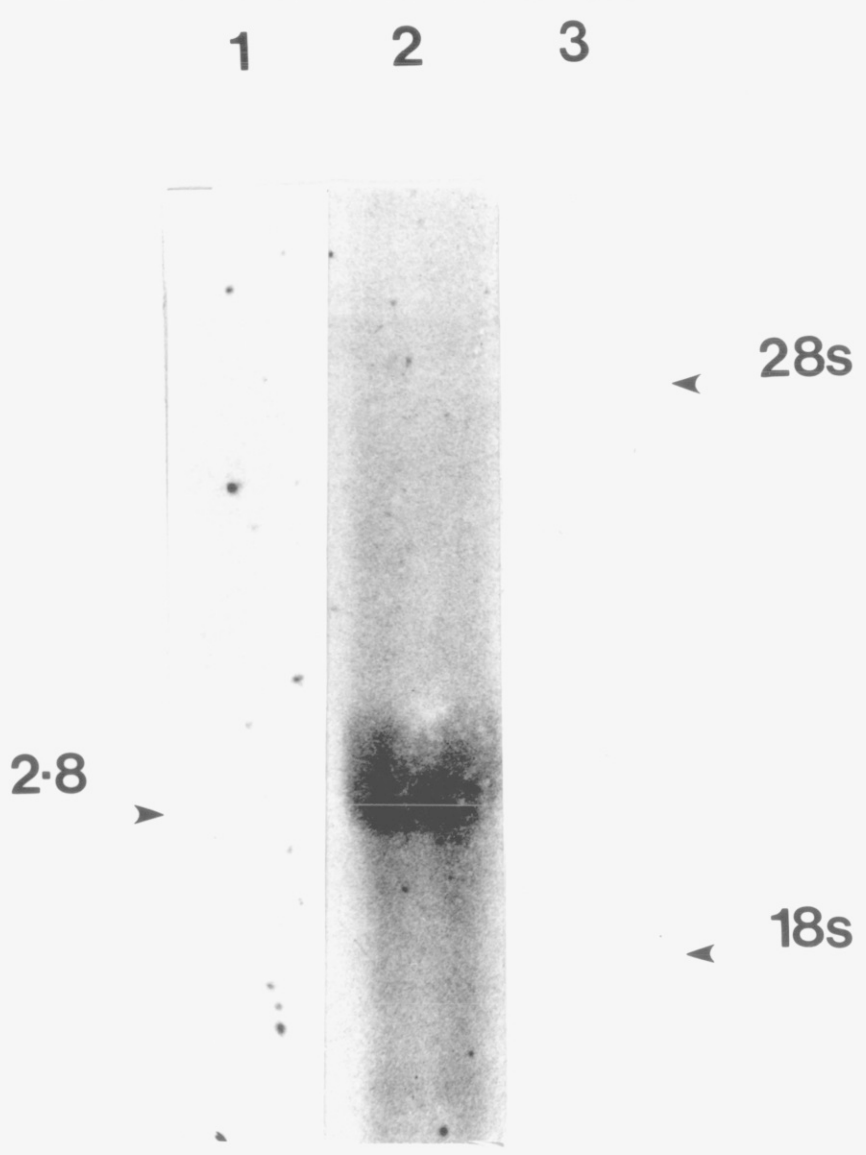


FIGURE (3.5)

Determination of size of mRNA encoding the γ subunit in E13 pectoral muscle.

Track 1 10μg of heart mRNA

Track 2 10μg of E13 pectoral muscle mRNA

Track 3 10μg of fibroblast mRNA

Conditions were as described in Figure (3.3) except that the blot was hybridised with insert derived from plasmid PCSMγs, labelled by random hexamer priming to 8.0×10^8 cpm/μg.

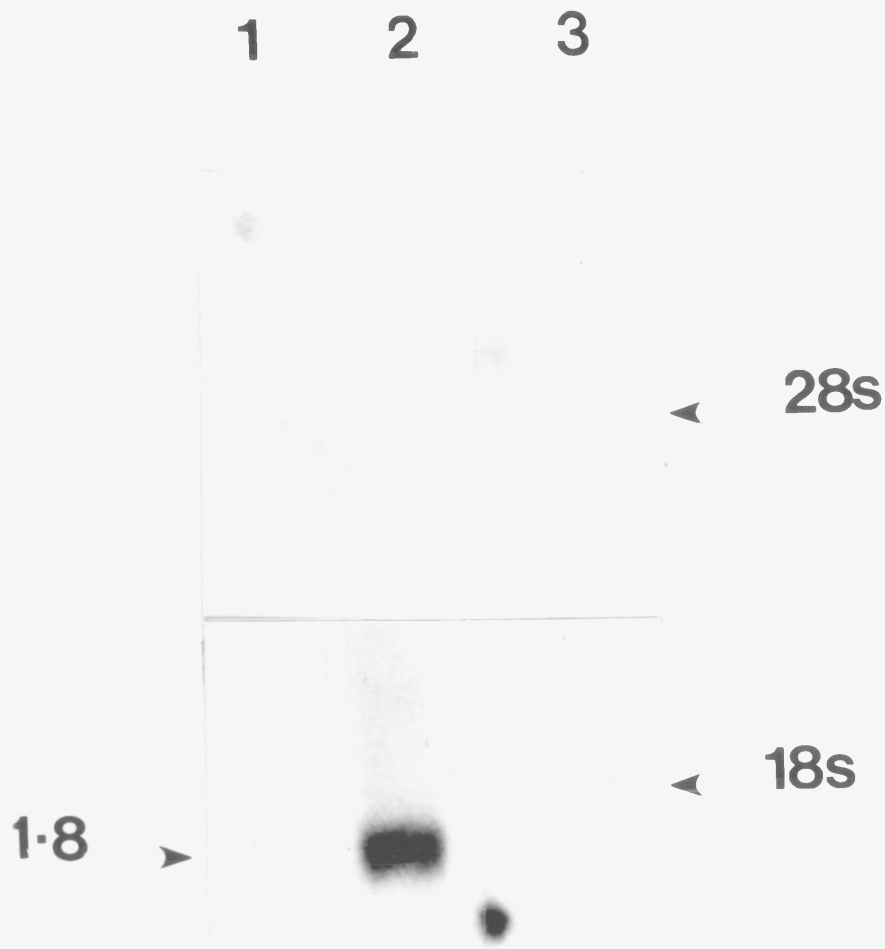


FIGURE (3.6)

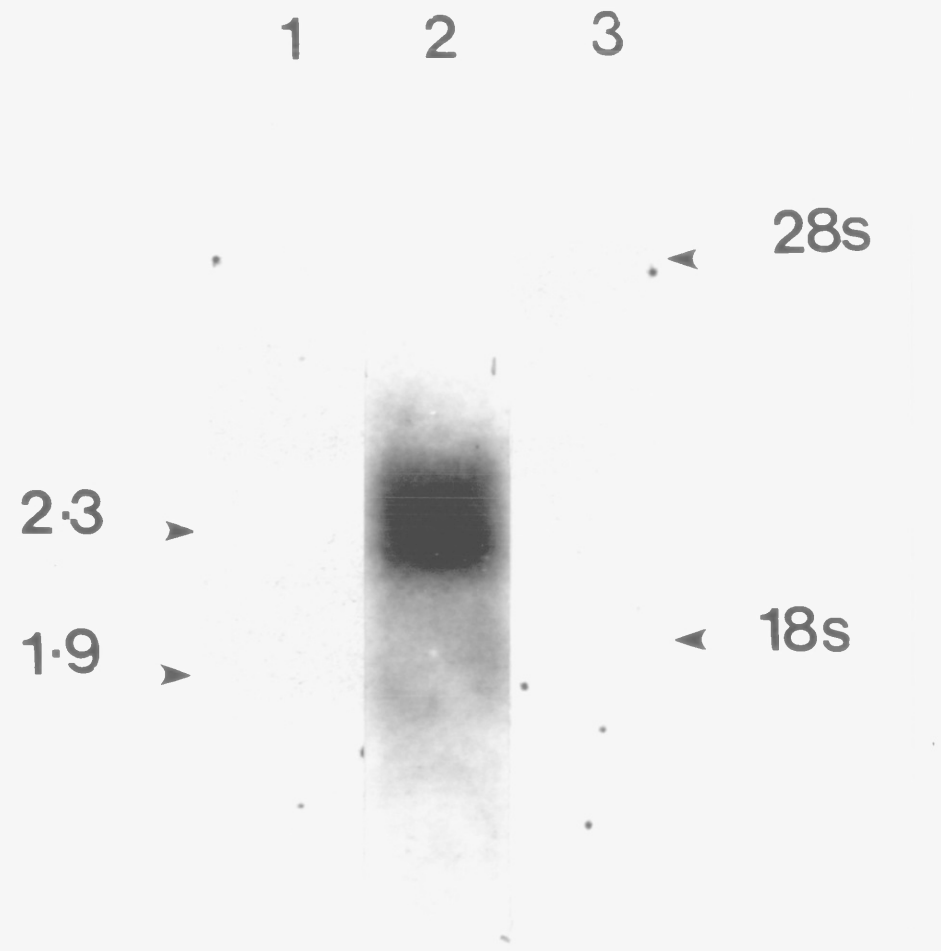
Determination of size of mRNAs encoding the δ subunit in E13 pectoral muscle.

Track 1 10μg of heart mRNA

Track 2 10μg of E13 pectoral muscle mRNA

Track 3 10μg of fibroblast mRNA

Conditions were as described in Figure (3.3) except that the blot was hybridised with insert derived from plasmid PCSMδs labelled by random hexamer priming to 6.5 x 108 cpm/μg.



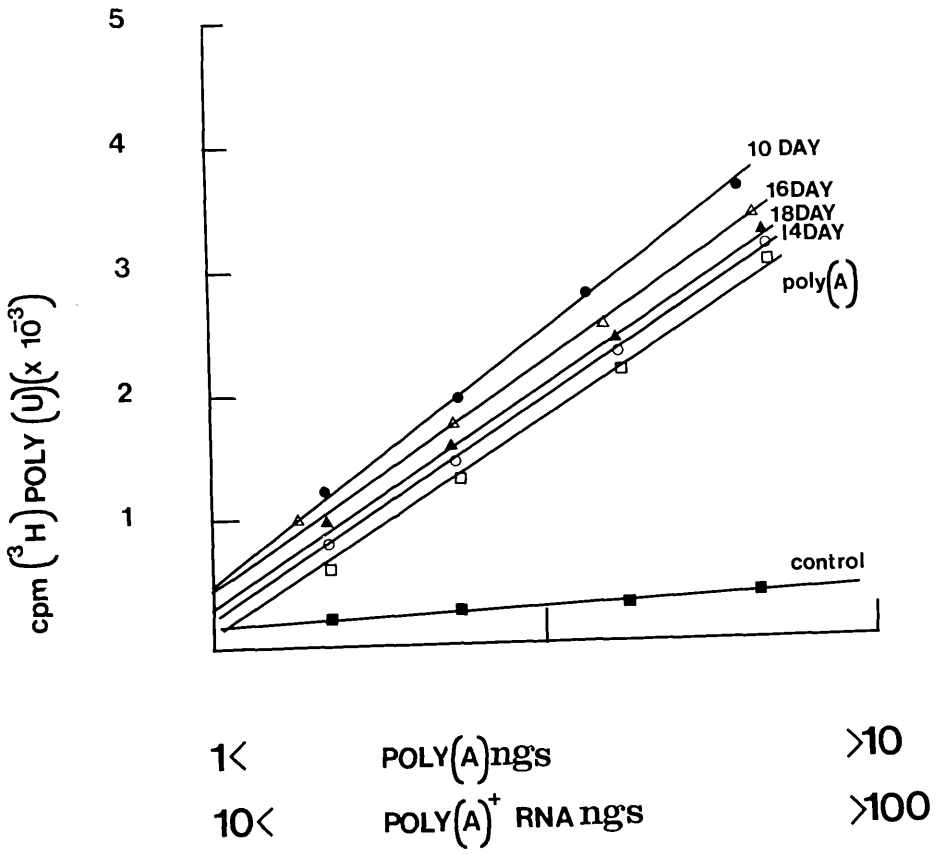
hybridising species in other tissues used in conjunction with pectoral muscle mRNA demonstrates the tissue specificity of AChR expression. Finally, these results confirm that the clones isolated and sequenced in Chapter 2 encode subunits of the AChR expressed in chick muscle.

Developmental Expression of the α, β, γ and δ mRNAs within Chick Pectoral Muscle

As a prerequisite for comparing the levels of the receptor subunit mRNAs in developing muscle, it was necessary to demonstrate that the level of poly(A)$^+$ within each mRNA preparation was equivalent at each time point. For comparative analysis, mRNA recovered from poly(A)$^+$ selection was quantified by spectroscopy and then standardised by poly(U) assay. This method measures the hybridisation of poly Uridine to the poly(A) tails present within mRNA preparations. Hybridisation is linear only over a limited concentration range. The linear range of hybridisation was determined by reference to poly(U) - poly(A) standards within the range of 0 - 10ng. Messenger RNA preparations were assayed from 0.1 - 0.01μg. The result of a typical calibration is shown in Figure (3.7). Assuming a mean mRNA size of 1000bp, then 1ng poly(A) = 10ng mRNA. As is shown in Figure (3.7) the profile of the mRNA preparations is similar to that for the standards. The slight difference in slope indicates that either the average size of the mRNA species is greater than 1000bp or that the preparations contain

FIGURE (3.7)

[^{3}H] Poly(U) calibration of mRNA prepared from E10, E12, E14, E16, E18 and 1 day post hatch pectoral muscle. The data for E12, and 1 day post hatch mRNA is not shown.



residual amounts of ribosomal RNA. However, it is clear that the developmental mRNA preparations contain equal amounts of poly(A)$^+$ and can therefore be used for comparative analysis.

Developmental Expression of the α Subunit mRNA

Chick pectoral muscle mRNA (E10 - 1 day post hatch) was denatured and transferred to a Gene Screen membrane. Blots were then hybridised with a labelled antisense probe derived from in vitro transcription of plasmid PCSMαs. The results of such a blot are shown in Figure (3.8). Hybridisation signals were quantified by scanning densitometry. A major mRNA species of 3.2Kb was observed at all developmental stages, while minor mRNA species of 4.2 and 5.9Kb were detected only during early developmental stages. The proportion of the larger mRNA species to the major 3.2Kb species remained constant at 15%. Values derived from densitometric scanning for all developmental time points were normalised to that for E12 the peak of α subunit mRNA expression, which was given an arbitrary value of 1.0. These values do not include the contribution of the larger mRNA species. The data is presented in Figure (3.9) and represents the mean of 3 independent observations. α subunit mRNA levels increase slightly over the period E10 - E12 and dropped dramatically over the period E16 - E18. The level of α subunit mRNA detected at 1 day post hatch was approximately 1/10 of that seen at the peak of α subunit

FIGURE (3.8)

Developmental expression of α subunit mRNA. 10μg of mRNA isolated from E10, E12, E14, E16, E18 and 1 day post hatch pectoral muscle was denatured using 1M glyoxal/50% DMSO electrophoresed on a 1.1% Agarose gel and transferred to a Gene Screen membrane. The blot was then hybridised at 60°C for 18 hrs in 50% formamide, 5 x SSPE, 0.5% SDS, 100μg/ml salmon sperm DNA, 100μg/ml yeast tRNA and radiolabelled probe at 1 x 10^6 cpm/ml. Probe was produced by *in vitro* transcription of plasmid PCSMαs, (specific activity 8.9 x 10^8 cpm/μg). Prehybridisation was in the same buffer at 50°C without probe for 2 hrs. The blot was then washed in 0.1 x SSPE at 70°C for 2 hrs, before being exposed to Fuji X-ray film overnight at -70°C using an intensifying screen. The position of chick 28S and 18S ribosomal RNA is indicated.

FIGURE (3.8)

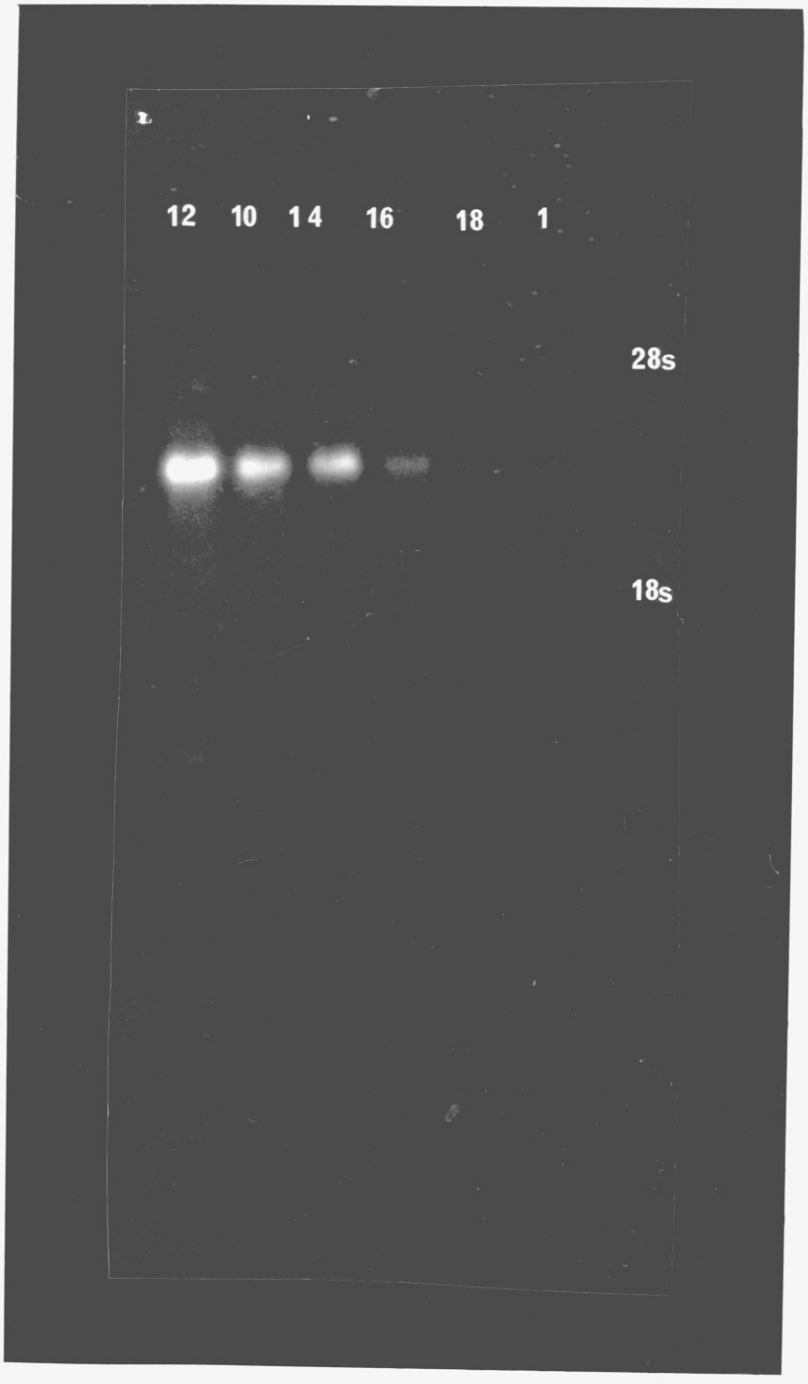
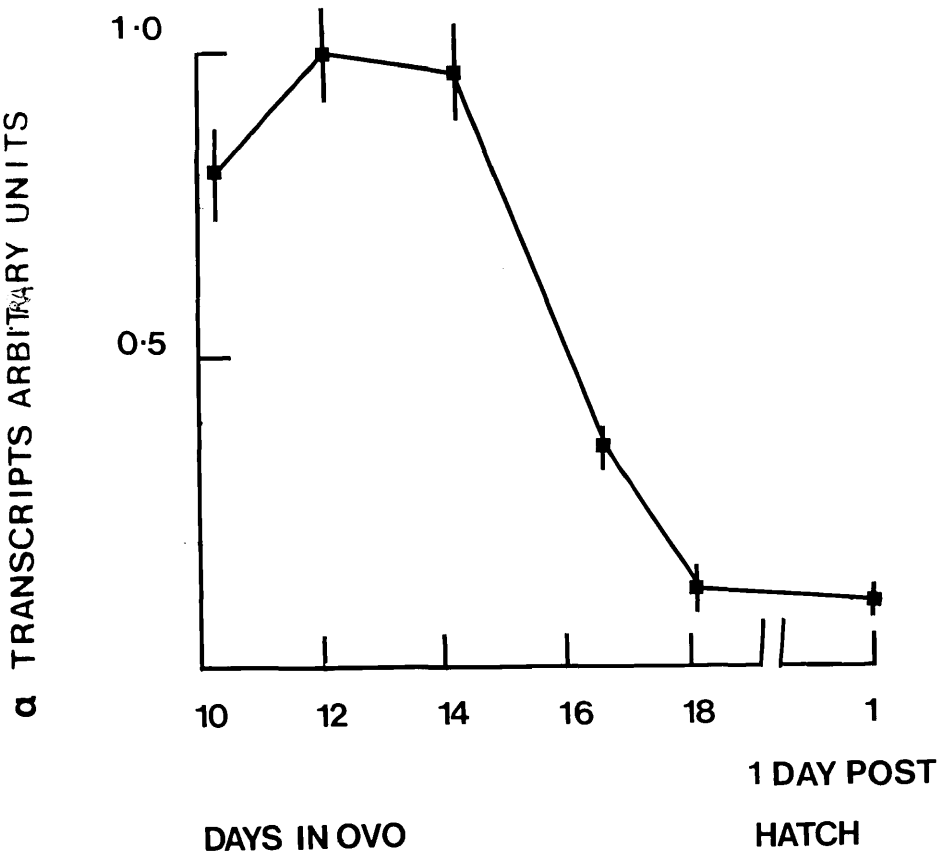


FIGURE (3.9)

Developmental expression of α subunit mRNA. Northern blot hybridisation signals (Figure (3.8)) were quantified by scanning densitometry. Data (from 3 independent blots) were then normalised to the peak of α subunit mRNA expression (E12) which was given an arbitrary value of 1.0.



Error bars are +- s.e.m. of three scans.

mRNA expression (E12).

Developmental Expression of the β and γ Subunit mRNAs

The method used to examine the pattern of expression of the β and γ subunit mRNAs was as outlined for the α subunit mRNA. Antisense probes were prepared by *in vitro* transcription of plasmids PCSMβs and PCSMγs respectively. Typical blots for the β and γ subunits are shown in Figures (3.10) and (3.12). Single mRNA species were observed at all developmental stages of 2.8 and 1.8Kb for the β and γ subunit mRNAs. As for the α subunit mRNA. the data derived from Northern blots using β and γ subunit probes were normalised to the peak of expression of the subunit mRNA, E12 and E14 for the β and γ subunit mRNAs respectively (Figures (3.11) and (3.13)).

The level of β subunit mRNA increased slightly over the period E10 - E12, and remained high until E16. The levels of β subunit mRNA observed at 1 day post hatch were 6.5 fold lower than detected at E12. γ subunit expression was maximal at E14. γ subunit mRNA levels decreased rapidly after E14, to reach a value 1/15 of that detected at the peak of γ subunit expression.

FIGURE (3.10)

Developmental expression of β subunit mRNA. 10μg of mRNA from E10, E12, E14, E16, E18 and 1 day post hatch pectoral muscle was treated as described with probe produced by *in vitro* transcription of plasmid PCSMβs (specific acitivity 7.5 x 10^8 cpm/μg).

FIGURE (3.10)

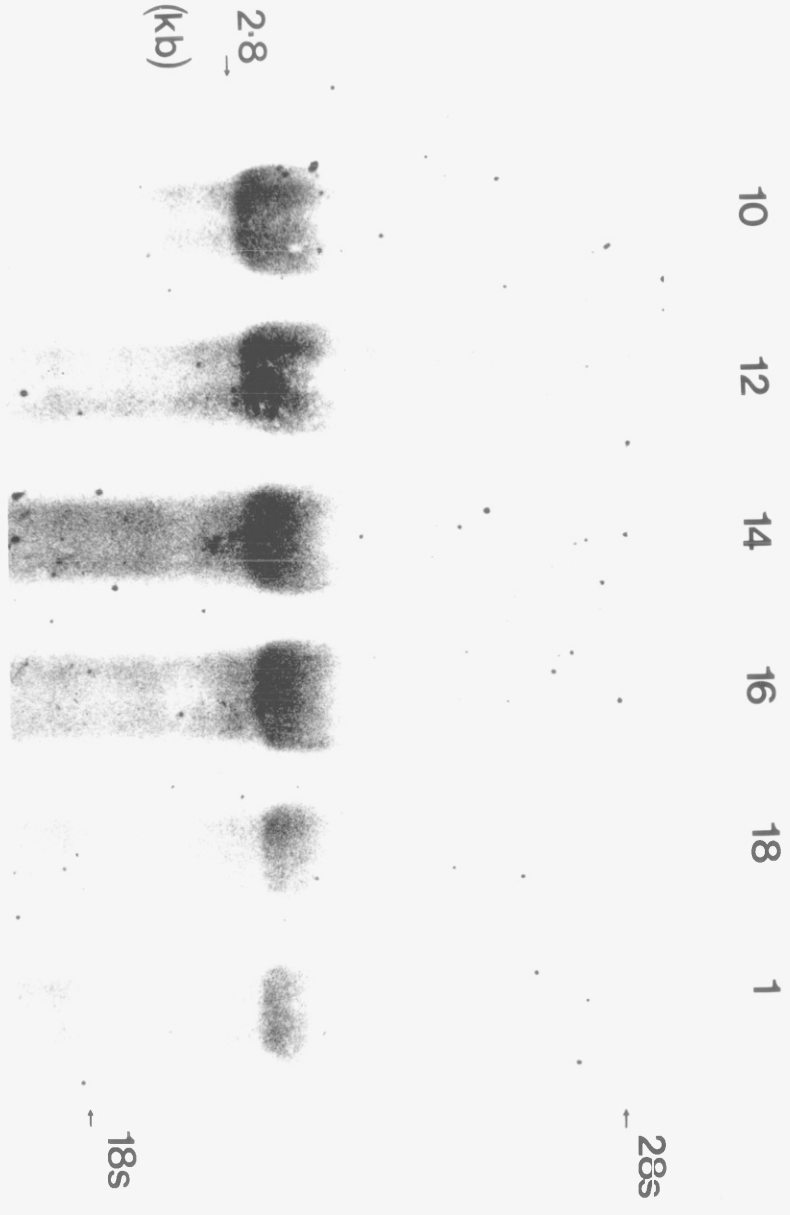
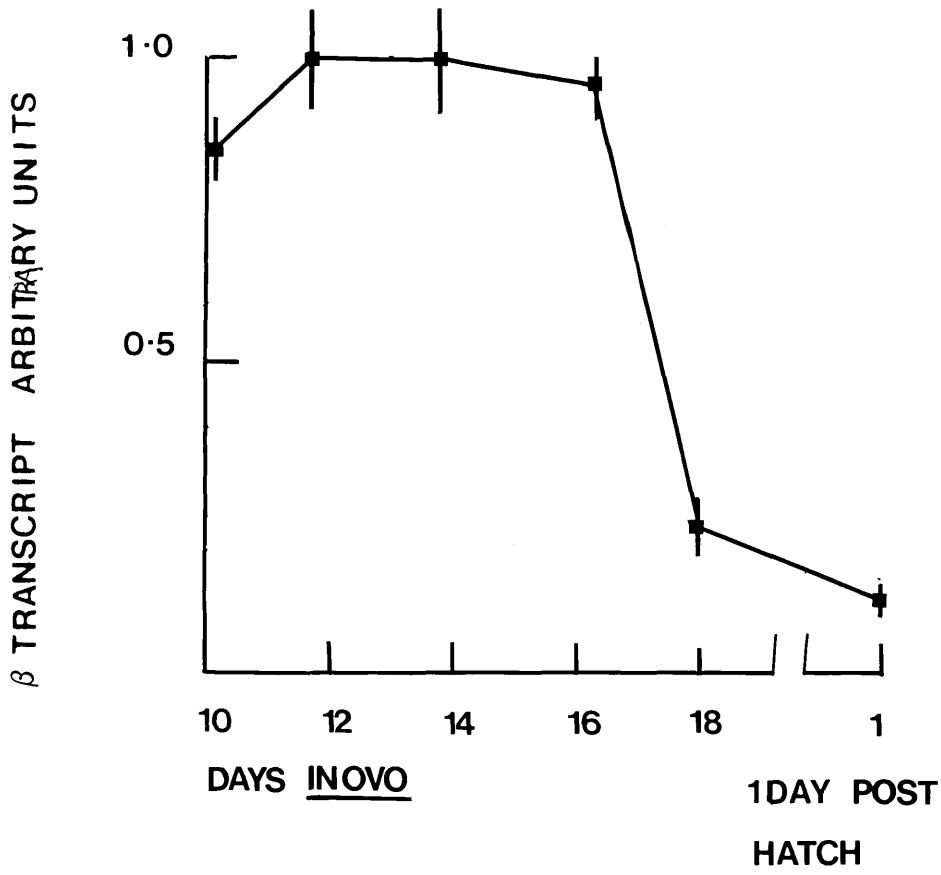


FIGURE (3.11)

Developmental expression of β subunit mRNA. Northern blot hybridisation signals (Figure (3.10)) were quantified by scanning densitometry. Data (from 3 independent blots) was then normalised to the peak of β subunit mRNA expression (E12) which was given an arbitrary value of 1.0.



Error bars are +- s.e.m. of three scans.

FIGURE (3.12)

Developmental expression of γ subunit mRNA. 10μg of mRNA isolated from E10, E12, E14, E16, E18 and 1 day post hatch pectoral muscle was treated as described in Figure (3.8). The blot was, however, hybridised with probe produced by *in vitro* transcription of plasmid PCSMγs (specific activity 6.9 x 10^8 cpm/μg).

FIGURE (3.12)

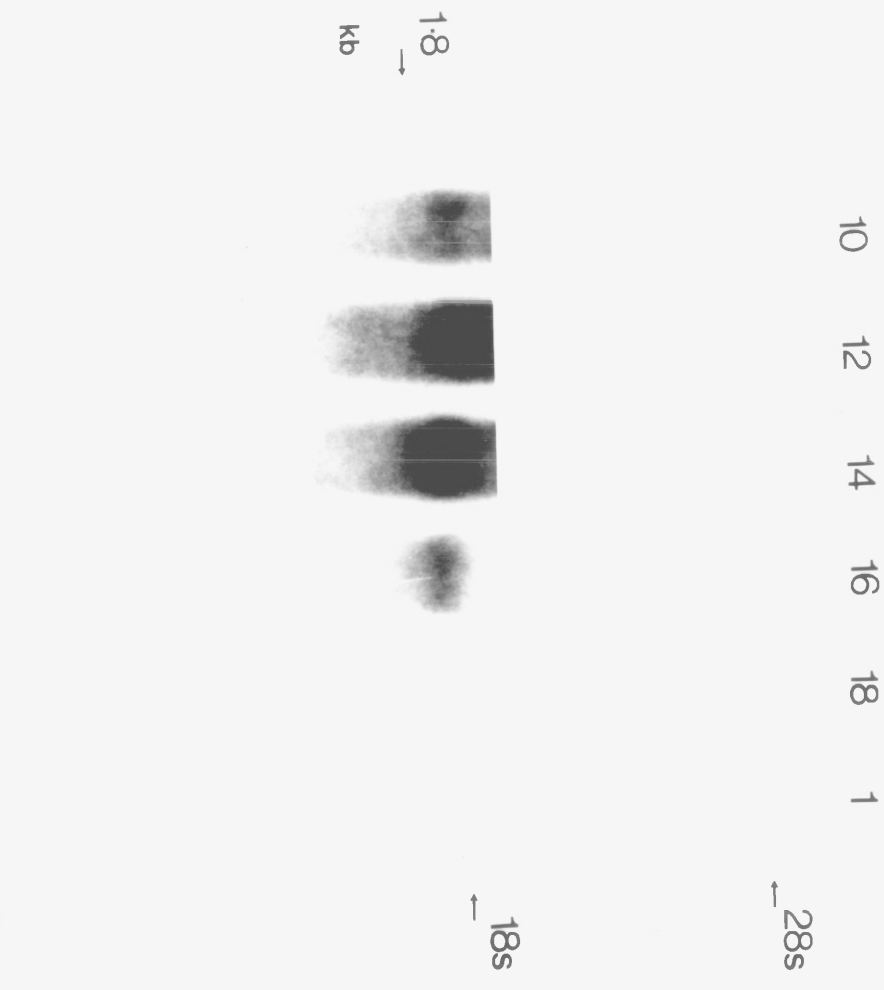
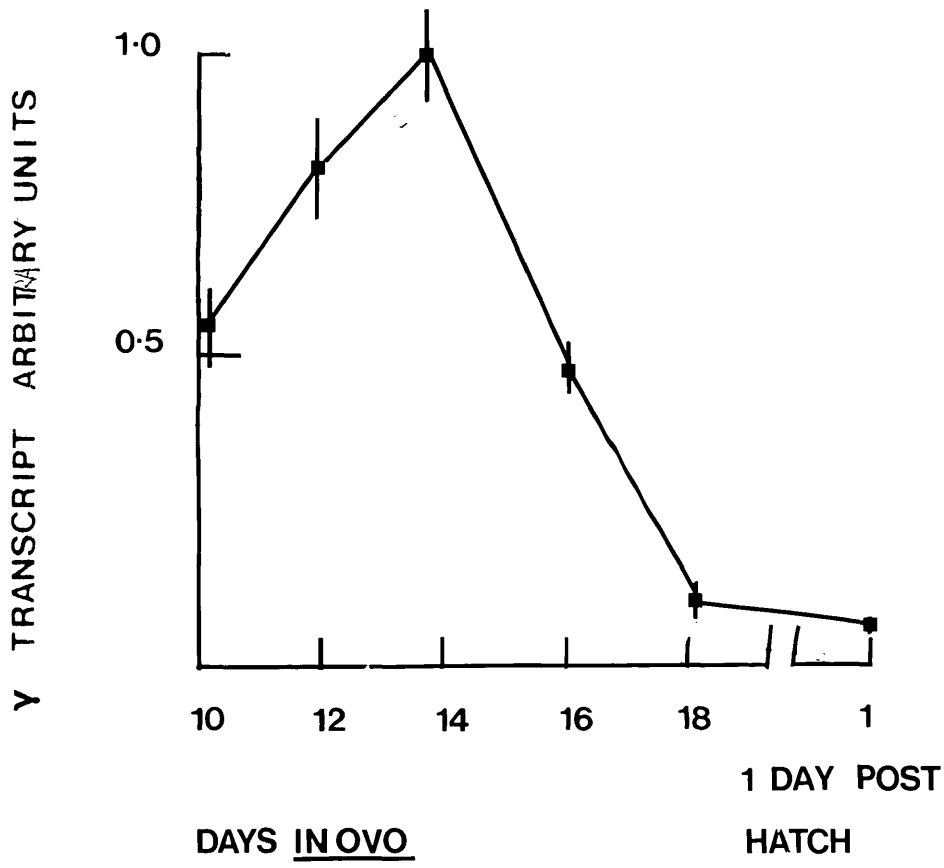


FIGURE (3.13)

Developmental expression of the γ subunit mRNA. Northern blot hybridisation signals (Figure (3.12)) were quantified by scanning densitometry. Data (from 3 independent blots) was then normalised to the peak of γ subunit mRNA expression (E14), which was given an arbitrary value of 1.0.



Error bars are +- s.e.m. of three scans.

Developmental Expression of the δ Subunit mRNAs during Chick Pectoral Muscle Development

The developmental expression of the δ subunit mRNA was determined as for the α subunit mRNA. Blots were hybridised with an antisense probe derived from *in vitro* transcription of plasmid PCSMδs. Two distinct mRNA species of 2.3 and 1.9Kb were detected at all developmental stages as shown in Figure (3.14). Data was processed as outlined for the α, β and γ subunit mRNAs. The values obtained for the 2.3Kb mRNA were normalised to E12, while the values obtained for the 1.9Kb mRNA were normalised to E14. The respective peaks of mRNA expression (Figure (3.15)), the developmental expression of total δ subunit mRNA (2.3 and 1.9Kb mRNAs), is shown in Figure (3.16). The two distinct δ subunit mRNAs were observed to have differing patterns of temporal expression. The larger mRNA species was more abundant at earlier developmental stages e.g. - E12, being almost undetectable in 1 day post hatch muscle. The smaller species was more abundant at later stages of development. The level of total δ subunit mRNA (2.3 and 1.9Kb mRNAs) detected in 1 day post hatch pectoral muscle, was 8 fold lower than that observed at its developmental peak (E12 or E14).

The developmental expression of the δ subunit mRNAs was examined further by using probes encoding different parts of the mature δ subunit. The structure of the chick δ

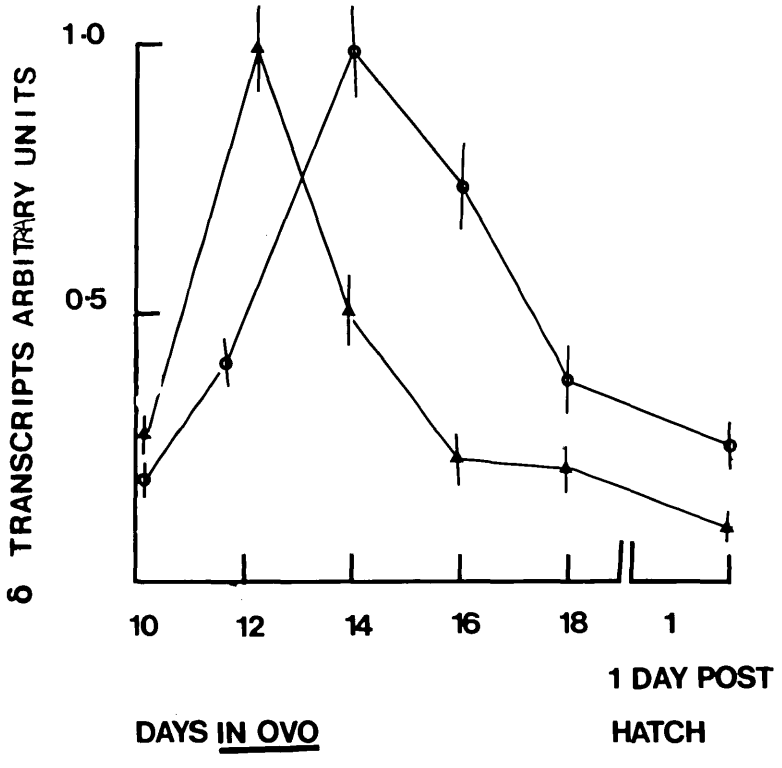
FIGURE (3.14)

Developmental expression of δ subunit mRNAs. 10μg of mRNA isolated from E10, E12, E14, E16, E18 and 1 day post hatch pectoral muscle was treated as described in Figure (3.8). The blot was, however, hybridised with probe produced by *in vitro* transcription of plasmid PCSMδs (specific activity 8.5×10^8 cpm/μg).

FIGURE (3.14)

FIGURE (3.15)

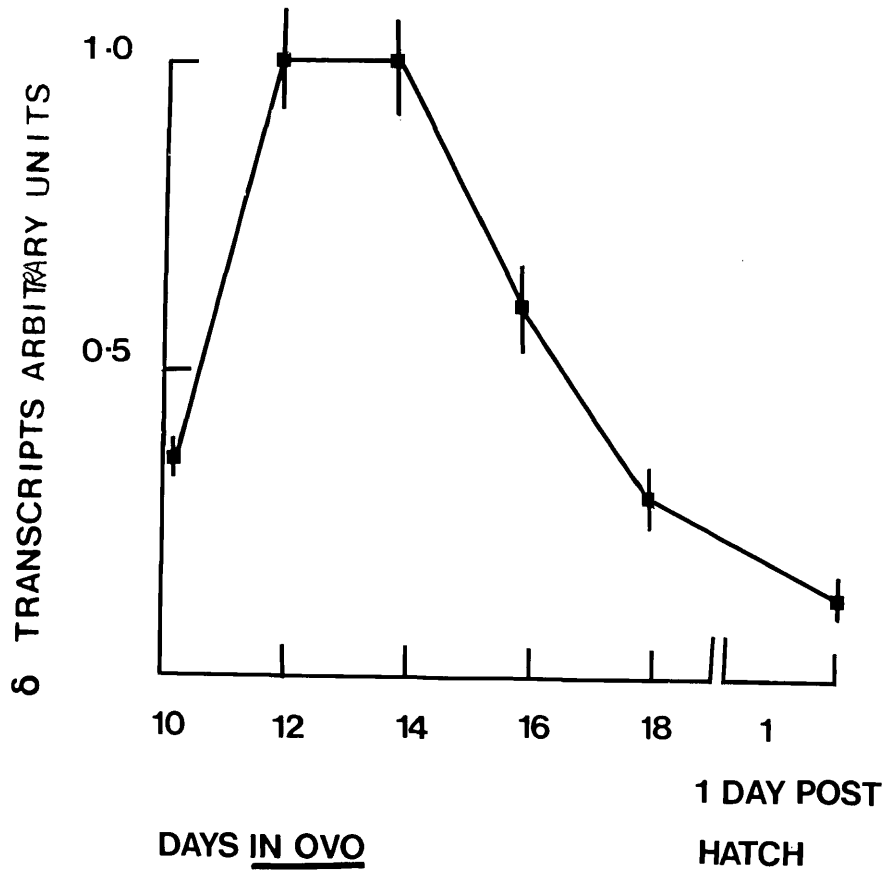
Developmental expression of δ subunit mRNAs. Northern blot hybridisation signals (Figure (3.14)) were quantified by scanning densitometry. Data derived from 3 independent blots was normalised to the peak of δ subunit mRNAs expression : 1.9Kb mRNA E14, 2.3Kb mRNA E12, which were given arbitrary values of 1.0. ▲ = 2.3Kb mRNA, ● = 1.9Kb mRNA



Error bars are +- s.e.m. of three scans.

FIGURE (3.16)

Developmental expression of total δ subunit mRNA. The sum of the hybridisation signals quantified by scanning densitometry (Figure (3.14)) were normalised to the peak of δ subunit mRNA expression (E12), which was given an arbitrary value of 1.0.



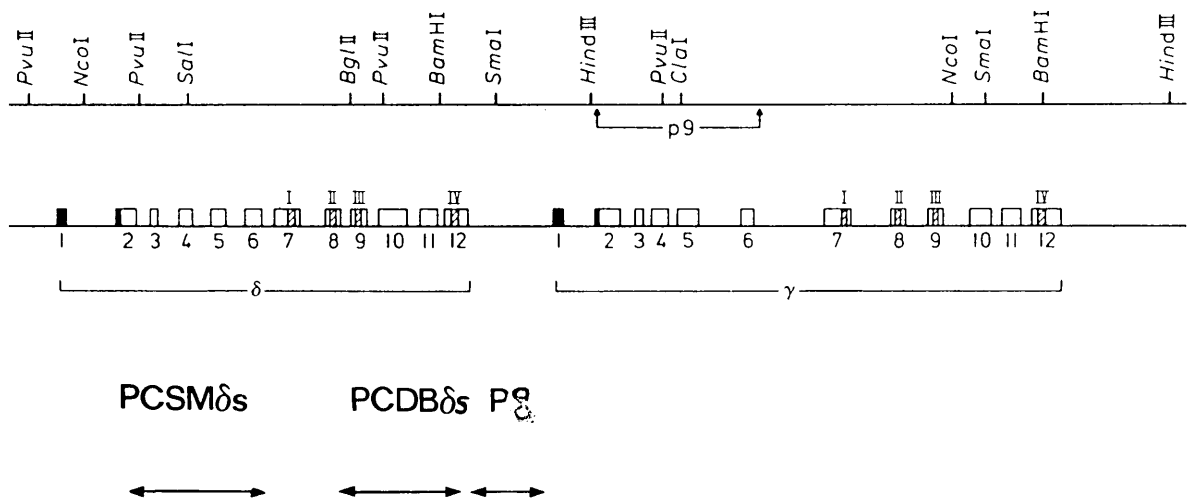
Error bars are +- s.e.m. of three observations.

subunit gene has been determined; it is closely linked to the γ subunit gene, and separated by an intergenic region of 740bp (Figure (3.17)), (Nef et al 1984).

Developmental Northern blots were probed with PCDBδs (a generous gift from D. Beeson, Royal Free Hospital, London) a cDNA encoding the C-terminal region of the mature δ subunit (encoding amino acid 248s - 462). An identical developmental profile to that shown in Figure (3.17) was observed. This suggests that both the 2.3 and 1.9Kb mRNA species have the capacity to encode the entire mature δ subunit. Developmental Northern blots were also probed with clone p8 containing the intergenic region of the δ and γ genes (Nef et al 1984). This probe failed to hybridise to either δ mRNA species. This suggests that there is no run through transcription between the δ and γ genes. An other explanation for the pattern of developmental expression of these two mRNAs could be incomplete processing, the 2.3Kb mRNA species perhaps being an incompletely processed form of the 1.9Kb mRNA species. Alternate splicing in the 5′ or 3′ untranslated region is another possibility. The use of different polyadenylation signals can be discounted in this case, since Nef et al (1984) reported only one conical polyadenylation signal within the 3′ region of the δ subunit gene. Multiple mRNA species encoding the δ subunits of other species have been reported (Hershey et al 1983, LaPolla et al 1984, Kubo et al 1985). The use of intron probes and sequence information on the 5′ and 3′ untranslated regions of the δ subunit gene should

FIGURE (3.17)

The structure of the γ and δ subunit genes (after Nef et al 1984). The position of the γ and δ probes used in this study is shown.



clarify this interesting observation.

The pattern of developmental expression of the α, β, γ and δ subunit mRNAs during chick pectoral muscle has been demonstrated above. The 4 subunit mRNAs have distinct patterns of temporal expression. Discrepancies between the patterns of temporal expression of the subunits of the bovine AChR have also been reported (Mishina et al 1986).

Quantitation of the Steady State of the α, β, γ and δ Subunit mRNAs during Chick Pectoral Muscle Development

The methods hitherto used for the measurement of specific mRNAs species suffer from the disadvantage that they are not strictly quantitative. Single stranded DNA fragments cannot provide accurate standards since the melting temperature of a DNA.DNA hybrid differs from that of the corresponding RNA.DNA hybrid (Cox et al 1984). Thus, only pure sense strand RNA can serve as an appropriate standard. It is now feasible to generate considerable amounts of pure RNA from a defined sequence by in vitro transcription of a cloned DNA template using viral RNA polymerases (Melton 1984).

Quantitation of the α Subunit mRNA within E12 Pectoral Muscle by SP6-T7-Directed Dot Blotting

Sense standards were produced by in vitro transcription

of plasmid PCSMαs utilising the SP6 promoter, denatured and transferred directly to a Gene Screen membrane with 10, 5, 2.5 and 1.25μg of E12 pectoral muscle mRNA as described in Methods and Materials. Blots were then hybridised with a complementary antisense probe derived from plasmid PCSMαs utilising the T7 promoter. A typical blot for the α subunit is shown in Figure (3.18). Hybridisation signals were then quantified by scanning densitometry (Figure (3.19)): α sense standards gave a linear hybridisation signal over the range 1ng to 1pg. This range was therefore used to calculate the level of α subunit mRNA present within E12 pectoral muscle mRNA. Pectoral muscle mRNA from 10 - 1.25μg also gave a linear hybridisation response, and α subunit mRNA abundance was therefore calculated at each concentration used. The results are shown in Table (3.1): these represent the mean of 3 independent observations. The level of α mRNA is expressed in terms of ngs/μg$(A)^+$ and also in attomoles, assuming a mRNA transcript size of 3.2Kb.

Quantitation of the β, γ and δ Subunit mRNA Levels in Pectoral Muscle by SP6-T7-Directed Dot Blotting

Sense standards were produced for quantification of the β, γ and δ subunit transcripts by in vitro transcription of plasmids PCSMβs, PCSMγs and PCSMδs respectively utilising the SP6 promoter. These standards were used in conjunction with mRNA derived from E12 pectoral mRNA (β subunit) and E14 in the case of the γ and δ subunits, as

FIGURE (3.18)

Quantification of α subunit mRNA levels in E12 pectoral muscle by SP6-T7 dot blotting.

Sense standards (2ng → 1pg) produced by *in vitro* transcription of plasmid PCSMαs and mRNA derived from E12 pectoral muscle was denatured using 1M glyoxal, 50% DMSO and transferred to a Gene Screen membrane using a Biorad Biodot apparatus. The blot was then hybridised in 50% formamide, 5 x SSPE, 0.5% SDS, 5 x Denhardts, 100μg/ml salmon sperm DNA and radiolabelled probe produced by *in vitro* transcription from plasmid PCSMαs labelled to 7.5 x 10^8cpm/μg, at 60°C for 24 hrs. Prehybridisation was in the same buffer without probe for 4 hrs. The blot was then washed in 0.1 x SSPE at 70°C for 4 hrs before exposure to Fuji X-ray film at -70°C using an intensifying screen for 12 hrs.

FIGURE (3.18)

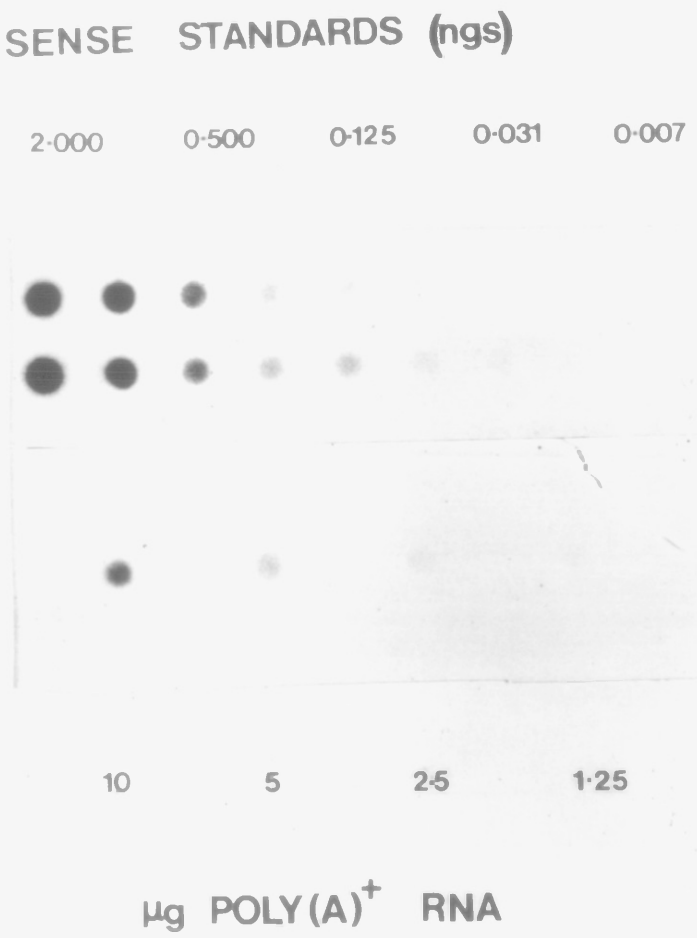

SENSE STANDARDS (ngs)
2·000
0·500
0·125
0·031
0·007
10
5
2·5
1·25
µg POLY(A)+ RNA

FIGURE (3.19)

Calibration curve for α subunit standards.

The autoradiograph in Figure (3.18) was subjected to scanning densitometry. The hybridisation signals were then plotted vs sense standard

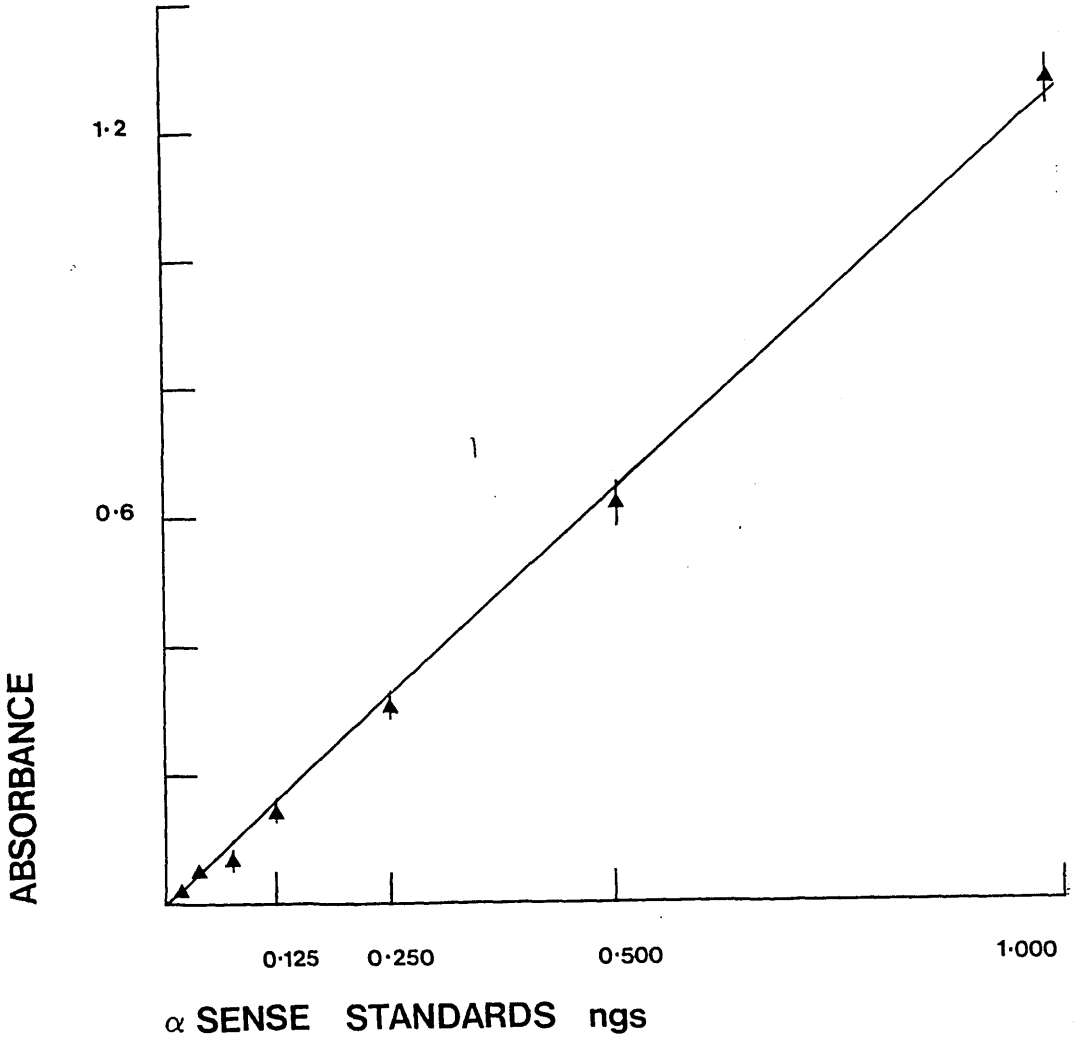


FIGURE (3.20)

Quantification of β subunit mRNA levels in E12 pectoral muscle by SP6-T7 dot blotting.

Sense standards (2ng → 1pg) produced by *in vitro* transcription of plamid PCSMβs and mRNA derived from E12 pectoral muscle. Conditions were as described in Figure (3.18), except that the blot was hybridised by *in vitro* transcription of plasmid PCSMβs, labelled to 8.5 x 10^8cpm/μg.

FIGURE (3.20)

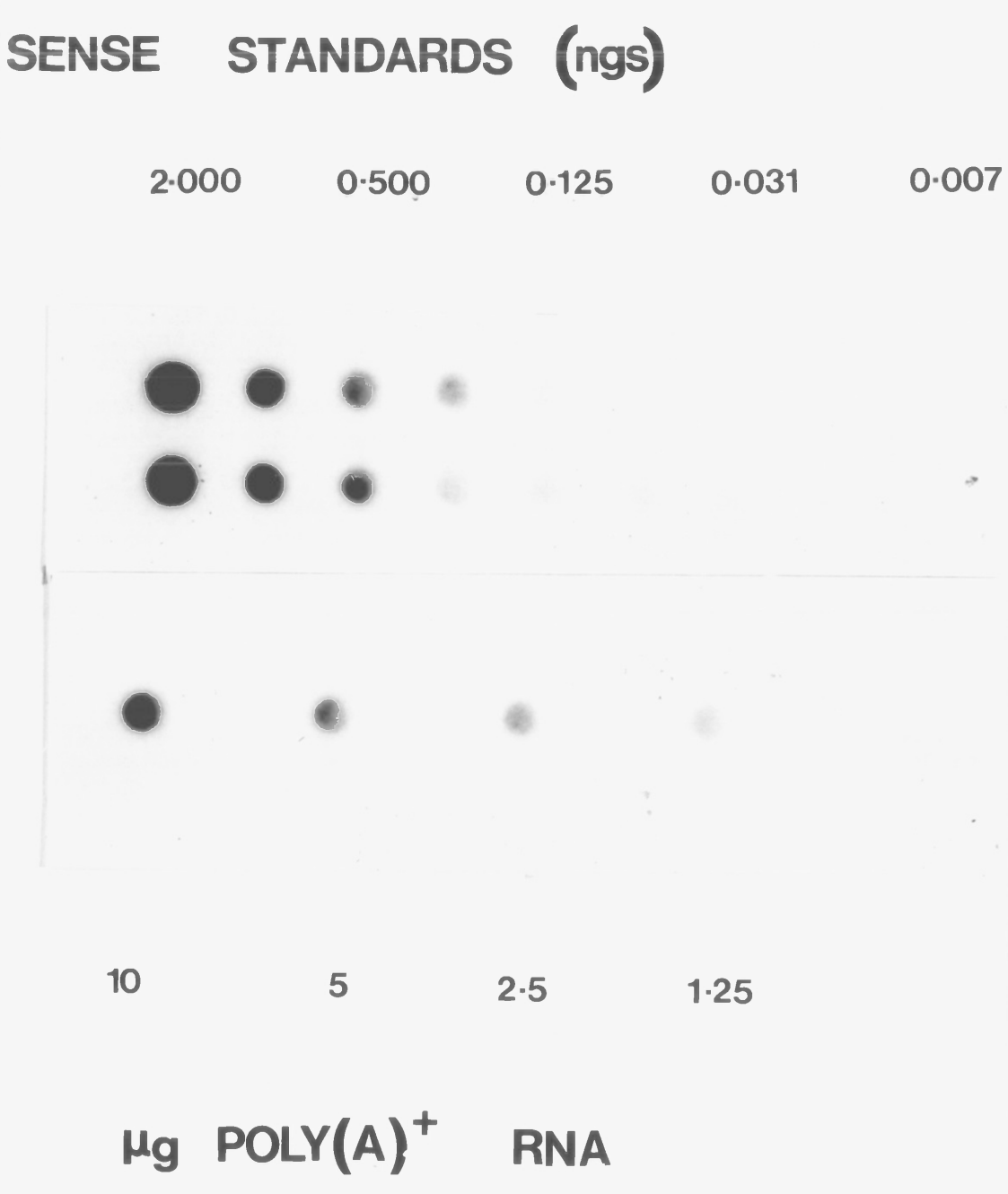

SENSE STANDARDS (ngs)
2·000
0·500
0·125
0·031
0·007
10
5
2·5
1·25
µg POLY(A)+ RNA

FIGURE (3.21)

Quantification of γ subunit mRNA levels in E14 pectoral muscle by SP6-T7 dot blotting.

Sense standards (2ng → 1pg) produced by in vitro transcription of plasmid, PCSMγs. Conditions were as described in Figure (3.18), except that the blot was hybridised with probe produced by in vitro transcription of plasmid PCSMγs, labelled to 8.0×10^8 cpm/μg.

FIGURE (3.21)

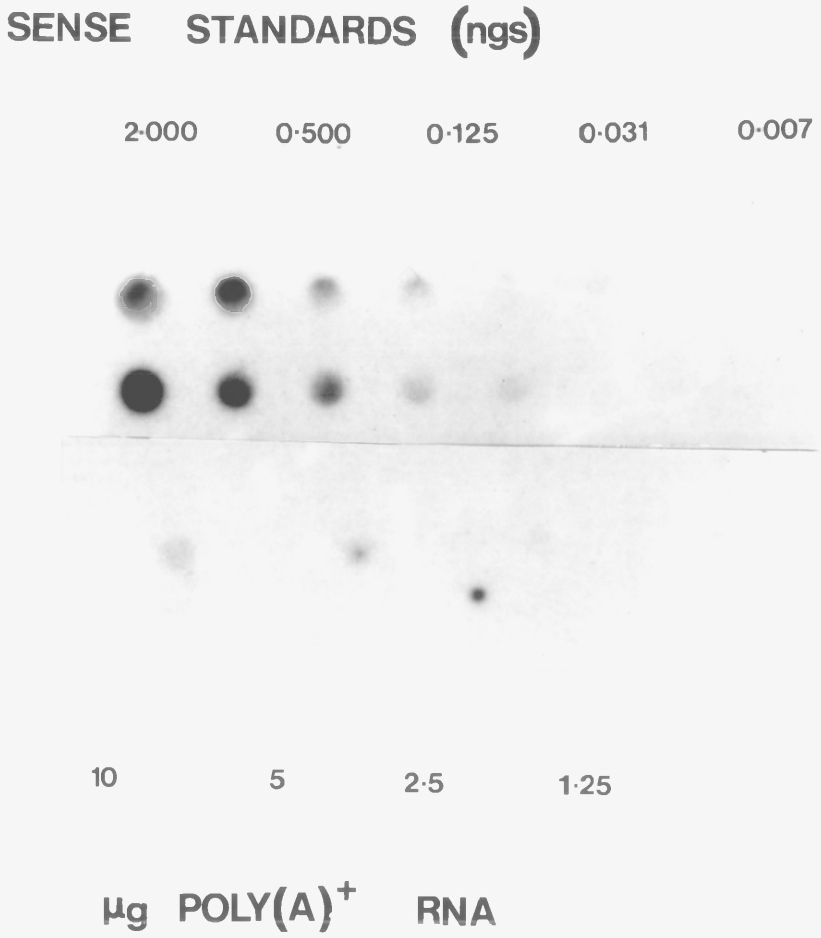
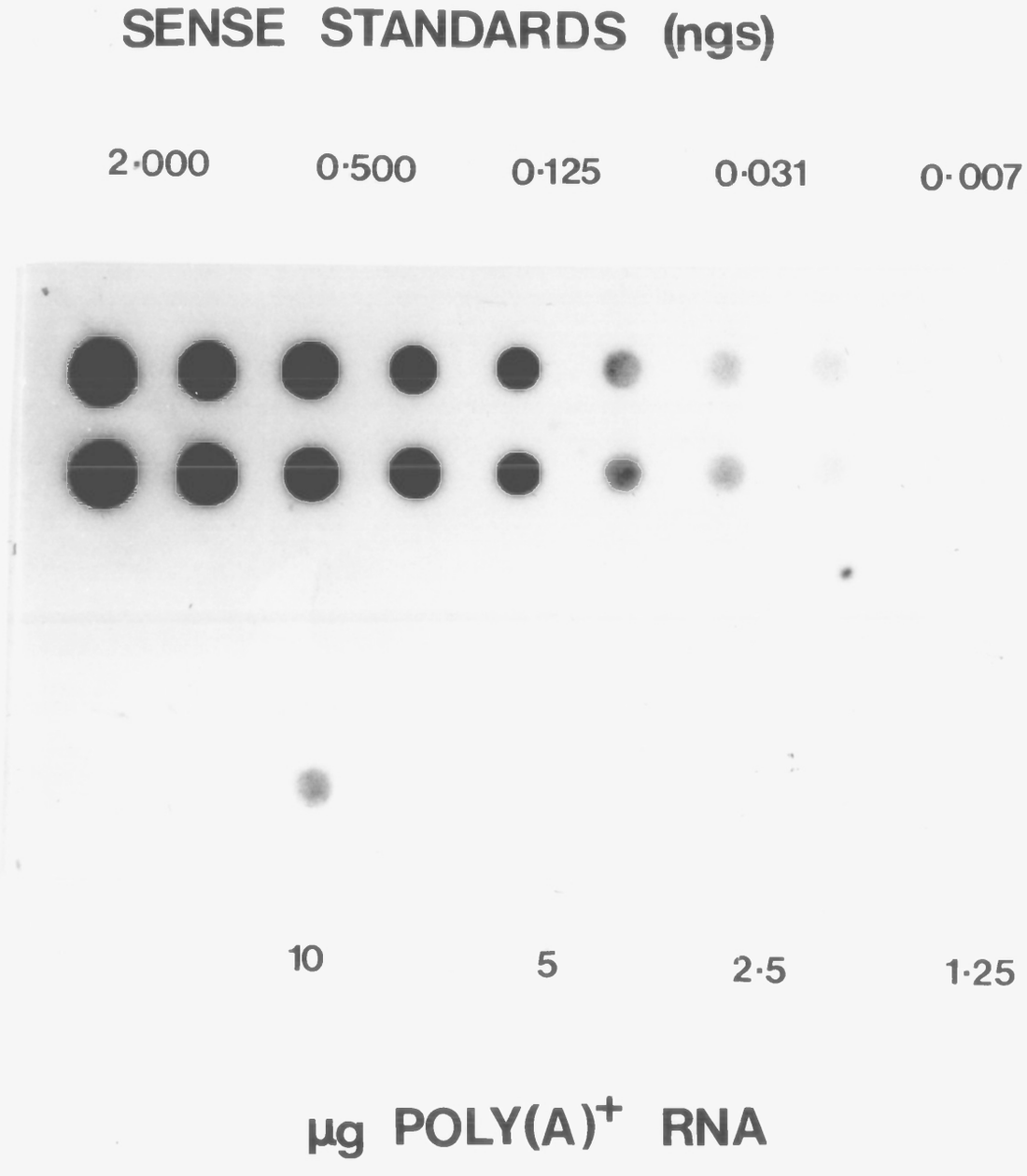

SENSE STANDARDS (ngs)
2·000
0·500
0·125
0·031
0·007
10
5
2·5
1·25
µg POLY(A)+ RNA

FIGURE (3.22)

Quantification of δ subunit mRNA levels in E14 pectoral muscle by SP6-T7 dot blotting.

Sense standards (2ng → 1pg) produced by *in vitro* transcription of plasmid PCSMδs. Conditions were as described in Figure (3.18), except that the blot was hybridised with probe produced by *in vitro* transcription of plasmid PCSMδ labelled to 9.5 x 10^8cpm/μg.

FIGURE (3.22)


SENSE STANDARDS (ngs)
2·000
0·500
0·125
0·031
0·007
10
5
2·5
1·25
µg POLY(A)+ RNA

described for the α subunit. Antisense probes were then produced by transcription of plasmids PCSMβs, PCSMγs and PCSMδs utilising the T7 promoter. The results of typical blots are shown in Figure (3.20) for the β subunit, Figure (3.21) for the γ subunit and Figure (3.22) for the δ subunit. As with the α sense standards, a linear hybridisation response was seen for all sense standards within the range of 1ng to 1pg (results not shown). Thus this linear range was used in calculating the level of the β, γ and δ mRNAs in pectoral muscle mRNA (E12 β, E14 γ and δ). Data was processed as for the determination of α subunit abundance. The results are shown in Table (3.1), expressed in terms of both ng/μg Poly(A)$^+$ and also in terms of attomoles, assuming mRNA sizes of β 2.8, γ 1.8, δ 2.3 and 1.9Kb. The ratio of the two δ transcripts was determined by reference to data obtained by Northern blotting.

Using the values obtained from this series of experiments, it was therefore possible to determine the molar levels of each receptor subunit at the peak of receptor expression, (Table (3.2)), E12 and E14. The data obtained from this series of experiments indicates that the level of the δ subunit mRNA is significantly lower than for either the α, β or γ subunit mRNAs. It also indicates that the ratio of the 4 receptor mRNAs does not reflect the stoichiometry of the receptor subunits, α_2, β, γ and δ. To confirm this observation, a portion of

TABLE (3.1)

Steady state levels of the α, β, γ and δ subunit mRNAs in E12 and E14 pectoral muscle mRNA.

Subunit Poly(A)$^+$	ng/μg Poly(A)$^+$	Attomoles/μg Poly(A)$^+$
α (12 day)	0.051 ± 0.005 (n = 3)	49.5 ± 0.23
β (12 day)	0.061 ± 0.004 (n = 4)	65.4 ± 0.40
γ (14 day)	0.072 ± 0.010 (n = 3)	72.5 ± 0.65
δ (14 day)	0.012 ± 0.002 (n = 5)	19.2 ± 0.19
δ 1.9Kb (14 day)	0.007 ± 0.002 (n = 5)	12.0 ± 0.10
δ 2.3Kb (12 day)	0.005 ± 0.001 (n = 5)	7.02 ± 0.09 ± sem

Assuming transcript sizes α 3.2, β 2.8, γ 1.8, δ 1.9, 2.3Kb
Mole of nucleotide = 330 grams
mRNA steady state levels were determined by SP6-T7 dot blotting. Values represent the mean of three independent experiments. Ratio of δ mRNA was determined by Northern blotting.

TABLE (3.2)

Subunit mRNA ratios in E12 and E14.

Day	α : β : γ : δ^
12	1.0 : 1.2 : 1.2 : 0.2^
14	1.0 : 1.2 : 1.4 : 0.4^

(^ sum of 2.3 and 1.9Kb mRNA species)

Data was derived from SP6-T7 dot blots as shown in Table 3.1, and data derived from Northern blotting experiments (Figures (3.8) to (3.16)). Subunit mRNA levels were normalised to that for the α subunit mRNA abundance.

the cDNA library described in Chapter 2 was screened with the α and δ cDNAs described above. 200,000pfu were screened in duplicate, using the cDNA probes labelled to high specific activity by random hexamer priming. After overnight exposure, 7 duplicating positives could be detected using the α subunit cDNA probe, while only 3 duplicating positives could be detected using the δ subunit cDNA probe . This confirms the discrepancy in abundance between the α and δ subunit mRNAs. However, values obtained for mRNA abundance derived from the latter method may not be accurate estimates, since differing efficiencies of reverse transcription and secondary mRNA structure may have an effect on the representation of a particular mRNA species in a cDNA library. Furthermore, the amplification of libaries may also lead to gross under-representation of certain sequences (Maniatis et al 1982).

DISCUSSION

The increased appearance of αButx binding sites (E10 to E14), reflecting an increase in AChR content of chick pectoral muscle demonstrated in Chapter 2, is concurrent with an increase in the steady state of each receptor mRNA. The magnitude of this increase is different for each subunit mRNA: the α γ and δ mRNAs showed larger increases than the β subunit mRNA. Furthermore, the decrease in steady state levels of the 4 subunit mRNAs (6.5 - 15 fold) from E14 onwards, parallels a decrease in the level of AChR expressed within pectoral muscle. Again the patterns of these decreases were distinct for each subunit mRNA. These results strongly suggest that the availability of the α, β, γ and δ mRNAs plays a regulatory role in controlling receptor appearance during chick pectoral muscle development. Messenger RNA steady state levels are a function of the rate of gene transcription and also mRNA half life. The most frequent method employed by eukaryotic cells to increase levels of specific mRNAs is to increase the rate of transcription of the relevant gene (Darnell 1982). There are, however, examples where changes in mRNA half life have been demonstrated to be of importance in controlling specific mRNA levels (Brock and Shapiro 1984).

Buonanno and Merlie (1986) have demonstrated that the rate of transcription of the α and δ subunit mRNAs of the

murine AChR increased 9-fold on mouse myoblast fusion by using nuclear run- off analysis (Marzluff and Huang 1984). Evans et al (1987) have also reported large increases in the steady state levels of the α, β, γ and δ mRNAs on murine myoblast fusion. Confirmation of increased AChR gene transcription during pectoral muscle development awaits nuclear run-off data for each subunit gene. Nuclear run-off data have proved difficult to obtain for muscle-specific genes in vivo, due to the problems of isolating intact nuclei from muscle. The detection of unprocessed intermediates, as for the case of the α subunit mRNA, is a good indication of the transcriptional control of AChR expression. Sheih et al (1986) have reported an increased level of unspliced intermediates of the α subunit mRNA encoding the chicken AChR on denervation of leg muscle. It would be of great interest to examine the levels of each receptor mRNA encoding the chick AChR on myoblast fusion. Myoblast fusion occurs early in chick gestation, and by E4 AChRs can be detected by α Butx binding activity (Dennis 1981). Nerve muscle contacts are also established early (E6 - E8), and synaptic activity can be detected also at this stage (Dennis 1981). At these early stages of chick development the size of the chick embryo makes accurate dissection of muscle populations difficult. One possible means of overcoming this problem is the use of in situ hybridisation.

The effects of muscle innervation on the level of AChR

expressed within muscle and on the level of its encoding mRNAs are demonstrated in this study. Thus the process of muscle innervation results in an increase in the level of all four subunit mRNAs (E10 - E14). Burden and Simon (1987) have reported similar observations during Xenopus muscle development. After this initial stimulation a large decrease in the level of each subunit mRNA is observed. The effects of neuronally evoked activity on the level of AChR expressed during muscle development has been examined in the chick (Betz et al 1980). Chronic paralysis of the developing chick embryo using the nicotinic antagonist flaxedil inhibits the natural reduction of AChR content of muscle that occurs during development, (which mirrors a decreased rate of biosynthesis (Burden 1977b), without modifying the rate of AChR degradation (Betz et al 1980). The half life of chick AChRs remains low until 3 weeks post hatch (Burden 1977b). The conclusion drawn from this series of observations was that neuronally evoked activity of muscle inhibits AChR biosynthesis. Conclusive evidence of this is provided by denervation experiments (reviewed in Chapter 4). The removal of the innervating neuron causes increased receptor biosynthesis (Brockes and Hall 1975). From the results presented within this Chapter it appears that the innervating neuron represses AChR biosynthesis by decreasing the level of the subunit mRNAs, presumably by affecting the rate of gene transcription. This observation could be further clarified by directly studying the effects of chronic

paralysis of the developing chick pectoral muscle on the levels of the α, β, γ and δ subunit mRNAs. In the bovine it has been demonstrated that the γ subunit of the AChR is replaced in the adult muscle by a novel subunit termed ε (Mishina et al 1986). In this study the level of the γ subunit, mRNA showed the greatest decrease during embryonic development. This is consistent with the existence within the chicken of a developmental isoform of the γ subunit analogues to the bovine ε subunit. This is discussed in detail in Chapter 4 in the light of data derived from denervation studies. It is also evident that the innervating neuron is capable of exerting positive effects on AChR biosynthesis as reflected by stimulation of AChR subunit mRNA levels early during myogensis. This phenomena is discussed in Chapter 5.

The determination of the steady state levels of the α, β, γ and δ mRNA levels during muscle development has important implications regarding AChR biosynthesis and also on the mechanisms controlling AChR gene transcription. The observation that the level of the δ subunit mRNA was lower than either the α, β or γ may be highly significant. This discrepancy in mRNA levels may reflect a difference in mRNA half life, or a difference in the rate of δ gene transcription compared to that of the α, β or γ subunit genes. The level of δ mRNA may therefore be rate limiting in receptor biosynthesis and a possible point of regulation. The requirement for two α subunits in the protein suggests also that the level of α

subunit mRNA may be important in controlling receptor expression.

Further evidence of the role of α subunit mRNA levels in regulating receptor appearance is demonstrated by the results of Harris et al (1988), who demonstrated a similar discrepancy between α subunit mRNA levels and the requirement for 2 copies of the α subunit within the AChR oligomer. Significantly, Harris et al (1988) also report that a 42Kd protein, termed ARIA, increases AChR levels by modulating α subunit mRNA levels. The determination of the α : β : γ : δ subunit mRNA ratio reveals that there may be differences in translation efficiency between the four receptor subunit mRNAs. Harris et al (1988), Burden et al (1988) and Sheih et al (1988) have all commented upon discrepancies between the mRNA levels of the α, γ and δ mRNAs in Xenopus and chicken muscle. Transcriptional control of AChR biosynthesis has been suggested by Merlie and co-workers in a number of papers studying AChR biosynthesis in the mouse muscle cell line BC3H-I (Merlie et al 1981, Merlie and Lindstrom 1983, Merlie et al 1983).

The patterns of temporal expression of the α, β, γ and δ mRNAs differ. Differences in temporal expression for the α, β, γ and δ subunit mRNAs encoding the bovine AChR have also been reported (Mishina et al 1986), and more recently the α, γ and δ subunits of the Xenopus AChR. The implication of these observations is that the

coordination of AChR gene expression is not achieved by a universal mechanism.

CHAPTER 4

THE EFFECTS OF MUSCLE INNERVATION AND DENERVATION ON THE LEVELS OF THE 4 SUBUNIT mRNA s ENCODING THE CHICK PECTORAL MUSCLE AChR

INTRODUCTION

The importance of the innervating neuron in maintaining the number and distribution of AChR in muscle is demonstrated by the effects of muscle denervation. Denervation of vertebrate muscle reverses the effects of muscle innervation by causing a large increase in extra-junctional receptors as judged by α Butx binding (reviewed by Fambrough 1979, Salpeter and Loring 1985). The magnitude of this increase in AChR number is dependent on the time of denervation before assay and also on muscle type, but is typically between 5 - 50 fold. This increase in receptor density is responsible for the "supersensitivity" of denervated muscle to ACh (Miledi 1960). Extra-junctional receptors that appear after muscle denervation are metabolically unstable and have biochemical, biophysical and antigenic properties of embryonic receptors, (Fambrough 1979).

The appearance of extra-junctional receptors after muscle denervation has been demonstrated to be due to de-novo synthesis (Brockes and Hall 1975). Futhermore, the observation that chronic electrical stimulation of denervated muscle represses the receptor appearance after denervation (Hall and Reiness 1977), demonstrates that the level of electrical activity within muscle can regulate the level of receptor biosynthesis. The availability of cloned cDNA probes has greatly enhanced the comprehension of the mechanisms underlying the

electrical repression of receptor biosynthesis. In both mouse and rat muscles the steady state level of each receptor subunit mRNA has been shown to increase on muscle denervation (Merlie et al 1984, Goldman et al 1985, Evans et al 1987). These results suggest that AChR biosynthesis is regulated, in part, by the availability of the receptor subunit mRNAs.

In the only such experiment reported on chicken muscle (Klarsfeld and Changeux 1985) only the α subunit was studied and a 17 fold increase in the level of its mRNA was found. In this Chapter the cDNA and genomic clones outlined in Chapter 2 were used to determine the effects of denervation of chicken pectoral muscle on the steady state levels of the α, β, γ and δ subunit mRNAs encoding the chicken AChR.

METHODS AND MATERIALS

Denervation of Chicken Pectoral Muscles

New Hampshire chickens (Davis Line 412) bred at Imperial College, London, were anesthetized using halothane. Denervation was performed unilaterally on the pectoral muscles of 8 week old chickens by removal of segments of the efferent nerve trunk. Muscles were removed 8 days after denervation and stored at -70°C before use. This period of denervation was chosen since in a previous study, maximal α Butx binding was seen 8 days post denervation (Sumikawa 1981). Contralateral muscles were also removed and these served as a control. Age matched chickens were used to provide an unperturbed innervated control. All surgical operations were performed by D. Green (Imperial College, London).

RNA Isolation

Total RNA was extracted from innervated (8 week), contralateral innervated, 8 day denervated, and embryonic pectoral muscle by the method of Chirgwin et al (1979) as described in Chapter 2. Messenger RNA was isolated by oligo (d)T cellulose chromatography, also as described in Chapter 2.

Poly(U) Calibration of mRNA Preparations

Messenger RNA purified by oligo (d)T chromatography was quantified by UV spectroscopy. Messenger RNA preparations were standardised by [^{3}H]poly(U) assay as described in Chapter 3.

Northern Blots

Northern blots were essentially as described in Chapter 2.

Hybridisation Probes

Probes for the α, β, γ and δ subunit mRNAs were as described in Chapter 3. A chicken muscle α actin probe was a generous gift from J. Rogers (Laboratory of Molecular Biology, Cambridge). The probe was a 1.5Kb PstI fragment encoding the entire α actin protein coding sequence, and was provided in plasmid pBR322. Probes were labelled to high specific activity by either random hexamer priming or *in vitro* transcription of the respective cDNA fragment, as described in Chapter 3.

Densitometric Analysis

Hybridisation signals were quantified by scanning densitometry as outlined in Chapter 3.

RESULTS

The effects of innervation and denervation on the levels of the α, β, γ and δ subunit mRNAs as compared to the levels in embryonic pectoral muscle were investigated by Northern blotting. Prior to investigating the effects of innervation/denervation on receptor mRNAs, mRNA isolated from each muscle type was standardised by poly(U) assay, to ensure that the amounts of poly(A)$^+$ RNA in each sample were comparable.

Quantification of the Levels of Poly(A)$^+$RNA in Embryonic Innervated, Denervated and Contralateral mRNA Preparations

The yields of mRNA after oligo (d)T chromatography were quantified by spectroscopy and mRNA preparations from different muscle types were first standardised by poly(U) assay. The results showed that the mRNA preparations isolated from the different muscle types contained identical amounts of poly(A)$^+$ RNA, and could therefore be used for quantitative analysis (the results of a typical calibration is shown in Chapter 3, Figure (3.7)). Essentially identical results were found using mRNA derived from the muscle types above (results not shown).

Detection of the α, β, γ and δ mRNAs Within Contralateral Innervated, Denervated and Embryonic Pectoral Muscle

Northern blots of mRNA from E12 or E14 (α, β E12) (γ, δ E14) at the developmental peak of expression of each mRNA), contralateral innervated and denervated pectoral muscle were probed with radiolabelled subunit specific probes. Strong hybridisation signals corresponding to the α, β, γ and δ mRNAs were detected in embryonic and denervated mRNA α Figure (4.1), β Figure (4.2), γ Figure (4.3) and δ Figure (4.4). The subunit mRNAs present within denervated muscle were determined to be of the same size as those observed within embryonic muscle α, 3.2Kb (minor RNA species being detected of 4.2 and 5.9Kb), β, 2.8Kb, and γ 1.8Kb. Within embryonic muscles two δ mRNAs of 2.3 and 1.9Kb were observed as discussed in detail in Chapter 3; the smaller mRNA being more abundant at E14. Within denervated muscle only the 1.9Kb mRNA was detected. The hybridisation signals obtained from contralateral muscle mRNA were much weaker than those from denervated or embryonic muscle mRNA. No γ subunit mRNA could be detected at all in contralateral muscle mRNA. The sensitivity of the hybridisation analysis of the γ subunit mRNA was determined to be between 0.1 - 2pgs as determined by reference to data obtained in Chapter 3 by SP6 dot blotting.

FIGURE (4.1)

Effects of muscle denervation on α subunit mRNA levels as determined by Northern blotting. 10μg of mRNA isolated from E13 muscle (lane E), 8 week contralateral muscle (lane C) and denervated pectoral muscle (D) were denatured in 1M glyoxal 50% DMSO, electrophoresed on a 1% agarose gel and transferred to a Gene Screen membrane. The blot was then hybridised for 24 hrs at 42°C in 50% formamide, 5 x SSPE, 1 x Denhardts, 0.5% SDS, 100μg/ml salmon sperm DNA, 100μ/ml yeast tRNA, and labelled insert derived from plasmid PCSMαs (see Chapter 3) at 10^6cpm/ml. Prehybridisation was in the same buffer without probe for 2 hrs. Insert DNA was labelled by random hexamer priming to 1.3 x 10^9cpm/μg. The blot was then washed in 0.1 x SSPE at 65°C for 1 hr, before exposure to Fuji film at -70°C using an intensifying screen for 24 hrs.

FIGURE (4.1)

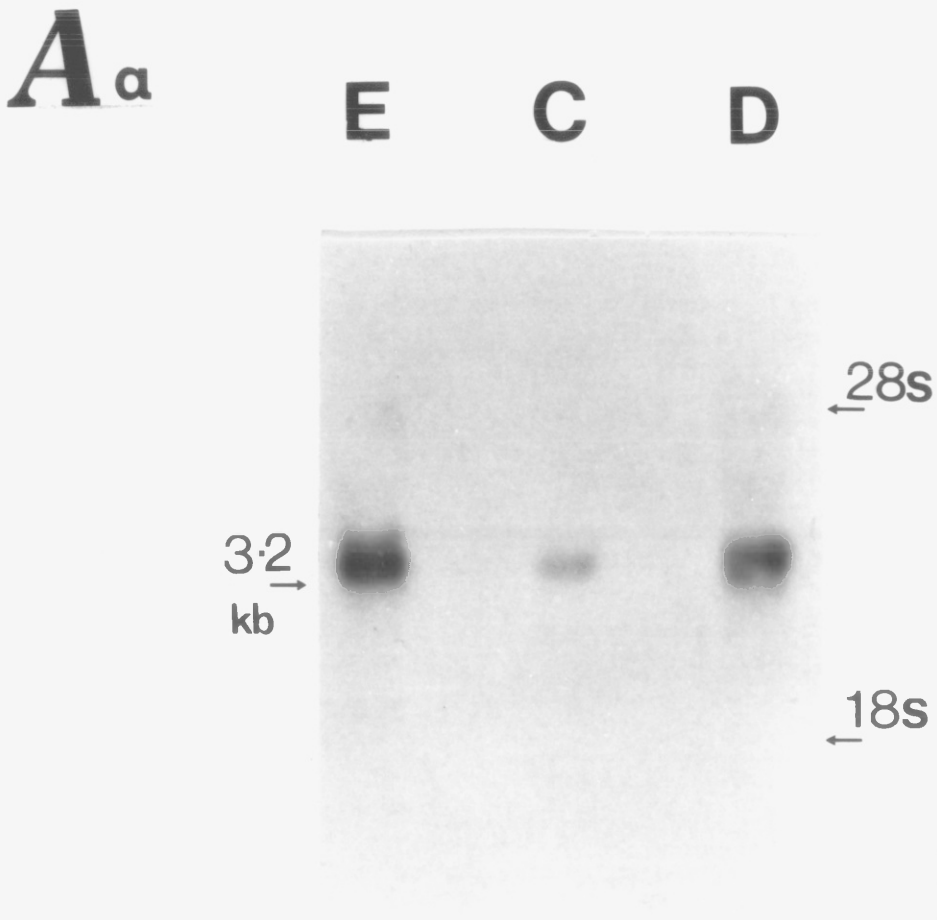


FIGURE (4.2)

Effects of muscle denervation on β subunit mRNA levels as determined by Northern blot analysis. Conditions were as described in Figure (4.1), except that the blot was hybridised with insert derived from plasmid PCSMβs (see Chapter 3) labelled by random hexamer priming to 7.0 x 10^8 cpm/μg.

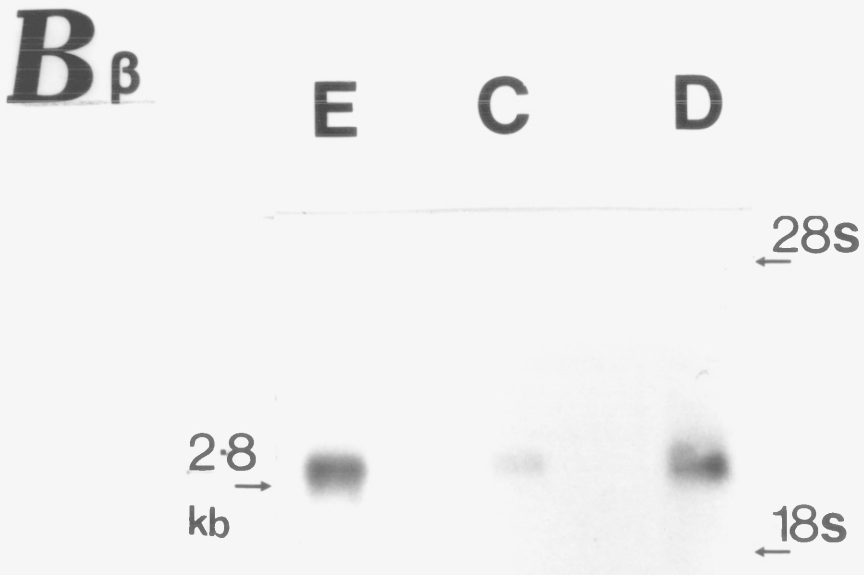


FIGURE (4.3)

Effects of muscle denervation on γ subunit mRNA levels as determined by Northern blotting. Conditions were as described in Figure (4.1), except that the blot was hybridised with insert derived from plasmid PCSMγs (see Chapter 3) labelled by random hexamer priming to 8.0 x 10^8cpm/μg.

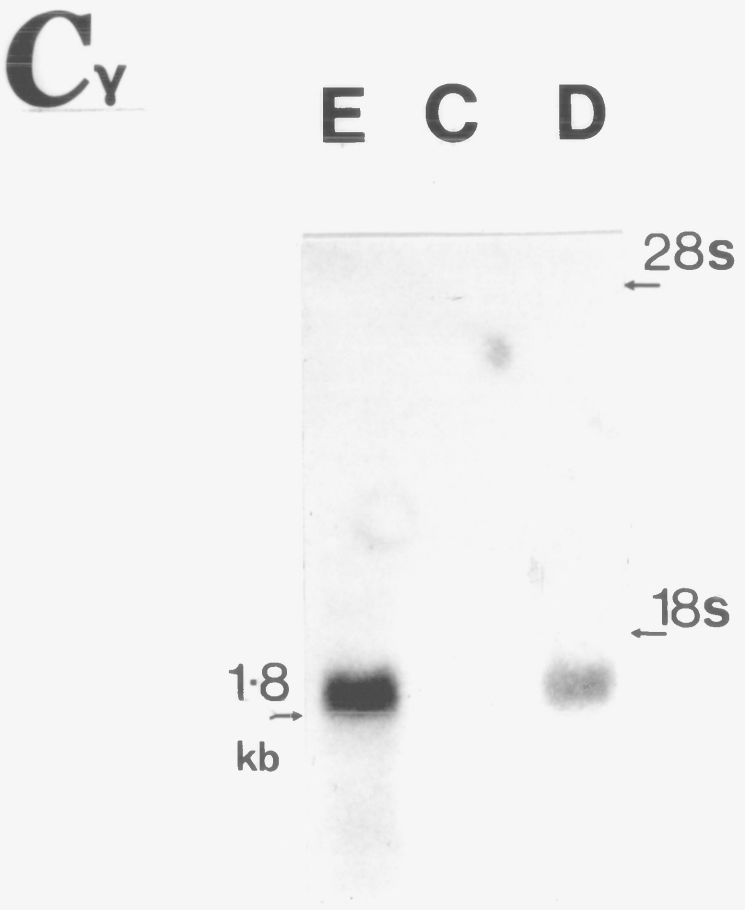
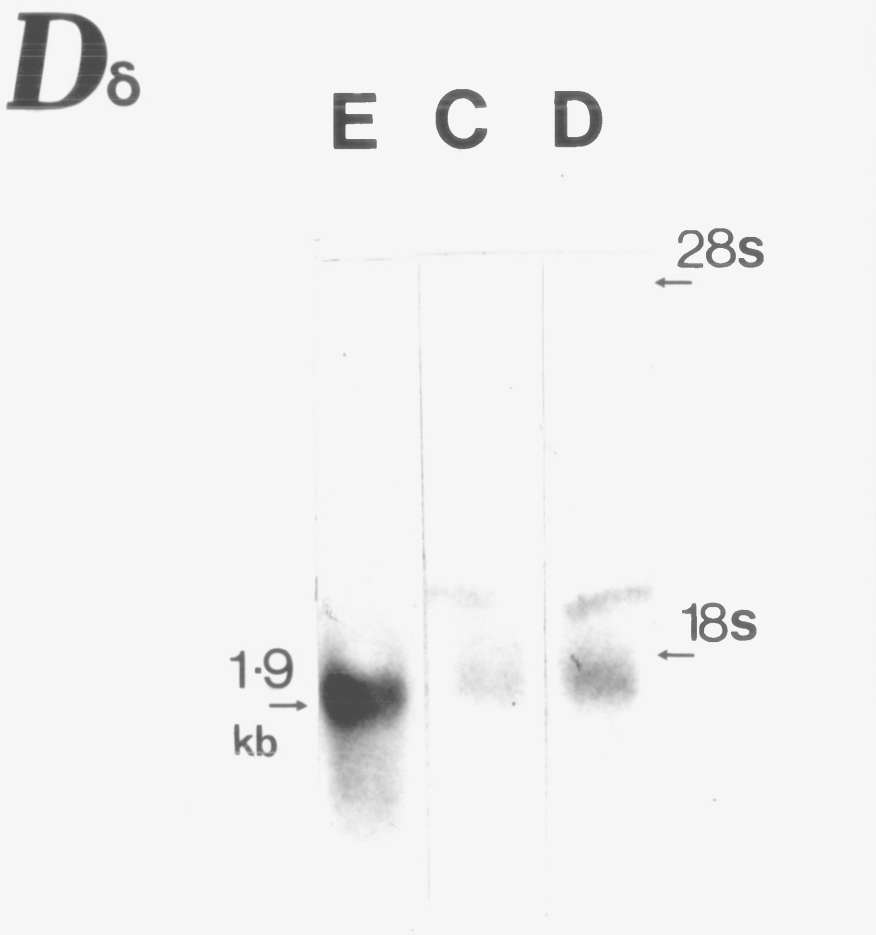


FIGURE (4.4)

Effects of muscle denervation on δ subunit mRNA levels as determined by Northern blotting. Conditions were as described in Figure (4.1) except that the blot was hybridised with insert derived from plasmid PCSMδs (see Chapter 3) labelled by random hexamer priming to 1.0 x 10^9cpm/μg.



Quantification of the Subunit mRNA Levels Expressed within Contralateral Innervated, Denervated and Embryonic Pectoral Muscle

The relative steady state levels of the receptor subunit mRNAs expressed within contralateral innervated, denervated and embryonic pectoral muscle were quantified by densitometric scanning of Northern blots, followed by digitised integration of the hybridisation signals. In order to confirm that the relationship between signal intensity and mRNA concentration was linear at the concentrations used in this study, dot blots of varying amounts of mRNA were probed with the α subunit cDNA probe (Figure (4.5)). The autoradiogram was then subjected to densitometry and a plot of signal vs mRNA concentration (Figure (4.6)) shows that linearity holds for the amounts of mRNA analysed from all three muscle types. Values obtained from densitometric scanning of Northern blots were normalised to that obtained for denervated muscle, which was given an arbitrary value of 1.0. The results are shown in Table (4.1), and represent the mean of 3 independent experiments. For the α, β and δ subunit mRNAs denervation resulted in a 5.9 to 8.3 fold increase in the respective mRNA level. This augmentation is even greater in the case of the γ subunit mRNA; the magnitude of this increase could not be estimated since the γ subunit mRNA could not be detected in contralateral innervated pectoral muscles. The levels of each subunit mRNA present within embryonic and 8 day denervated muscle

FIGURE (4.5)

Dot blot analysis of the level of α subunit mRNA in 13 day embryonic (E), contralateral (C) and denervated (D) pectoral muscle. Messenger RNA (1·25- 20μg) was denatured in 1M glyoxal/50% DMSO and applied directly to a Gene Screen membrane using a Bio-dot (Bio-rad) manifold. Hybridisation, prehybridisation and autoradiography were as described in Figure (4.1).

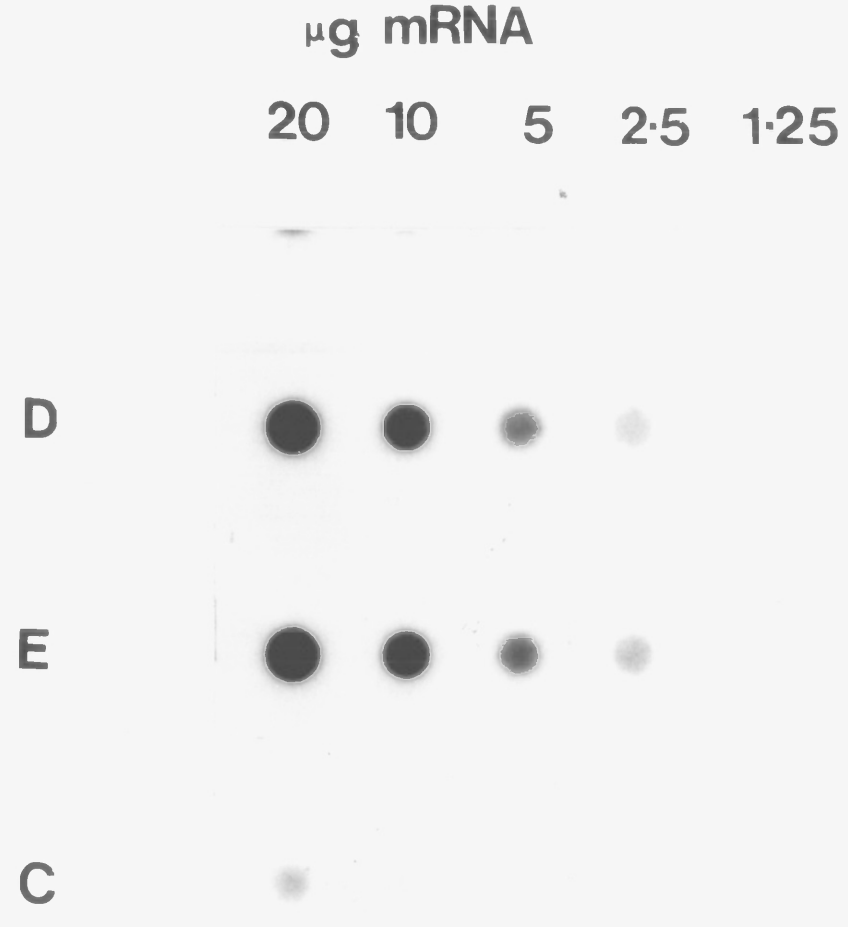


FIGURE (4.6)

Quantification of the level of α subunit mRNA in 13 day embryonic (E), contralateral (C) and denervated (D) pectoral muscle. Signals were quantified by densitometric scanning of Figure (4.5).

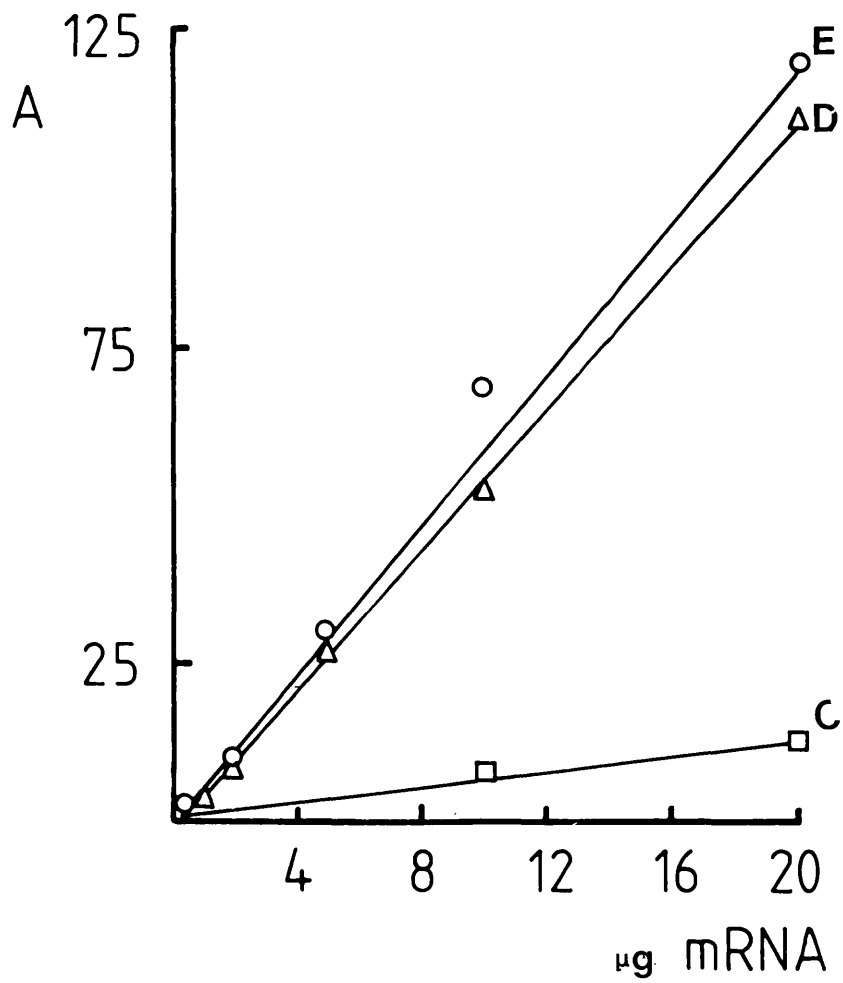


TABLE (4.1)

Effects of Maturation and of Denervation on AChR Subunit mRNA Levels

Subunit mRNA probed	Innervated (I)	Embryonic (E)	Denervated (D)	D/I	D/I corrected ratio
α	0.12 ± 0.01	1.16 ± 0.20	1.0 ± 0.11	8.3	11.6
β	0.17 ± 0.02	1.59 ± 0.19	1.0 ± 0.09	5.9	8.3
γ	ND	1.46 ± 0.25	1.0 ± 0.18	ND	ND
δ	0.15 ± 0.03	1.47 ± 0.21	1.0 ± 0.21	6.7	9.4

In each Northern blot all three tissues were compared simultaneously (as in Figure (4.1)). The innervated muscle used here was the contralateral control pectoral from the denervated bird. After hybridising with each of the four subunit probes in turn, scanning densitometry of each lane provided the integrated density value for each of the subunit mRNAs. Each determination was the mean (± SD) of three blots. Using those mean values, all of the other values given for the muscles have been normalised to that for the denervated muscle, which for each subunit is assigned a relative value of 1.0. The ratio of the steady-state mRNA levels, denervated to innervated muscle (D/I), is also shown. In the last column, the D/I ratio has been corrected for the fact that the contralateral

muscle itself shows a small increase over a true control innervated muscle (Figure (4.7)), so that it is now expressed for true innervated muscle. ND indicates that no signal above background was detectable. Under the conditions used, the lower limit of sensitivity for the detection of RNA was between 1 and 5pg.

were similar, suggesting that similar processes control AChR gene expression in these two muscle states.

Differences in the Level of Expression of the α Subunit mRNA between Contralateral Innervated and Normal Innervated Pectoral Muscles

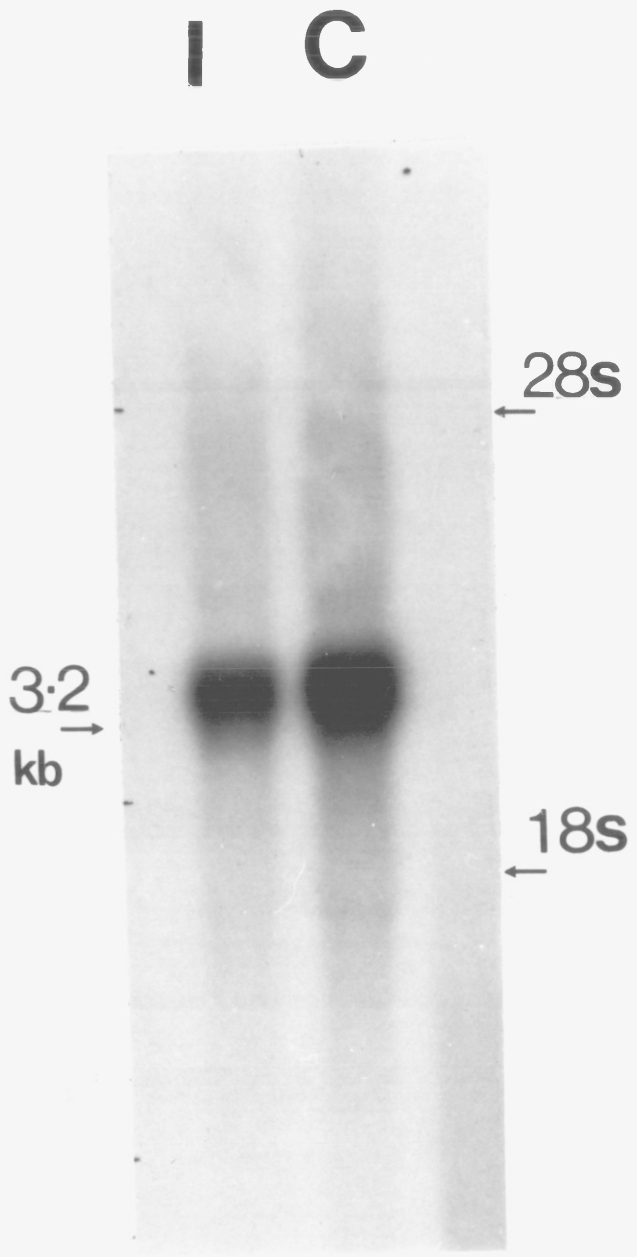
It is known that unilateral denervation of a bilateral muscle, such as the pectoral, results in some compensatory change in the contralateral side, due to the increased load on the latter. For example, a mild hypertrophy of the contralateral muscle occurs (Holly et al 1980). The effect of these changes on the level of α subunit mRNA were investigated by Northern blotting of contralateral innervated and normal age matched innervated pectoral muscle mRNA (Figure (4.7)). Quantitation of the autoradiographs revealed a 1.4 (mean of three observations) fold increase in α subunit mRNA in contralateral muscle compared to normal innervated muscle. Assuming that this holds for all subunit mRNAs, then the true increases in mRNA levels in denervated compared with innervated muscle is in the range of 8.3 - 9.4 fold for the β and δ and 12 fold for α (Table (4.1)).

Determination of the Levels of α Actin mRNA in Denervated, Contralateral Innervated, and Normal Innervated Pectoral Muscles

The level of α actin mRNA in the different muscle types

FIGURE (4.7)

Northern blot analysis of the α subunit mRNA from contralateral innervated (C) and normal aged matched innervated (I) pectoral muscle 20μg of mRNA was denatured in 1M glyoxal/50% DMSO electrophoresed on a 1% agarose gel and transferred to a Gene Screen membrane. Prehybridisation, hybridisation and autoradiography was as described in Figure (4.1), except that the blot was exposed for 1 week.

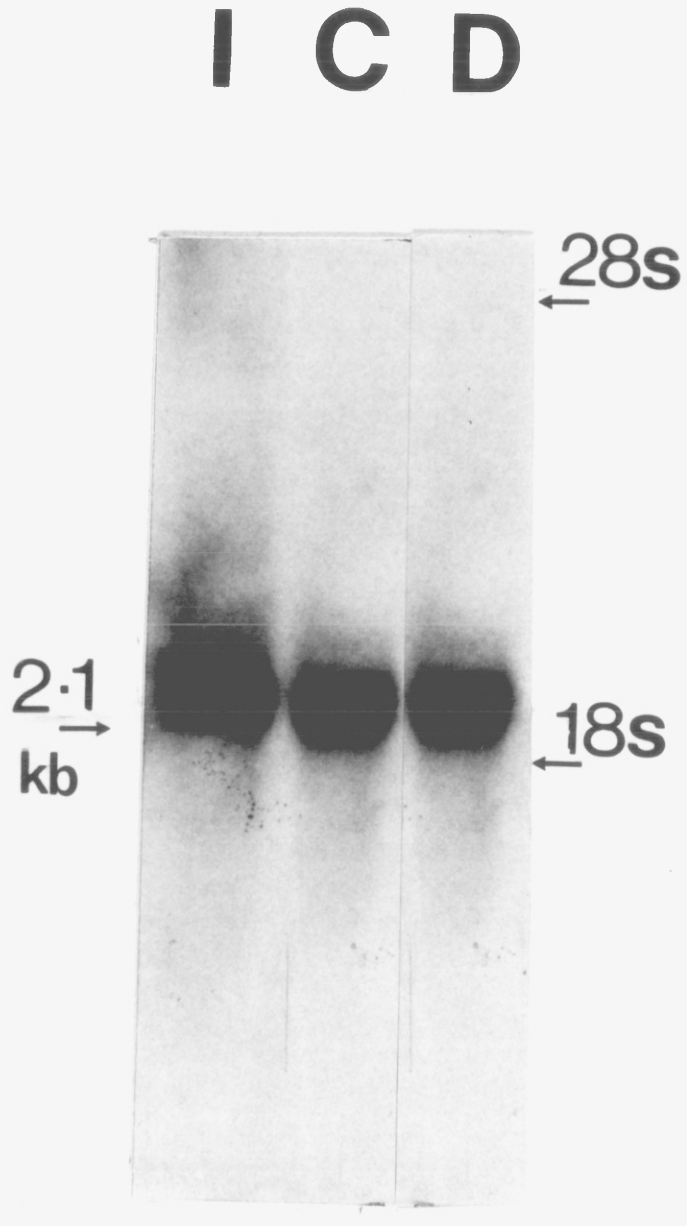


was examined by Northern blotting (Figure (4.8)). The level of α actin mRNA was quantified as for AChR subunit mRNAs. The α actin mRNA levels were not significantly different between the three muscle types.

FIGURE (4.8)

Northern blot analysis of α actin mRNA. 2μg of mRNA isolated from denervated muscle (D), contralateral innervated (C), and normal aged matched innervated pectoral muscle (I) was denatured in 1M glyoxal/50% DMSO electrophoresed on a 1% Agarose gel and transferred to a Gene Screen membrane. The blot was then hybridised at 42°C for 24 hrs in 50% Formamide, 5 x SSPE, 1 x Denhardts, 0.5% SDS, 100μg/ml salmon sperm DNA, and radiolabelled probe encoding the entire α actin protein coding sequence labelled to 7.0 x 10^8 cpm/μg. Prehybridisation was in the same buffer without probe to 2 hrs at 42°C. The blot was then washed at 65°C in 0.1 x SSPE for 1 hr before exposure to Fuji film at -70°C for 2 hrs using an intensifying screen.

FIGURE (4.8)



DISCUSSION

The increased level of AChR expressed within muscle upon its denervation (Fambrough 1979) is concurrent in the case of the chicken pectoral muscle with an 8 to 12 fold increase in the steady state level of the α, β and δ subunit mRNA levels. A large increase in the γ subunit mRNA was also observed, but the magnitude of this increase could not be determined as no γ subunit mRNA could be detected in innervated muscle. Thus, denervation of chicken pectoral muscle results in a large increase in the steady state level of all four AChR subunit mRNAs, in parallel with a 30 - 40 fold increase in AChR receptor protein as determined by α Butx binding (Sumikawa et al 1982b). The parallel increase of both AChR and its encoding mRNAs suggests that receptor expression is controlled by transcriptional mechanisms. This change in AChR subunit mRNAs is not due to a general increase in transcription since the level of α actin mRNA, a major muscle protein, did not change significantly upon denervation. The increased level of AChR content of a muscle upon denervation can be repressed by chronic electrical stimulation (Hall and Reiness 1977). From the data presented above, the innvervating neuron is able to supress AChR biosynthesis by modulating the level of all 4 subunit mRNAs, suggesting in turn that the innervating neuron is capable of modulating either the level of AChR gene transcription or the half lives of the various AChR subunit mRNAs. The

results of Sheih et al (1986) suggest that the innervating neuron is capable of controlling the level of receptor biosynthesis by modulation of the transcription of the 4 subunit genes. Furthermore, it is clear that translational factors must be involved since the increase in AChRs is 4 - 5 times larger than the increase in subunit mRNA. Translational factors controlling receptor biosynthesis upon muscle denervation have been implicated from the experiments of Goldman et al (1985), and Sheih et al (1986). Both of these groups report anomalies between surface AChR levels and increases in receptor subunit mRNA levels. The increases upon denervation of the steady state levels of the α, β, γ and δ mRNAs are different, suggesting that AChR gene expression may not be coordinated by a universal mechanism. Interestingly, the levels of each subunit mRNA present within embryonic muscle (E12 or E14) are similar to those present within denervated muscle. Furthermore, the levels of each receptor mRNA present within 8 week innervated muscle are comparable with those obtained for 1 day post hatch. Both of these observations suggest that the mechanisms controlling the level of receptor mRNAs within denervated and embryonic muscle are common.

The excess loading of the contralateral innervated pectoral muscle when unilaterally denervated leads to a small rise in AChR synthesis as reflected by a rise in the α subunit mRNA levels. This does not reflect a general increase in transcription since the level of α

actin did not change significantly in this situation. It will be of great interest to know if the AChR mRNAs are of a limited class which respond in this manner to muscle stress as represented in this state.

The 8.3 fold increase in α subunit mRNA determined for denervated muscle when compared to contralateral innervated is less than half that observed by Klarsfeld and Changeux (1985), who measured the α subunit mRNA within the same species. This discrepancy may reside within the period of denervation prior to mRNA extraction (8 days and 4 days respectively), or in age of the chickens used (8 weeks versus 6 days) may contribute to this discrepancy. The period of denervation, namely, 8 days prior to mRNA extraction, was based on that used by Sumikawa et al (1982b) who showed that α Butx binding was maximal 8 days post denervation. Alternatively, the fact that Klarsfeld and Changeux (1985) used leg muscles may account for it, denervation of different muscles may result in differential increases in rates of gene transcription, since within rat diaphragm or mouse leg muscle (Merlie et al 1984 Goldman et al 1985) increases of 7 fold, and 50 - 100 fold have been reported. Variation in subunit gene response to muscle denervation is also evident from these results; namely, the α subunit mRNA level appears to be more responsive to muscle denervation than either the β or δ, while the magnitude of this increase for the γ could not be estimated. Evans et al (1987) report the effects of muscle denervation in both

mouse and rat muscles on the steady state levels of the α, β, γ and δ subunit mRNAs. The β subunit mRNA within the murine was much less responsive to muscle denervation than either the α, , γ or δ subunit mRNAs. Clearly more extensive studies are required to determine the factors involved in these differences.

A final point raised by this series of experiments is the possible existence in the chicken of a fifth AChR muscle subunit as has been demonstrated for the bovine receptor (Takai et al, 1985). As demonstrated above the γ subunit mRNA could not be detected in contralateral innervated chicken pectoral muscle, but was present within embryonic and denervated muscle. This is consistent with the results of Mishina et al (1986), who showed that the bovine γ subunit mRNA was abundant in fetal muscle but was essentially undetectable in adult muscle. In parallel with this decline in γ subunit mRNA during development an increase in ε subunit mRNA was observed. Furthermore, Witzemann et al (1987) have shown that upon muscle denervation the γ subunit is again produced.

Using combinations of subunit specific RNAs, derived from cloned cDNAs and the Xenopus oocyte system, Mishina et al (1986) showed that the two types of receptor (α_2, β, γ, δ and α_2, β, δ, ε) have different channel conductances and gating properties, which resemble the receptor channels found in fetal and adult muscles respectively. A marked decrease in the mean channel open

time of the AChR during development has also been reported in a number of vertebrate species (reviewed by Schuetze and Role 1987), with the notable exception of the chicken. Presumably, therefore, should a chicken muscle ε subunit exist, a receptor containing this polypeptide would be indistinguishable from a receptor containing the γ subunit. A second less likely interpretation of this result is that the chicken receptor comprises just α, β and δ subunits. Since the charge and size of the AChR purified from innervated and denervated pectoral muscle are the same (Sumikawa et al 1982b), this would require two copies of both α and another subunit in the oligomer. Recently Takai et al (1987) have demonstrated, using subunit specific RNAs derived from cloned cDNAs, that the Torpedo AChR channel can be formed from just α, β and δ polypeptides, but the gating efficiency is 1/10 that of the α_2, β, γ, δ oligomer.

CHAPTER 5

EFFECTS OF CGRP ON THE LEVELS OF AChR RECEPTOR AND ITS ENCODING mRNAs IN EMBRYONIC MUSCLE

INTRODUCTION

The maintenance of the number and distribution of AChRs within adult innervated muscle is dependent on a number of factors. The activity of the innervating neuron is important in controlling the number of extra-junctional AChRs by directly modifying the level of receptor biosynthesis (reviewed in Chapters 3 and 4). AChRs within adult innervated muscle are densely packed in clusters at the end plate regions. AChRs within this region must be continuously replaced since they are labile and turnover, albeit at much reduced rates in comparison to embryonic or extra-junctional receptors (Burden 1977a,b). Continued synthesis of AChRs at endplate regions of innervated muscle, while synthesis of extra-junctional receptors is repressed, requires the intervention of a signal of neuronal origin to override the effects of electrical repression on AChR biosynthesis (Changeux et al 1987).

Local synthesis of AChR within innervated muscle has been implicated by experiments designed to examine the distribution of the mRNAs that encode the AChR oligomer. The α and δ mRNAs encoding the murine AChR are found in higher concentrations in junctional regions as compared to extra-junctional regions as demonstrated by microdissection (Merlie and Sanes 1985). Furthermore, Burden and Simon (1987) and Fontaine et al (1988) have shown that the α and δ mRNA within innervated muscle are

more abundant in junctional regions as compared to extra-junctional regions, by in-situ hybridisation.

A number of possible anterograde factors that stimulate AChR biosynthesis have been reported (reviewed by Salpeter 1987). Jessel et al (1979) have isolated a low molecular weight component of 1674 daltons of neuronal origin that increases α Butx binding in cultured chick myoblasts. The identification of this factor or its method of action have yet to be elucidated. Recently Fischbach and co-workers (Harris et al 1988) have isolated a 42Kd protein (termed ARIA) from chick brain that increases the level of AChR and α subunit mRNA levels in muscle cultures. Another good candidate for a neuronally derived factor responsible for modulating AChR biosynthesis is the neuropeptide CGRP. CGRP is a widely distributed peptide generated by differential RNA processing from a single genomic locus, which also encodes calcitonin (Rosenfeld et al 1983). CGRP exerts several biological actions such as vasoconstriction, inhibition of gastric secretion, and ingestive behaviour (Fischer and Born 1985) and has been proposed to act as a neurotransmitter in the sensory tract (Tschopp et al 1985).

CGRP has been localised by immunohistochemistry to chicken motorneurons (New and Mudge 1986a,b, Fontaine et al 1986), and to murine motor end plates (Takami et al 1985). Moreover the peptide has been co-localised with

ACh in rat neurons (Takami et al 1985). CGRP may, therefore, serve as a co-existing neuronal messenger with the classical neurotransmitter ACh (Hokfelt et al 1984). Consistent with such a role, CGRP is released from rat trigeminal cells in a calcium dependent manner (Mason et al 1984). Futhermore, it has recently been shown that addition of CGRP to chick muscle cells in primary culture significantly increases the level of surface AChR, without changing the rate of AChR degradation or modifying the overall rate of protein synthesis (New and Mudge 1986b, Fontaine et al 1986). CGRP has also been shown to increase the intracellular level of cAMP in primary chick myotubes (Laufer and Changeux 1987). It has been previously shown that cAMP or analogues of this nucleotide, as well as activators of adenylate cyclase, stimulate AChR biosynthesis (Betz and Changeux 1979, Blosser and Appel 1980). CGRP may have additional effects on the AChR other than those postulated above, since CGRP has been demonstrated to increase the rate of AChR desensitization in rat embryonic muscle cultures (Huganir and Greengard 1987). Phosphorylation of the AChR in Torpedo electroplaque and rat myotubes has been previously shown to be mediated by activation of cAMP-dependent protein kinase which specifically phosphorylates the α and δ subunits of the AChR (Huganir 1984, Huganir et al 1986, Miles et al 1987).

In a collaborative project with Dr. A. Mudge (MRC Neuroimmunology Programme, University College, London),

the effects of CGRP on AChR biosynthesis were further investigated. The cDNA and genomic clones described in Chapters 3 and 4 were used to monitor the effects of CGRP and activators of adenylate cyclase on the steady state levels of the α, β, γ and δ subunit mRNAs in primary cultures of chick myotubes.

METHODS AND MATERIALS

Cell Culture

Pectoral muscle from E11 chicks was dissociated in a Ca^{2+} -Mg^{2+} -free balanced PBS at 37°C, the cell suspension was plated onto tissue-culture plastic previously incubated with a solution of 1% gelatin and then air dried. Cells were plated at a density of 4 x 10^6 cells per 100mm dish, 5 x 10^5 cells per 35mm dish or 10^5 cells per 16mm well. Growth medium was DMEM (Gibco) containing 25mM $NaHCO_3$ and supplemented with 10% v/v horse serum, 5% v/v chick embryo extract (CEE) plus 50 units/ml penicillin, 50μg/ml streptomycin, 10mM glutamine and 10mM glucose. The cultures were grown in a humid atmosphere with 5% CO_2 and after 2 days the CEE was reduced to 2%. When cultures were grown for longer than 3 days, the cells were treated with 5 x 10^{-6}M cytosine arabinoside for 24 hrs between days 2 and 3 of culture to reduce the number of fibroblasts and subsequently were fed every 2 days.

α Butx Binding Assay on Cell Cultures

The number of surface AChRs was measured by binding ^{125}I - α Butx (Amersham International; sp. act. 2000Ci/mmol). Myotube cultures were incubated at 37°C for 60 mins with 5 x 10^{-9}M ^{125}I - α Butx in DMEM plus 1% bovine serum albumin. The cultures were then washed 4 times for a

total of 15 mins in phosphate buffered saline plus 4% calf serum and the cells were then collected from the dishes or wells with 0.25% w/v trypsin and counted in a gamma counter (Nuclear Enterprises). Non-specific binding was estimated in sister cultures by preincubation with 10mM carbachol (Sigma) and inclusion of 10mM carbachol during the ^{125}I - α Butx incubation.

Determination of cAMP Levels in Chick Myotube Cultures

Cells cultured in wells were washed twice with DMEM, twice with PBS and then 100μl of 6% trichloroacetic acid was added to the cell monolayer; plates were then stored at -20^{o}C until cellular cAMP levels were measured. cAMP was measured using a competitive radioimmunoassay kit (Amersham International); duplicated sample aliquots were acetylated, as recommended in the kit protocol, in order to use the more sensitive scale.

RNA Isolation

Total RNA was prepared from pools of 4 to 20 culture dishes. Cells were harvested after digestion with 0.25% trypsin as described above. Total RNA was isolated by the method of Chirgwin et al (1979) as described in Chapter 2. Yields of RNA varied between 50 - 125μg of total RNA per dish. Where yields of total RNA were sufficient poly(A)$^{+}$ RNA was selected by oligo (d)T cellulose chromatography (Aviv and Leder 1972) as described in

Chapter 2.

Poly(U) Calibration of RNA Preparations

The yields of both total RNA and poly(A)$^+$ RNA were first quantified by spectroscopy at 260nm. RNA preparations were then calibrated by ^{3}H-poly(U) assay as described in Chapter 3.

Northern Blots

Northern blots were performed as described in Chapter 3. Hybridisation probes for the α, β, γ and δ subunit mRNAs encoding the chick AChR were as described in Chapter 3. The α actin probe was as described in Chapter 4. The β/γ actin probe P x 55 was a generous gift from M. Goedert (Laboratory of Molecular Biology, Cambridge). The probe PX55 encodes a murine β/γ pseudogene and was provided as a 2.5Kb PstI fragment cloned in plasmid PBR322.

RNase Protection Analysis

α subunit mRNA levels were measured in some experiments by RNase protection.

(1) Probe synthesis and plasmid construction.

The α subunit cDNA described in Chapter 2 was digested with EcoRI and PstI to yield a 169bp

fragment encoding the first exon and part of the second exon of the chick α subunit gene (Figure (5.1)), Nef et al (1988). This fragment was cloned into vector p-GEM2. Ligation, transformation and large scale plasmid preparation were as described in Chapter 3. The plasmid PCSMα2 was then linearised with EcoRI and labelled probe was produced by transcription using the SP6 promotor as described in Chapter 3. Probe was then purified on a 6% acrylamide/7M urea gel as described in Chapter 3. Probe was then localised by autoradiography. A small gel slice containing the labelled probe was removed and incubated at 45°C for 2 hrs in 300μl of Maxam and Gilbert elution buffer:-

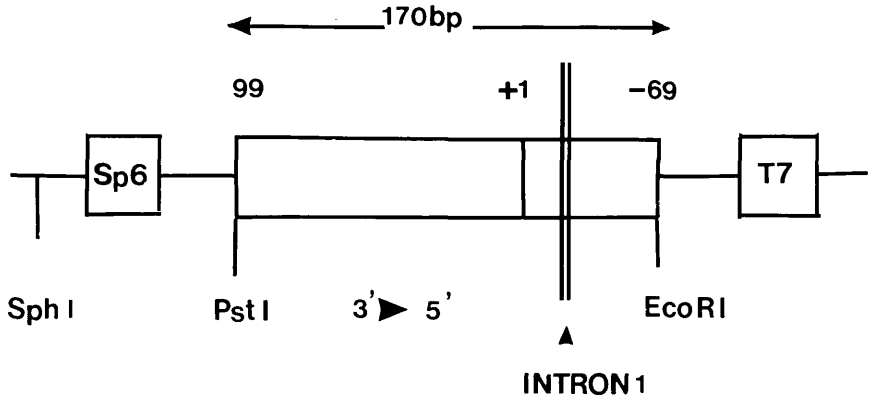
0.5M ammonium acetate
1mM EDTA
0.5% SDS

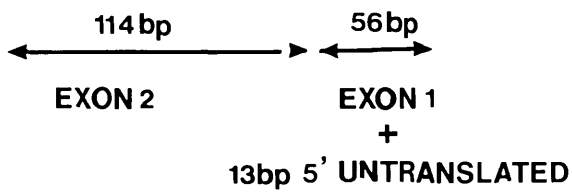
The solution was extracted with an equal volume of phenol (equilibrated with TE) and precipitated with 2 volumes of 95% ethanol. Probe was collected by centrifugation and resuspended in 50μl of hybridisation buffer.

Hybridisation buffer:-
80% Formamide
400mM NaCl

FIGURE (5.1)

Plasmid PCSMα2. Intron/exon boundries were based on those defined for the chick α subunit gene, as described by Nef et al (1988).





+1=ATG

40mM PIPES pH 6.4

1mM EDTA

(2) RNase protection assays

Total RNA between 50μg and 25μg from E11 pectoral muscle and from cultures treated with CGRP and other agents was resuspended at 1mg/ml in hybridisation buffer. Probe was then added (5×10^5 cpm). Samples were then mixed and incubated at 85°C for 5 mins before overnight hybridisation at 45°C. 350μl of digestion buffer was then added.

Digestion buffer:

10mM Tris-HCl pH 7.4

1mM EDTA

300mM NaCl

20μg/ml RNase A

0.5μg/ml RNase T

Digestion was at 37°C for 30 mins. In some experiments the concentration of RNase A was varied to optimise the signal to noise ratio (Between 5 -> 40μg/ml). After digestion 5μl of 20% SDS was added and 5μl of protenase K 10mg/ml, and digestion was then extended for a further 10 mins. Samples were then phenol extracted and ethanol precipitated after the

addition of 10μg of yeast tRNA. RNA protected fragments were recovered by centrifugation and suspended in 5μl of sequencing dyes (Chapter 2) and resolved on 6% acrylamide 7M urea gels. Gels were then processed as for sequencing gels in Chapter 2. Autoradiography was performed at -70^{o}C with intensifying screens.

Scanning Densitometry

Autoradiographs were quantified by scanning densitometry as described in Chapter 3.

Data were analysed using a students t-test.

RESULTS

Effects of CGRP, CT and Forskolin on the Levels of AChR Expressed on Chick Myotubes

The level of AChR expressed on the surface of chick myotubes was estimated by α Butx binding. CGRP caused a significant increase in the level of AChR expressed over control cultures (Table (5.1)). The CGRP stimulation was found to be variable and was between 25 - 50% of that in control cultures, with a mean value of 40%. Typically 2 - 6 fmoles of $^{125}I\alpha$Butx binding was detected per well in control cultures. Both Cholera Toxin (CT) and Forskolin caused significant increases in the surface levels of AChR (Table (5.1)), the magnitude of these increases being similar to those obtained with CGRP. The effects of CT were found to be more variable than those seen with either CGRP or Forskolin. These results are consistent with the earlier observations of New and Mudge (1986b) and Fontaine et al (1986). Both these groups report similar effects of CGRP on the level of AChR expressed on the surface of chick muscle cultures. Furthermore, both these groups demonstrated that this increase was not due to changes in rates of AChR degradation. CGRP has also recently been shown to increase AChR levels in rat and mouse myotube cultures (J. P. Merlie - personal communication). CT has previously been shown to increase the level of AChR within chick myotube cultures (Betz and Changeux 1979,

Table (5.1)

Effects of CGRP, CT, and Forskolin on the Level of Surface α Butx Binding in Chick Primary Muscle Cultures

(+) (CGRP) (10^{-7}M)	(+) (CT) (25ng/ml)	(+) (FORSKOLIN) (10^{-9}M)	CONTROL (UNSTIMULATED)
1.4^	1.35^	1.49^	1.0
± 0.09	± 0.20	± 0.10	± 0.09

^ = values significantly different at 0.99 confidence interval

The effects of CGRP, CT and Forskolin on the level of AChR as reflected by α Butx binding was measured on sister cultures after 24 hrs exposure to the pharmacological agents. The results represent the mean of four experiments.

Fontaine et al 1986). Consistent with the effects of both Forskolin and CT, large increases in the levels of cAMP were observed over control cultures (Table (5.2)). CGRP also significantly increased the levels of cAMP in chick myotube cultures, but this increase was smaller than that observed for Forskolin or CT. Laufer and Changeux (1987) have recently demonstrated the potency of CGRP in activating adenylate cyclase. The existence of a receptor for CGRP is implicated by these observations. Recently specific high affinity binding sites have been demonstrated in chick muscle (A. Mudge - personal communication). Furthermore, the CGRP receptor has been identified as a 65K dalton glycoprotein in chick myotubes (H. Poo - personal communication).

Effects of CGRP on the Levels of the α, β, γ and δ Subunit mRNAs Encoding the AChR in Chick Muscle Cultures

Northern blots were performed on mRNA derived from pooled cultures, after 3 days in culture following exposure to CGRP for 24 hrs. Typical blots for the α, β, γ and δ subunit mRNAs are shown in Figures (5.2), (5.3), (5.4), and (5.5). The size of the respective subunit mRNAs were α 3.2Kb (with minor bands at 4.2 and 5.9Kb), β 2.8Kb, γ 1.8Kb, and for the δ subunit mRNAs 1.9 and 2.3Kb, identical to those values obtained from fresh pectoral muscle (Chapters 3 and 4). Blots were subjected to scanning densitometry as outlined in Chapters 3 and 4. Signals were then normalised to that of unstimulated

FIGURE (5.2)

Effects of CGRP on α subunit mRNA levels as determined by Northern blot analysis. Messenger RNA was denatured using 1M glyoxal, 50% DMSO, electrophoresed on a 1.0% agarose gel and transferred to a Gene Screen membrane. The blot was then hybridised in 50% Formamide, 5 X SSPE, 1 x Denhardts, 0.5% SDS, 100μg/ml salmon sperm DNA, 100μg/ml yeast tRNA and labelled insert derived from plasmid PCSMαs (see Chapter 3). Insert DNA was labelled by random hexamer priming to 1 x 10^9cpm/μg, for 24 hrs at 42°C. The blot was then washed at 0.1 x SSPE at 65°C for 1 hr before exposure to Fuji film at -70°C using an intensifying screen for 24 hrs. The migration of chick 28S and 18S ribosomal RNA bands is also shown.

FIGURE (5.2)

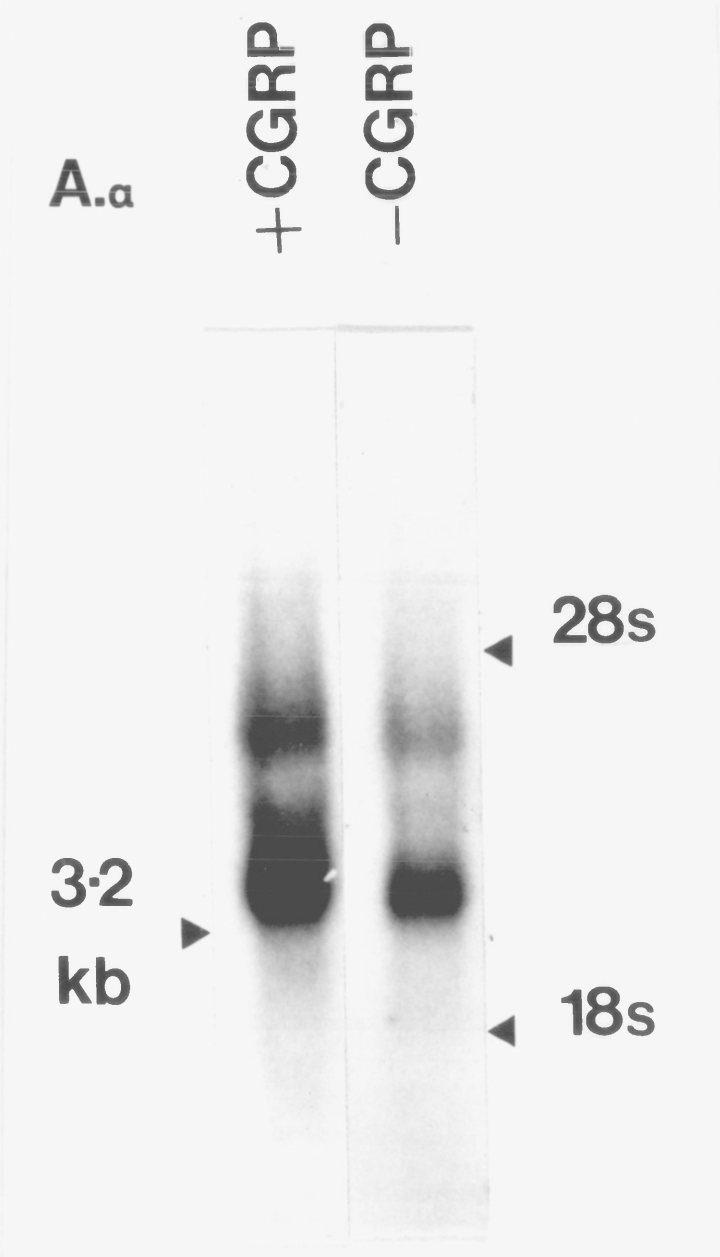


FIGURE (5.3)

Effects of CGRP on β subunit mRNA levels as determined by Northern blot analysis. Conditions were as described in Figure (5.2), except that the blot was hybridised with insert derived from plasmid PCSMβs (see Chapter 3), labelled by random hexamer priming to 8.5 x 10^8cpm/μg.

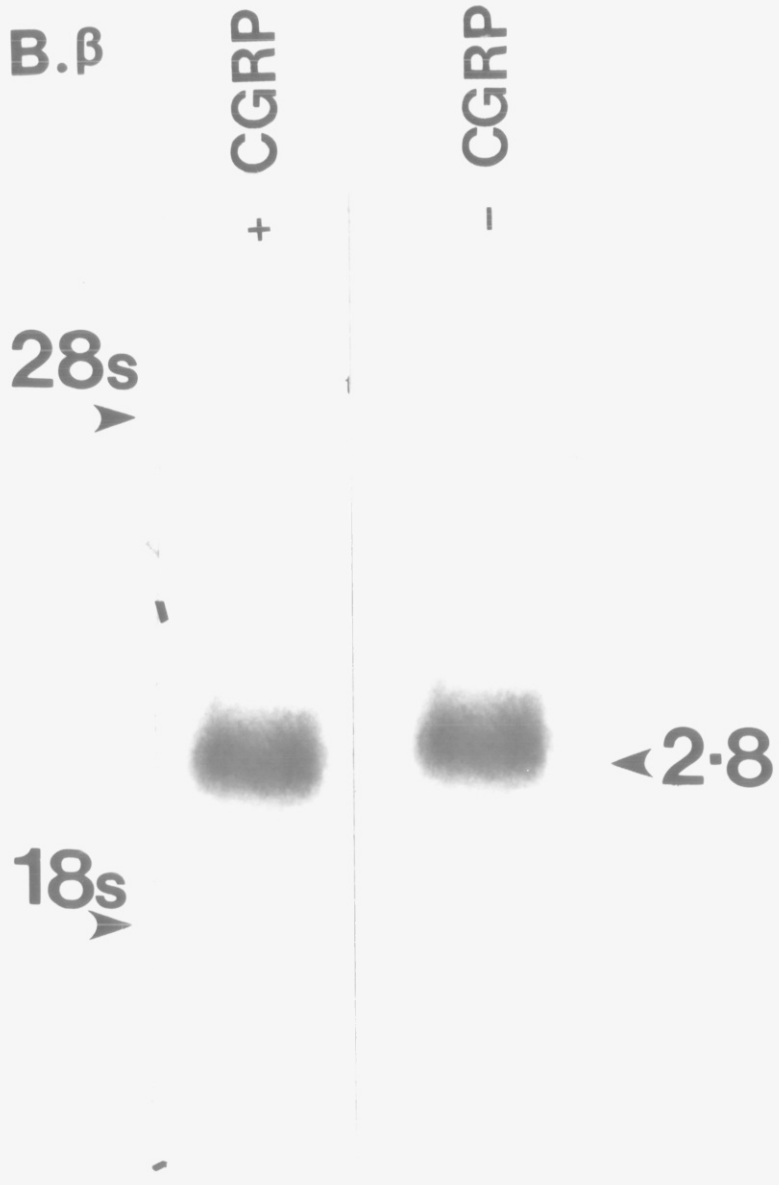


FIGURE (5.4)

Effects of CGRP on γ subunit mRNA levels as determined by Northern blot analysis. Conditions were as described in Figure (5.2), except that the blot was hybridised with insert derived from plasmid PCSMγs (see Chapter 3), labelled by random hexamer priming to 1.5 x 10^9cpm/μg.

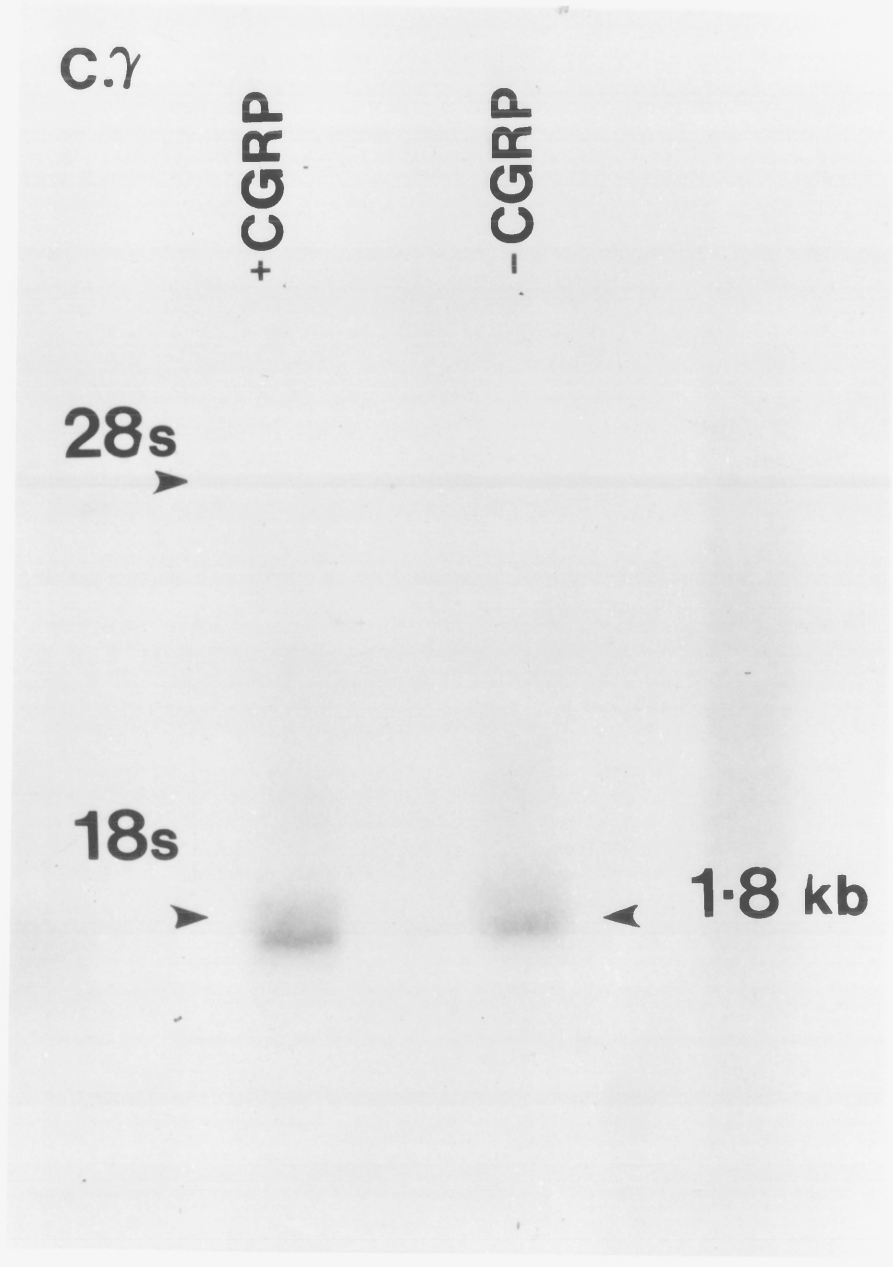


FIGURE (5.5)

Effects of CGRP on δ subunit mRNA levels as determined by Northern blot analysis. Conditions were as described in Figure (5.2), except that the blot was hybridised with insert derived from plasmid PCSMδs (see Chapter 3), labelled by random hexamer priming to 9 x 10^8cpm/μg.

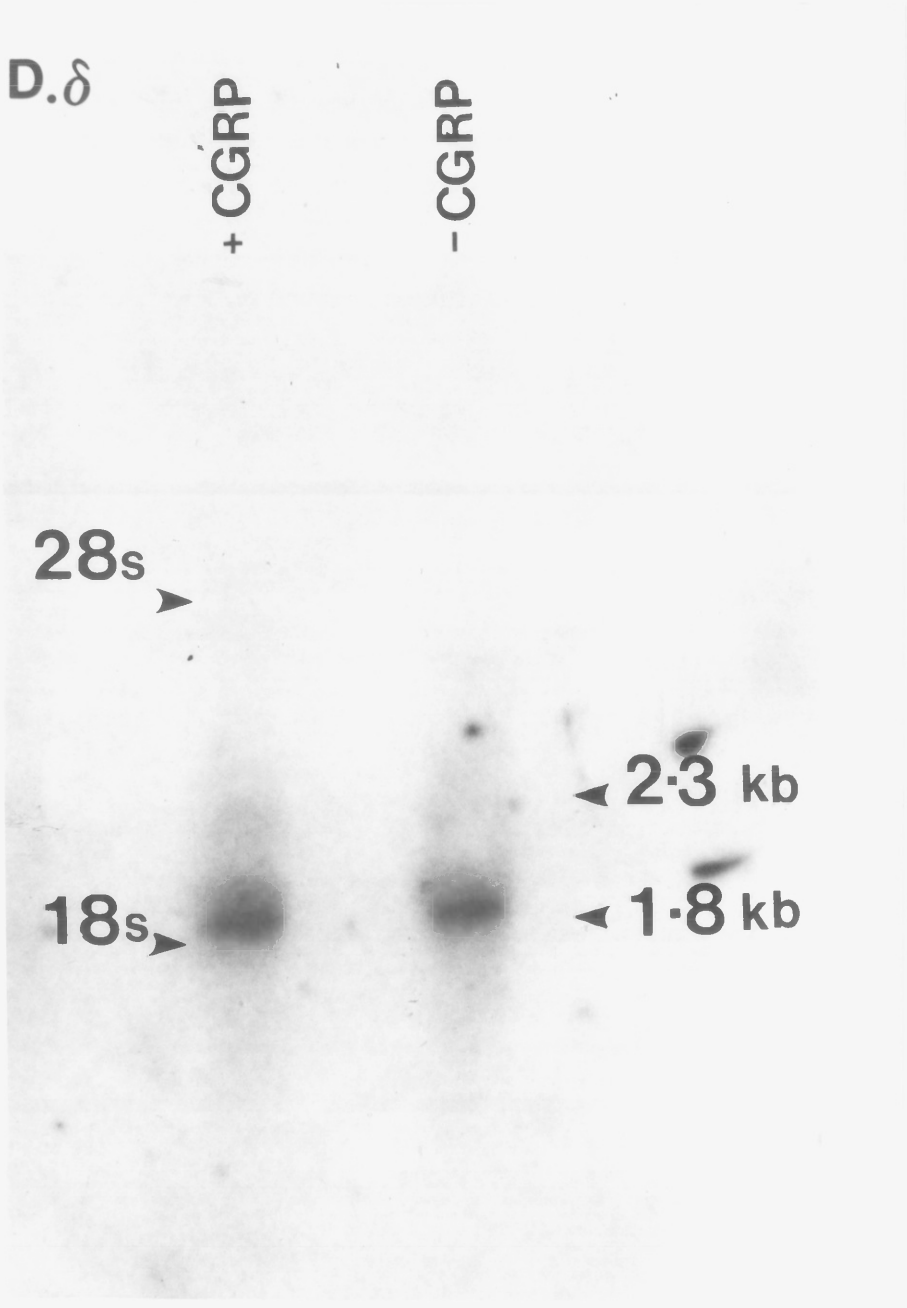


TABLE (5.2)

Effects of CGRP and Forskolin on the Level of cAMP in Chick Muscle Cultures

	+CGRP (10^{-7}M)	+FORSKOLIN (10^{-9}M)	+CT 25ngs/mL	CONTROL
cAMP Fm/dish	230 ± 31	720 ± 80	650 ± 120	75 ± 5
Relative increase	(3.0)	(9.6)	(8.6)	(1.0)

Intracellular cAMP levels were determined by radioimmunoassay. Assays were performed on triplicate cultures, which were exposed to pharmacological agents for 30 mins prior to assay. Data is from a typical experiment which was repeated several times with similar results. Data was normalised to control cultures which were given an arbitrary value of 1.0.

cultures which were given an arbitrary value of 1.0. The data for the α, β, γ and δ subunit mRNAs are shown in Table (5.3) and represents the mean of 3 different experiments. In each case the CGRP stimulation of α Butx binding was always greater than 30%. Data was then compared by t-test. A 60% increase in the level of the α subunit mRNA was observed, but no statistically significant change in the level of either the β, γ or δ mRNAs was observed. Fontaine et al (1987) report a similar effect of CGRP on α subunit mRNA levels, but the magnitude of this increase was much greater (3 - 4 fold). CGRP, in this group's experiments, did not significantly change the level of α actin or β/γ actin. Furthermore, Fontaine et al (1987) also reported that CT caused a similar increase in α subunit mRNA. In parallel with measurements on the levels of AChR subunit mRNA, the levels of both α and β/γ actin were measured on exposure to CGRP by RNA dot blotting and the results are shown in Table (5.4). CGRP did not change the level of α actin, but a 2 fold decrease in the level of β/γ actin was observed on exposure to CGRP. These discrepancies between the results of Fontaine et al (1987) and those presented above, could possibly reside in differences in culture time before addition of CGRP (namely 3 days compared to 6 days) or variations between cultures masking larger increases in α subunit mRNA levels. To address these discrepancies between the results presented

TABLE (5.3)

Effects of CGRP on α, β, γ and δ Subunit mRNAs after 3 days in Culture

Subunit transcript	(+) CGRP (10^{-7}M)	(-) CGRP
α	1.60 ^ ± 0.09	1.0 ± 0.04
β	1.1 ± 0.05	1.0 ± 0.02
γ	1.4 ± 0.09	1.0 ± 0.08
δ 1.9	1.0 ± 0.1	1.0 ± 0.07
δ 2.3	1.3 ± 0.05	1.0 ± 0.09

^ = values significantly different at 0.99 confidence interval

Messenger RNA was extracted from pools of pectoral muscle cultures, and AChR subunit mRNA levels were analysed by Northern blotting after poly(U) calibration. Hybridisation signals were quantified by scanning densitometry. Results represent mean of 3 independent experiments, values were normalised to that of (-) CGRP.

TABLE (5.4)

Effects of CGRP on the levels of α skeletal actin and β/γ actin mRNAs

	(+) CGRP (10^{-7}M)	(−) CGRP
* β/γ actin	1.0 ± 0.20	2.1^ ± 0.18
X α actin	1.1 ± 0.24	1.0 ± 0.31

* values normalised to (+) CGRP

x values normalised to (−) CGRP

^ significantly different at 0.99 confidence interval

Messenger RNA was extracted from pools of pectoral muscle cultures and actin mRNA levels were determined by Dot Blotting. Hybridisation signals were quantified by scanning densitometry. The results represent the mean of 3 independent experiments.

above and those of Fontaine et al (1987), the effects of CGRP on α subunit mRNA levels was further investigated by the technique of RNase mapping. The sensitivity of this technique being such that 3 culture dishes could provide sufficient RNA for analysis.

Effects of CGRP and Forskolin on α Subunit mRNA Levels as Measured by RNase Protection

As a prerequisite to examining the effects of CGRP and Forskolin on α subunit mRNA levels, it was necessary to optimise RNase digest conditions to maximise the signal to noise ratio and to establish the linearity of the assay. RNase digest conditions from 5μg/ml to 40μg/ml were examined using 10μg of E12 pectoral muscle total RNA (Figure (5.6)). At all concentrations of RNase A used, a predicted fragment of 169 nucleotides was evident and fragments of 114, and 55bp were also protected. These fragments were not artifacts since they were detected in muscle mRNA, and absent from muscle poly(A)$^-$ preparations and also from liver mRNA and poly(A)$^-$ RNA (Figure (5.7)). The smaller protected fragments were present at lower abundance (10 fold) compared to the major fragment. The linearity of the assay was also tested using concentrations of total RNA from 50 - 5μg. The hybridisation signal was found to be linear over this concentration range (results not shown). The detection of smaller protected fragments indicates the presence of unspliced forms of the α subunit mRNA as suggested in

FIGURE (5.6)

Optimisation of RNase A digest conditions. E12 pectoral muscle total RNA at 1μg/ml in 80% Formamide 400mM NaCl, 40mM PIPES pH 6.4, was hybridised with 5 x 10^5cpm of probe produced by *in vitro* transcription of plasmid PCSMα2 encoding from nucleotide -69 to 99 of the chick muscle α subunit cDNA (Beeson *et al* 1988). Hybridisation was overnight at 45^{o}C. The hybridisation mixes were then digested with RNase A from 5 - 40μg/ml and RNase T_1 (0.5 μg/ml). Protected fragments were then separated on a 6% acrylamide 7M urea gel. Size markers were end labelled fragments of plasmid pBR322 digested with *MspI*. End labelling of markers was performed as described by Maniatis *et al* (1982). The gel was then exposed to Fuji film at -70^{o}C using an intensifying screen for 2 hrs.

FIGURE (5.6)

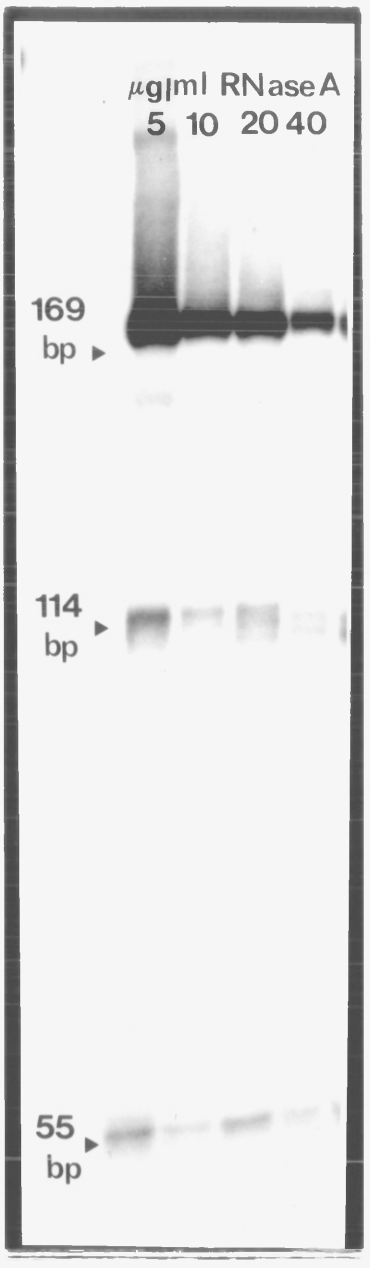


FIGURE (5.7)

Detection of α subunit mRNA precursors by RNase protection.

Track 1 10μg of E12 pectoral muscle total RNA

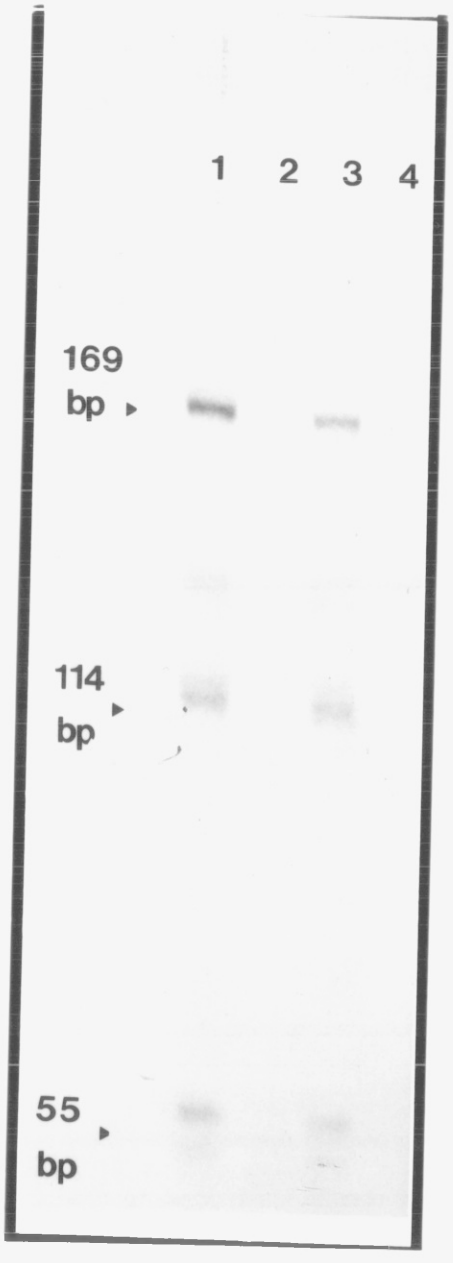
Track 2 10μg of E12 liver total RNA, 10μg of muscle poly(A)- RNA

Track 3 2μg of E12 pectoral muscle mRNA

Track 4 10μg of E12 liver mRNA

were hybridised with a probe encoding from nucleotide -69 to 99 of the chick muscle α subunit cDNA, as described in Figure (5.6). The hybridisation mixes were then digested with RNase A at 20μg/ml and RNase T_1 0.5 μg/ml. Denaturing gel electrophoresis and autoradiography were as described in Figure (5.6).

FIGURE (5.7)



Chapter 3. The genomic organisation of the chick α subunit gene has been characterised (Ballivet et al 1983, Nef et al 1988) and appears to be identical in its intron/exon pattern as the human α gene (Noda et al 1983c). Using this model, an intron is predicted at position -14 in the chick cDNA sequence (Beeson et al 1988, Nef et al 1988). Indeed this is verified by the production of protected fragments of 114bp and 55bp. The detection of these fragments confirms the presence of unspliced forms of the α subunit mRNA in chick muscle RNA preparations, as discussed in Chapter 3. Further confirmation of this is that the ratio of these smaller fragments to the major protected fragment is similar to the ratio of the larger mRNA species (4.2 and 5.9Kb) to the major RNA of 3.2Kb as determined by Northern blotting (Figure (5.2)).

Once optimal digestion conditions had been established using E12 pectoral muscle total RNA, the effects of CGRP and Forskolin on the levels of α subunit mRNA were investigated. Sufficient total RNA was obtained from 2 sister cultures, which both showed 35 - 40% increased α Butx binding compared to control cultures. Poly(U) calibrated total RNA from 2 day and 6 day pectoral muscle cultures exposed to CGRP or Forskolin for 24 hrs were assayed for α subunit mRNA content by RNase protection. The results of typical protection experiments are shown in Figures (5.8) and (5.9). A major protected fragment of 169bp was detected with minor fragments of 114 and

FIGURE (5.8)

Effects of CGRP and Forskolin on the level of α subunit mRNA in pectoral muscle cultures (3 days in culture before pharmacological challenge).
Track 1 10μg of total RNA from control cultures
Track 2 10μg of total RNA from cultures treated with 10^{-7}M CGRP for 24 hrs
Track 3 10μg of total RNA from cultures treated with Forskolin (10μM) for 24 hrs
were hybridised with an α subunit probe as described in Figure (5.6). RNase digest conditions, denaturing gel electrophoresis and autoradiography were as described in Figure (5.6).

FIGURE (5.8)

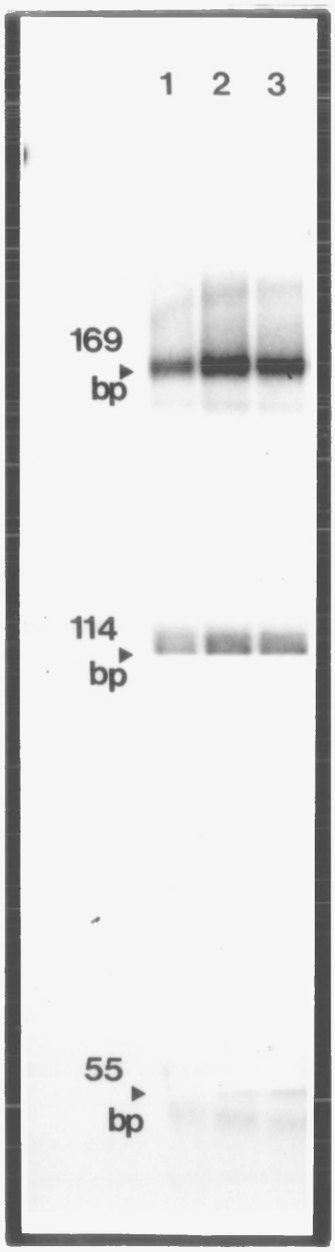
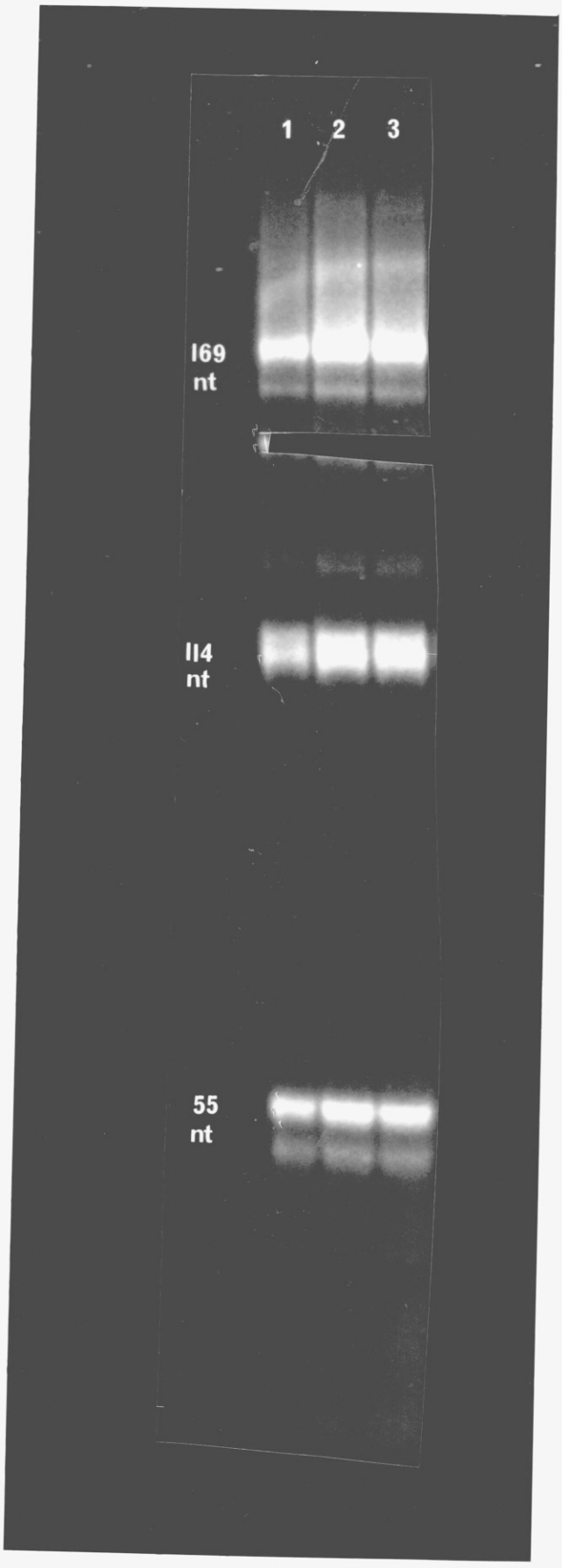


FIGURE (5.9)

Effects of CGRP and Forskolin on the level of α subunit mRNA in pectoral muscle cultures (6 days in culture before pharmacological challenge).
Track 1 10μg of total RNA from control cultures
Track 2 10μg of total RNA from cultures treated with 10^{-7}M CGRP for 24 hrs
Track 3 10μg of total RNA from cultures treated with Forskolin (10μM) for 24 hrs
were hybridised with an α subunit probe as described in Figure (5.6). RNase digest conditions, denaturing gel electrophoresis and autoradiography were as described in Figure (5.6).

FIGURE (5.9)



55bp also being present (as discussed above). Autoradiograms were then subjected to densitometric scanning and values were again normalised to control cultures, which were given an arbitrary value of 1.0. The results in Table (5.5) are the mean of 3 independent experiments. CGRP and Forskolin both caused significant increases in the level of α subunit mRNA at both culture times (see table 5.5). The magnitude of these increases in α subunit levels were larger than those detected by Northern blotting. This is probably due to the fact that Northern blots were performed on mRNA derived from a large number of cultures, while the RNase protection experiments were performed on RNA derived from 3 sister cultures. Variation between cultures in Northern blotting experiments mask the larger increase seen on RNase protection. The effect of culture time and CGRP stimulation on β/γ actin levels was also investigated by RNA dot blotting. CGRP caused a 2 - 3 fold decrease in β/γ actin at both time points without effecting the level of α actin mRNA (results not shown).

This series of experiments confirms that CGRP stimulates the level of α subunit transcript as does Forskolin as demonstrated above. It does not, however, explain the differences between these results and those of Fontaine et al (1987). Furthermore, the decrease in β/γ actin mRNA on CGRP stimulation was also seen with increasing culture time. Differences may reside within other aspects of methodology. Perhaps importantly the RNA

TABLE (5.5)

Effects of CGRP and Forskolin on the α Subunit Transcript Encoding the AChR after 3 and 6 days in Culture

DAYS IN CULTURE	CGRP (10^{-7}M)	CONTROL	(+) FORSKOLIN (10μM)
3 Days	2.50 ± 0.21 ^	1.0 ± 0.04	1.75 ± 0.06 ^
6 Days	1.80 ± 0.15 ^	1.0 ± 0.05	1.59 ± 0.07 ^

α subunit levels were measured by RNase protection on poly(U) calibrated total RNA derived from 2 cultures. Hybridisation signals were quantified by densitometry. The results represent the mean of three separate experiments.

^ = values significantly different at 0.99 confidence interval

preparations above were standardised by poly(U) calibration. Fontaine et al (1987) do not report the method they utilised for RNA calibration.

DISCUSSION

CGRP, a neuropeptide that has been shown to coexist with ACh in chick motor neurons, has further been shown to increase the number of AChR in chick muscle primary cultures (New and Mudge 1986a,b, Fontaine et al 1986). The results presented in this Chapter demonstrate that CGRP significantly increases the level of α subunit mRNA as determined by Northern blotting and RNase protection, the magnitude of this increase was ~150%. CGRP, however, did not effect the levels of the β, γ or δ subunit mRNAs as determined by Northern blotting. CGRP also decreased the levels of β/γ actin mRNA, the magnitude of this change being similar to that seen for the α subunit mRNA. CGRP has been further shown to increase the level of cAMP, consistent with the results of Laufer and Changeux (1987), this suggests that CGRP may be important in controlling AChR biosynthesis. Consistent with this mechanism Forskolin, a potent activator of adenylate cyclase, also increases α subunit mRNA levels. Fontaine et al (1987) have demonstrated that CGRP and CT enhance α subunit mRNA levels by a mechanism that also enhances cAMP levels. Such an increase may result from specific stimulation of α subunit gene transcription or from a stabilization of the α subunit mRNA. The observation that the smaller protected fragment (114 + 55bp) also increase suggests that α subunit mRNA intermediates increase on exposure to CGRP. This indicates that increased α subunit gene transcription is

responsible for the change in α subunit mRNA levels. Nuclear run-off experiments should provide conclusive evidence of the mechanism of action of CGRP in modulating α subunit mRNA levels. The effects on the other subunit mRNAs encoding the AChR of CGRP and Forskolin are not clear from this series of experiments. The effects of CGRP stimulation may not be specific to AChR genes, since β/γ actin mRNA levels were sensitive to CGRP. The mechanism by which CGRP regulates α subunit mRNA levels is unknown. The possibility that cAMP regulates gene transcription by directly binding to, or stimulating, the phosphorylation of a protein which interacts with specific DNA sequences, as demonstrated in prokaryotes (De Combruggle et al 1984), is attractive. Highly conserved cAMP regulated DNA elements have been demonstrated to flank several eukaryotic genes such as those for phosphoenolpyruvate carboxykinase, corticotropin releasing factor, somatostatin and proenkephalin (Comb et al 1986, De Bustro: et al 1986). The 5′ end and promoter region of the chick AChR α subunit gene has been isolated and functionally characterised (Klarsfeld et al 1987). It will thus be possible to establish whether similar cAMP regulated sequences control the expression of AChR genes.

The magnitude of the CGRP stimulation of α subunit mRNA levels is small but this must be considered with possible local effects. All of the data above was derived from embryonic muscle cultures, where α subunit mRNA levels

have been shown to be high as compared to innervated muscle (see Chapters 3 and 4). Furthermore, extra-junctional receptors are abundant in such systems. In innervated muscles, AChR subunit mRNAs are localised to junctional regions (Merlie and Sanes 1985, Burden and Simon 1987, Fontaine et al 1988). It will be of interest to determine the localisation of CGRP receptors within innervated muscles. Localisation of CGRP receptors to junction areas would be predicted to promote local junctional AChR synthesis. Tentatively, the results support the idea that during development CGRP, co-released with ACh from motor neurons, would increase the expression of the genes encoding the AChR via a cAMP dependent mechanism in junctional regions. The synthesis of extra-junctional receptors would, meanwhile, be repressed by neuronally derived muscle activity (see Chapter 4). Whereas the in vivo role of muscle electrical activity in AChR gene regulation is well established, the relevance of CGRP to the development of the neuromuscular junction remains to be demonstrated.

CHAPTER 6

GENERAL DISCUSSION

The major theme that has developed within this thesis has been the control of AChR expression in different muscle states. The control of AChR biosynthesis is known to be closely linked to embryonic muscle development and also to muscle innervation (Fambrough 1979, Schuetze and Role 1987). The natural development of the chick embryo provides a convenient and accessible system to study developmental receptor regulation.

The effects of innervation on the level of AChR expressed within muscle and on its encoding mRNAs has been demonstrated. Thus, during early embryonic pectoral muscle development, the level of AChR increases with parallel increases in the steady state level of all four receptor mRNAs. As innervation proceeds, a drastic decrease in the level of all four receptor mRNAs occurs (8 - 15 fold) with a concurrent decrease in AChR.

Denervation of adult muscle leads to a large increase in the level of extra-junctional receptors (Fambrough 1979, Schuetze and Role 1987). As demonstrated in this thesis, in the chick pectoral muscle the steady state level of the α, β, γ and δ mRNAs all dramatically increase approaching embryonic values. Taken together, these results suggest that AChR expression is controlled by transcriptional mechanisms. This interpretation is dependent on the presumption that changes in half lives of the α, β, γ and δ mRNAs are negligible in these different muscle states.

Chronic electrical stimulation of denervated muscle represses the normal increase in receptor biosynthesis that occurs on muscle denervation (Hall and Reiness 1977). From the results presented in this thesis, the innervating neuron is capable of supressing receptor biosynthesis by modifying the rate of transcription of the α, β, γ and δ genes encoding the AChR. Recently Goldman et al (1988) have demonstrated that chronic electrical stimulation of denervated muscle supresses the normal increase of the steady state level of the 4 receptor mRNAs that normally occurs upon muscle denervation. This result is based on the presumption that the half lives of the subunit mRNAs are constant during muscle development. In the case of the α subunit, Sheih et al (1986) have demonstrated that innervation specifically decreases α subunit gene transcription. Similar conclusions have been drawn by other groups who have examined the control of AChR expression in these states (Merlie et al 1984, Klarsfeld and Changeux 1985, Buonanno and Merlie 1986, Sheih et al 1986, Evans et al 1987). As suggested by Fambrough (1979), the denervated state represents a reversal of the normal repression of AChR biosynthesis that occurs upon innervation and a return to the levels of AChR synthesis in embryonic muscle. Supporting Fambrough's suggestion, as demonstrated in this thesis, the levels of 4 mRNAs encoding the AChR in embryonic muscle and denervated muscle are similar.

The observation that in 8 week post hatch pectoral muscle the γ subunit mRNA is undetectable, suggests that in the chicken an equivalent to the bovine ε subunit exists (Mishina et al 1985). Mishina et al (1986) have demonstrated that the ε subunit replaces the γ subunit during bovine muscle development. This developmental switch is reversed by muscle denervation, the γ subunit mRNA again being produced in denervated muscle (Witzemann et al 1987). The switch of a γ subunit for an ε subunit is sufficient to account for differences that exist between fetal and adult AChRs, in respect to channel open time and channel conductance (Sakmann and Brenner 1978). Schuetze et al (1980) report that the chick channel open time and channel conductance remain constant up to 10 weeks post hatch. Presumably should a chick ε subunit exist, then the properties of the α_2, β, δ, ε form of the receptor are indistinguishable in terms of their physiology to the α_2, β, γ, δ form. Cloning of full length cDNA clones encoding all 4 subunits of the chick AChR, and cloning of a putative chick ε subunit cDNA should clarify these ambiguities in the structure of chick muscle AChRs. However, this developmental switch is insufficient to account for the stabilisation and clustering of the AChR in adult muscle. Post translational modification of the AChR such as glycosylation and phosphorylation have been implicated in these changes (Hall et al 1983, Schuetze et al 1986).

It is evident from results presented in this thesis and

those of others (Merlie et al 1984, Klarsfeld and Changeux 1985, Buc nanno and Merlie 1986, Evans et al 1987, Goldman et al 1988) that the innervating neuron is capable of repressing the rate of AChR biosynthesis by a mechanism that decreases the steady state level of the mRNAs encoding the receptor, by presumably reducing the rate of AChR gene transcription (Sheih et al 1986). The signalling mechanisms involved in the process remain elusive. It is perhaps noteworthy that muscle activity has been shown to stimulate phosphatidylinositide metabolism, enhancing protein kinase C levels (Vergerra et al 1985). Interestingly, Fontaine et al (1987) have demonstrated that phorbol esters, potent activators of protein kinase C, decrease AChR α subunit mRNA levels in culture. This suggests that the electrical repression of AChR gene expression could be mediated by activators of phosphatidylinositide metabolism.

AChRs within innervated muscle are localised to dense clusters in endplate regions. Continued synthesis in the innervated state is still required to maintain receptor density in junctional regions, as junctional receptors still turnover (Burden 1977b). The continued synthesis of the AChR in junctional regions, while the synthesis of extra-junctional receptors is repressed by electrical activity, requires the intervention of a neuronal anterograde signal to promote local synthesis of the AChR (Changeux et al 1988). The local nature of AChR synthesis in innervated muscle has been implicated by the

observations of Merlie and Sanes (1985) and Fontaine et al (1988), who have demonstrated that receptor mRNAs are localised to junctional regions in innervated muscle. In this thesis, CGRP, a neuropeptide that co-exists with ACh in chick motor neurons (New and Mudge 1986a), was shown to elevate levels of AChR in chick muscle cultures in accordance with the observations of New and Mudge (1986b) and Fontaine et al (1986). Furthermore, CGRP also increased specifically the level of α subunit mRNA by a cAMP dependent mechanism in common with the observation of Fontaine et al (1987), without effecting the level of either the β, γ or δ subunit mRNAs. The observation that α subunit mRNA precursors increase on exposure to CGRP suggests that gene transcription is responsible for this change in α subunit mRNA levels. Thus CGRP may act to increase AChR synthesis in junctional regions by increasing α subunit mRNA levels, suggesting that α subunit mRNA levels are important in controlling receptor appearance. However, changes of both AChR and AChR α subunit mRNA were both small (as compared to those obtained on muscle denervation), and confirmation of the role of CGRP as a positive signal modulating AChR biosynthesis in vivo awaits the demonstration of the relevance of CGRP to neuromuscular development.

The developmental expression of the AChR and its encoding mRNAs has been demonstrated in this thesis. It has also been possible, by the use of novel RNA "sense standards" produced by in vitro transcription of cloned cDNAs (Zing

et al 1987), to compare directly the steady state levels of the α, β, γ and δ subunit mRNAs. The results have important implications on the control of receptor biosynthesis. It was possible to demonstrate that the level of each subunit mRNA differed. Moreover, it was evident that the δ subunit mRNA was present at significantly lower levels than either the α, β or γ mRNAs. This may represent a difference in the rate of δ subunit gene transcription or a difference in the half life of the δ subunit mRNA. The implication of this observation is that the level of the δ subunit mRNA could be rate limiting in receptor biosynthesis and therefore transcription of the δ subunit gene may be a possible point of regulating AChR biosynthesis. The requirement for two α subunits in the protein suggests also that the level of α subunit mRNA may therefore be important in controlling receptor expression. The modulation of α subunit mRNA levels by CGRP, as demonstrated in this thesis, and the resulting small but significant increase in AChR, may reflect the regulatory role of α subunit mRNA levels in controlling receptor appearance. Further evidence of the importance of α subunit mRNA levels in controlling receptor appearance is demonstrated by the results of Harris et al (1988). This group have demonstrated that a 42Kd protein, termed ARIA, enhances AChR levels by modulation of α subunit mRNA levels.

If receptor expression were simply controlled by transcriptional mechanisms, one might therefore predict

that the ratio of receptor mRNAs would be similar to that of the protein subunit stoichiometry: α_2, β, γ and δ. The mRNA ratio suggests some measure of translational control of receptor expression. Sheih et al (1987) have recently demonstrated discrepancies between mRNA levels and receptor protein levels in denervated muscle. Merlie et al in a series of papers (Merlie et al 1981, Merlie and Lindstrom 1983, Merlie et al 1983) have proposed that the fine tuning of AChR expression is at post translational levels. While the translation of the individual receptor mRNAs takes place rapidly, the formation of the AChR in a functional form in the muscle membrane takes up to 3 hrs (Devreotes et al 1977, Fambrough 1979). This later period almost certainly represents a rate limiting step in the overall biosynthetic pathway and a potential point at which regulation could occur. Clearly much more extensive evidence is required to elucidate post translational mechanisms. One promising approach is the isolation of mutants deficient in these mechanisms. Black et al (1987) have reported the isolation of two mutants of the C_2 cell line (Yaffe and Saxel 1977). These mutants seem to be deficient in the level of α subunit protein present, but this is not due to modifications of the α subunit mRNA primary structure as determined by S_1 mapping or changes in the steady state level of the α subunit mRNA.

Further points raised by this thesis are centered on the

initiation and control of AChR gene expression. AChRs are first detected by α Butx binding in vivo, when myoblasts fuse to form myotubes (reviewed by Dennis 1981). The expression of many contractile protein such as α skeletal actin (Grinkchnik et al 1986) and myosin (Medford et al 1983) are also enhanced on myoblast fusion. It is attractive to speculate that these genes are regulated by similar mechanisms. The appearance of AChR is due in part to increased gene transcription (Buonanno and Merlie 1986). Since all four subunits are required for expression of active AChR (Mishina et al 1984), a mechanism must therefore exist to coordinate AChR gene expression. The chromosomal location of the AChR genes has recently been elucidated (Heidmann et al 1986), the four genes are distributed in three different chromosomes (α chromosome 17, β chromosome 11, γ and δ chromosome 1). Coordination of expression must be achieved by the intervention of transacting factors. The examination of the promoter regions of these genes may lead to a better understanding of the control mechanisms underlying receptor gene transcription. The structure of the promoter and upstream regions of the chicken α subunit gene and the rat γ subunit gene have recently been reported (Klarsfeld et al 1987, Gardner et al 1987). Both of these promoters show common features of typical eukaryotic promoters such as TATA boxes and CAAT boxes (Breathnach et al 1981). The only significant homology between the two sequences is an inverted repeat of the Sp1 binding site (Dyan and Tjian 1985). Both of these

regions are sufficient to confer both tissue specificity and developmental specificity, as judged by transient expression using reporter gene vectors (Mulligan and Berg 1980). Furthermore, no significant homology has been detected between the upstream regions of the chick α, γ and δ subunit genes (M. Ballivet - personal communication). The existence of a developmental isoform of the γ subunit, the ε subunit has added implications for the control of AChR gene transcription. The γ and δ genes are closely linked within the chick (Nef et al 1984) and this structure is conserved in other vertebrates (Gardner et al 1987). However, the control of transcription of the γ subunit gene in adult muscle must differ from that of the δ, since the γ subunit gene is not expressed in adult muscle. The lack of homology between AChR gene upstream regions as yet has not provided universal targets for the putative second messenger systems implicated in either electrical repression or trophic factor activation of AChR gene expression (J.P. Changeux - personal communication). Differences in patterns of temporal expression of the α, β, γ and δ subunit mRNAs, and differing steady state mRNA levels of the respective mRNAs as demonstrated in this thesis, suggest also that the control of AChR gene expression is mediated by complex mechanisms. Coordination of the four subunit genes may not be mediated by a common mechanism.

In conclusion, molecular biology has yielded the complete

sequence of the AChR subunits along with plausible structural models, the genomic organisation of the receptor subunit genes and functional expression in a heterologous cell type. The immediate future should yield considerably more information as receptor function is explored by mutagenesis, and structure is explored by using antibodies to synthetic peptides predicted from the sequence. The recent analysis of denervation hypersensitivity at the level of mRNA, points to a period in which the regulation of AChR expression from gene transcription to subunit assembly will be elucidated in detail. This problem will be approached in heterologous animal cell lines stably transformed with cloned AChR cDNAs. Cloned genes, however, are clearly no panacea for understanding the complex developmental alterations of the receptor, in which post-translational modifications appear to play an important role.

REFERENCES

Abood, L.G., Latham, W. and Grassi, S. (1983) Proc. Natl. Acad. Sci. USA, 80 3536 - 3539.

Amara, S.G., Jonas, V., Rosenfeld, M.G. and Evans, R.M. (1982) Nature, 298 240 - 244.

Anderson, D.J. and Blobel, G. (1981) Proc. Natl. Acad. Sci. USA, 78 5598 - 5603.

Anderson, D.J. and Blobel, G (1983) Cold Spring Harbor Symposium on Quantitative Biology, 48 125 - 134.

Auffray, C. and Rougeon, F. (1980) Eur. J. Biochem. 107 303 - 314.

Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA, 69 1408 - 1412.

Ballivet, M., Patrick, J., Lee, J. and Heinemann, S. (1982) Proc. Natl. Acad. Sci. USA, 79 4466 - 4470.

Ballivet, M., Nef, P., Stalder, R. and Fulpius, B (1983) Cold Spring Harbor Symposium on Quantitative Biology, 48 83 - 86.

Barkas, T., Maron, A., Roth, B., Alliod, C., Tzartos, S.J. and Ballivet, M. (1987) Science, 235 77 - 80.

Barnard, E.A. and Dolly, O. (1982) Trends Neurosci. 5 325 - 327.

Barnard, E.A., Miledi, R. and Sumikawa, K. (1982) Proc. R. Soc. Lond. B., 215 241 - 246.

Barnard, E.A., Beeson, D.M.W., Bilbe, G., Brown, D., Constanti, A., Houamed, K. and Smart, T.G. (1984) Journal of Receptor Research, 4 681 - 704.

Barnard, E.A. and Bilbe, G. (1987) In Neurochemistry IRL Press, Oxford, p 140 - 175.

Beeson, D.M.W. (1987) PhD Thesis, University of London.

Betz, H., Bourgeois, J-P and Changeux, J-P (1977) Febs Letts, 77 219 - 224.

Betz, H. and Changeux, J-P (1979) Nature, 278 749 - 752.

Betz, H. and Changeux, J-P (1980) J. Physiol. (Lond.), 302 197 - 218.

Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res., 7 1513 - 1519.

Black, I.B., Alder, J.E., Drefus, C., Jonakait, C.M., Katz, D.M., LaGama, E.F. and Markey, K.M. (1984) Science, 225 1266 - 1270.

Black, R., Goldman, G., Hoschwender, S., Lindstrom, J. and Hall, Z. J. (1987) Cell Biol., 105 1329 - 1335.

Blosser, J.C. and Appel, S.M. (1980) J. Biol. Chem., 255 1235 - 1238.

Boulter, J., Evans, K., Goldman, D., Martin, G., Treco, D., Heinemann, S. and Patrick, J. (1986) Nature, 319 368 - 374.

Boulter, J., Luyten, W., Evans, K., Mason, P., Ballivet, M., Goldman, D., Stengelin, S., Martin, G. and Heinemann, S. (1985) J. Neurosci., 5 2545 - 2552.

Breathnach, R. and Chambon, P. (1981) Biochem., 50 349 - 583.

Brenner, H.R. and Sakmann, B. (1983) J. Physiol. (Lond.), 337 159 - 171.

Brisson, A. and Unwin, P. (1984) Nature, 315 474 - 477.

Brock, L. and Shapiro, K.L. (1984) Cell, 34 207 - 214.

Brockes, G.P. and Hall, Z.W. (1975) Biochemistry, 14 2100 - 2106.

Buonanno, A. and Merlie, J.P. (1986) J. Biol. Chem., 259 11452 - 11455.

Buonanno, A., Mudd, J., Shah, V. and Merlie, J.P. (1987) J. Biol. Chem., 261 16451 - 16458.

Burden, S. (1977a) Dev. Biol., 57 317 - 329.

Burden, S. (1977b) Dev. Biol., 61 79 - 85.

Burden, S. and Simon, A. Abstracts of EMBO Neurobiology Meeting (1987), p.86, Heidelberg.

Burden, S. (1987) In the Vertebrate Neuromuscular Junction, Alan Liss Ltd, New York p 115 - 150.

Chang, H.W. and Bock, E. (1977) Biochemistry, 16 4513 - 4520.

Changeux, J-P and Sealock, R.E. (1975) Biochem., Biophys. Acta, 415 215 - 249.

Changeux, J-P., Bon, F., Cartland, J., Devillers-Thiery, A., Giraudat, J., Heidmann, T., Holton, B., Nghiem, H-O., Popot, J-L, Van Rapenbusch, R. and Tzartos, S. (1983) Cold Spring Harbor Symposium on Quantitative Biology, 48 35 - 52.

Changeux, J-P, Devillers-Thiery, A. and Chemonilli, P. (1984) Science, 255 1335 - 1345.

Changeux, J-P, Giraudat, J. and Dennis, M. (1987) TIPS, 8 439 - 444.

Charnay, P., Trisman, R., Chao, M., Axel, R. and Maniatis, T. (1984) Cell, 38 251 - 263.

Chirgwin, J.M., Przbyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry, 18 5294 - 5299.

Claudio, T., Ballivet, M., Patrick, J. and Heinemann, S. (1983) Proc. Natl. Acad. Sci. USA, 80 1111 - 1115.

Claudio, T. (1987) Proc. Natl. Acad. Sci., USA, 84 5697 - 5971.

Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E. and Goodman, H. (1986) Nature, 323 353 - 356.

Cox, K.H., DeLeon, D.V. and Angerer, L.M. (1984) Dev. Biol., 101 485 - 502.

Craido, M., Hochschwender, S., Sarin, V., Fox, J.L. and Lindstrom, J. (1985) Proc. Natl. Acad. Sci. USA, 82 2004 - 2008.

Dale, H.H., Feldberg, W. and Vogt, M. (1936) J. Physiol., 86 353 - 380.

Damle and Karlin (1978) Biochemistry, 17 2039 - 2045.

Darnell, J.E. (1982) Nature, 297 365 - 371.

DeVreotes, D.N., Gardner, J.M. and Fambrough, D. (1977) Cell, 10 365 - 373.

DeCombruggle, B., Bushby, S. and Buch, S. (1984) In Biological Regulation, eds R. Goldberger, p 126 - 167 New York Plenum.

Denhardt, D.T. (1966) Biochem. Biophys. Res. Commun., 23 641 - 646.

Dennis, M.J. (1981) Ann. Rev. Neurosci., 4 43 - 68.

DeBustro, A., Baylin, S.B., Levine, M. and Nelkin, D. (1986) J. Biol. Chem., 261 8036 - 8041.

Devillers-Thiery, A., Giraudat, J., Bentaboulet, M. and Changeux, J-P (1983) Proc. Natl. Acad. Sci. USA, 80 2067 - 2071.

Dolly, O. and Barnard, E.A. (1987) Biochemistry, 16 5052 - 5060.

Dyan, W.S. and Tjian, R. (1985) Nature, 316 774 - 778.

Evan, K., Goldman, D., Heinemann, S. and Patrick, J. (1987) J. Biol. Chem., 262 4911 - 4916.

Fambrough, D.M. (1979) Physiol. Rev., 59 165 - 227.

Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem., 132 6 - 13.

Fertuck, H.C. and Salpeter, M.M. (1976) J. Cell Biol., 69 44 - 58.

Finer-Moore, J. and Stroud, R.M. (1984) Proc. Natl. Acad. Sci. USA, 81 155 - 159.

Fischer, J.A. and Born, W. (1985) Peptides, 6 265 - 271.

Fontaine, B., Klarsfeld, A., Hokfelt, T. and Changeux, J-P (1986) Neurosci. Letts., 71 59 - 65.

Fontaine, B., Klarsfeld, A. and Changeux, J-P Mol. Cell Biol., (1987), 105 1337 - 1342.

Gardner, J., Heinemann, S. and Patrick, J. (1987) Mol. Brain Res., 3 141 - 153.

Giraudat, J., Dennis, M., Heidmann, T., Chang, J-Y and Changeux, J-P (1986) Proc. Natl. Acad. Sci. USA, 83 2719 - 2723.

Goldman, D., Boulter, J., Heinemann, S. and Patrick, J. (1985) J. Neurosci., 5 2553 - 2558.

Goodman, L.S. and Gilman, A. (1975) The Pharmacological Basis of Therapeutics, MacMillan, New York.

Gration, K.A.F., Lamber, J.J., Romsey, R. and Usherwood, P.N.R. (1981) Nature, 291 423 - 425.

Green, M.R., Maniatis, T. and Melton, D.A. (1983) Cell, 32 681 - 694.

Grenningloh, G., Dienitz, L., Schmitt, B., Methfessel, C., Zensem, M., Gundlefinger, E.D. and Betz, H. (1987) Nature, 328 215 - 220.

Grinchnik, J.M., Bergsma, D.J. and Schwartz (1986) Nucleic Acids Res., 14 1683 - 1702.

Gurdon, J.B. (1974) The Control of Gene Expression in Animal Development, Clarendon Press, Oxford.

Guy, H.R. (1984) Biophys. J., 45 249 - 261.

Hall, Z.W. and Reiness, C.G. (1977) Nature, 268 655 - 657.

Hall, Z.W., Roisin, M.P., Gu, Y. and Gorin, P.D. (1983) Cold Spring Harbor Symposium on Quantitative Biology, 48 101 - 108.

Harris, D.A., Falls, D., Dill-Devor, B. and Fischbach, J. (1988) Proc. Natl. Acad. Sci. USA, 85 1983 - 1987.

Heidmann, T., Buonanno, A., Geoffroy, B., Robert, B., Guenet, J.L., Merlie, J.P. and Changeux, J-P (1986) Science, 234 21 - 25.

Heinemann, S., Boulter, J., Connolly, J., Goldman, D., Evans, K., Ballivet, M. and Patrick, J. (1986) In the AChR edited by A. Maelicke, p 360 - 387.

Hershey, N.D., Noonan, D.J., Mixter, K.S., Claudio, T. and Davidson, N. (1983) Cold Spring Harbor Symposium on Quantitative Biology, 48 79 - 82.

Hokfelt, T., Johansson, O. and Goldstein, M. (1984) Science, 225 7326 - 7334,

Holly, R.G., Ashmore, C. and Mole, P.A. (1980) J. Physiol., 238 C62 - C71.

Hucho, F., Oberthur, W. and Lottspeich, L. (1986) FEBS Lett., 205 137 - 142.

Huynh, T., Young, R.A. and Davis, R.W. (1985) In DNA Cloning, A Practical Approach ed. D. Glover IRL, Vol 1 p 49 - 78.

Huganir, R.L. and Greengard, P. (1983) Proc. Natl. Acad. Sci. USA, 80 1130 - 1134.

Huganir, R.L. and Greengard, P. (1984) Proc. Natl. Acad. Sci. USA, 80 1130 -1134.

Huganir, R.L., Delcor, A.H., Greengard, P. and Hess, G.P. (1986) Nature, 321 774 - 776.

Huganir, R.L. and Greengard, P. (1987) TIPS, 10 472 - 477.

Ivarie, R.D. and Jones, P.P. (1979) Biochem. Biophys. Acta., 126 525 - 535.

Imoto, K., Methfessel, C., Sakmann, B., Mishina, M., Mori, Y., Konno, T., Fukuda, K., Kurasaki, M., Bujo, H., Fujita, Y. and Numa, S. (1986) Nature, 324 670 - 674.

Jessel, J.M., Siegel, R.E. and Fischbach, G.D. (1979) Proc. Natl. Acad. Sci. USA, 76 5397 - 5401.

Kao, P., Dwork, A., Kaldang, R., Silver, L., Wideman, J. Stain, S. and Karlin, A. (1984) J. Biol. Chem., 259 11662 - 11665.

Katz, B. (1960) J. Physiol. (Lond.), 160 379 - 388.

Karlin, A. (1983) Neuroscience Comment, 1 111- 123.

Kistler, J., Stroud, R.M., Klymkowsky, R.A., Lalancette, R.T. and Fairclough, R.H. (1982) Biophys. J., 37 371 - 376.

Klarsfeld, A. and Changeux, J-P (1985) Proc. Natl. Acad. Sci. USA, 82 4558 - 4562.

Klarsfeld, A., Duabas, P., Bourachet, B. and Changeux, J-P Mol. Cell Biol. (1987), 7 951 - 955.

Kubo, T., Noda, M., Takai, T., Tanabe, T., Kayano, T., Shimizu, S., Tanaka, K., Takahashi, H., Hirose, T., Inayama, S., Kikuno, R., Miyata, T. and Numa, S. (1985) Eur. J. Biochem., 149 5 - 13.

Kurosaki, T., Fukuda, K., Komo, T., Mori, Y., Tanaka, K., Mishina, M. and Numa, S. (1987) FEBS, 217 152 - 260.

Laemmli, U.K. (1970) Nature, 227 680 - 685.

Lane, C.D., Shannon, S. and Craig, R. (1979) Eur. J. Biochem., 101 485 - 495.

La Polla, R.J., Mixter-Mayne, K. and Davidson, N. (1984) Proc. Natl. Acad. Sci. USA, 81 7970 - 7974.

Laufer, R. and Changeux, J-P (1987) EMBO Journal, 6 901 - 966.

Lee, C.Y. (1972) Ann. Rev. Pharmacol., 12 265 - 280.

Lindstrom, J., Merlie, J. and Yogeeswaran, G. (1979) Biochemistry, 18 4465 - 4470.

Littman, D.R., Thomas, Y., Madden, P.S., Chess, L. and Axel, R. (1985) Cell, 40 237 - 241.

Lowry, O.M., Rosebrough, N.J., Farr, A.L. and Randal, R.J. (1951), J. Biol. Chem., 193 265 - 275.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

Marzluff, W. and Huang, R.C. (1984) In Transcription/Translation eds Hames, D.B. and Higgins, S.F. IRL Press Oxford 89 - 129.

Mason, R.T., River, J. and Vale, W. (1984) Nature, 308 653 - 655.

Medford, R.M., Nguyen, H.T. and Nadal, B. (1983) J. Biol. Chem., 412 11063 - 11075.

Melton, D.A. (1984) Nucleic Acids Res., 12 7035 - 7056.

Merlie, J.P., Hofler, J.G. and Sebbane, R. (1981) J. Biol. Chem., 256 6995 - 6999.

Merlie, J.P., Sebbane, R., Tzartos, S. and Lindstrom, J. (1982) J. Biol. Chem., 257 2694 - 2699.

Merlie, J.P. and Lindstrom, J. (1983) Cell, 34 747 - 757.

Merlie, J.P., Sebbane, R., Gardner, S., Olson, E. and Lindstrom, J. (1983) Cold Spring Harbor Symposium on Quantitative Biology, 48 135 - 146.

Merlie, J.P., Isenberg, K.E., Russell, S.D. and Sanes, J.R. (1984) J. Cell Biol., 99 332 - 335.

Merlie, J.P. and Sanes, J.R. (1985) Nature, 317 66 - 68.

Miledi, R. (1960) J. Physiol. (Lond.), 151 24 - 30.

Miledi, R., Parker, I. and Sumikawa, K. (1982) EMBO Journal, 1 1307 - 1312.

Miles, K., Greengard, P., Delcour, R.M. and Huganir, R.L. (1987) Proc. Natl. Acad. Sci. USA, 84 6590 - 6595.

Mishina, M., Kurosaki, T., Tobimatsu, T., Morimoto, Y., Noda, M., Yamamoto, T., Terao, M., Lindstrom, J., Takashi, T., Kuno, M. and Numa, S. (1984) Nature, 307 604 - 608.

Mishina, M., Tobimatsu, T., Imoto, K., Tanaka, K., Fujita, Y., Fukuda, K., Kurasaki, M., Takahashi, H., Morimoto, Y., Hirose, T., Inayama, S., Takahashi, T., Kuno, M. and Numa, S. (1985) Nature, 313 364 - 369.

Mishina, M., Takai, T., Imoto, K., Noda, M., Takahashi, T., Numa, S., Methfessel, C. and Sakmann, B. (1986) Nature, 321 406 -411.

Mulligan, R.C. and Berg, P. (1980) Science, 209 1422 - 1427.

Nef, P., Mauron, A., Stalder, R., Alliod, C. and Ballivet, M. (1984) Proc. Natl. Sci. USA, 81 7975 - 7979.

Nef, P., Oneyser, C., Alliod, C., Couturier, S. and Ballivet, M. (1988) EMBO Journal, 7 595 - 601.

Neumann, D., Gershoni, J.M., Eridkin, M. and Fuchs, S. (1985) Proc. Natl. Acad. Sci. USA, 82 3490 - 3493.

New, H.V. and Mudge, A.W. (1986a) Dev. Biol., 116 337 - 346.

New, H.V. and Mudge, A.W. (1986b) Nature, 324 412 - 414.

Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T. and Numa, S. (1982) Nature, 299 793 - 797.

Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Hirose, T., Asai, M., Takashima, H., Inayama, S., Miyata, T. and Numa, S. (1983a) Nature, 301 251 - 255.

Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T. and Numa S. (1983b) Nature, 302 528 - 532.

Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Tanabe, T., Shimizu, S., Kikyotani, S., Kayano, T., Hirose, T., Mayama, S. and Numa, S. (1983c) Nature, 305 818 - 823.

Noda, M., Shimizu, S., Tanabe, T., Takai, T., Kayano, T., Ikoda, T., Takahashi, H., Nakayama, H., Kamaoka, Y., Minamino, N., Kangawa, K., Matsui, H., Raftery, M., Hirose, T., Inayama, S., Hayashida, H., Miyata, T. and Numa, S. (1984) Nature, 312 121 - 125.

O'Brien, R.D., Eldefrawi, M.E. and Eldefrawi, A.T. (1972) Ann. Rev. Pharmacol., 12 19 - 34.

Patrick, J. and Stallcup, W.B. (1977) Proc. Natl. Acad. Sci. USA, 74 859 - 872.

Patrick, J., Ballivet, M., Claudio, T., Forest, H. and Heinemann, S. (1983) Cold Spring Harbor Symposium, 71 - 77.

Pelham, H.R.B. and Jackson, R.J. (1976) Eur. J. Biochem., 67 247 - 256.

Popot, J.L. and Changeux, J-P (1984) Physiol. Revs., 64 1162 - 1184.

Raftery, M.A., Hunkapiller, M.W., Strader, C.D. and Hood, L.E. (1980) Science, 208 1454 - 1457.

Ratnam, M., Le Ngyen, D., Rivier, J., Sargent, P. and Lindstrom, J. (1986) Biochemistry, 25 2621 - 2632.

Rosenfeld, M.G., Mermod, J.J., Amara, S.G., Swanson, L.W. and Evans, R.M. Nature (1983), 304 129 - 135.

Sakmann, B. (1978) FASEB, 37 2654 - 2659.

Salpeter, M. and Loring, R.H. (1985) Prog. Neurobiol., 25 297 - 325.

Salpeter, M. (1987) In the Vertebrate Neuromuscular Junction, Alan Liss Ltd, p 55 - 115.

Sanger, F., Nicklen, S. and Coulson, A. (1977) Proc. Natl. Acad. Sci. USA, 74 5463 - 5467.

Schuetze, S.M. (1980) J. Physiol. (Lond.), 303 111 - 124.

Schofield, P.R., Darlison, M.G., Fujita, N., Burt, R., Stephenson, F.A., Rodriguez, H., Rhee, L.M., Ramachandran, J., Reale, V., Glencorse, T., Seeburg, P.H. and Barnard, E.A. (1987) (1987), 328 221 - 227.

Shibahara, S., Kubo, T., Perski, H.J., Takahashi, H., Noda, M. and Numa, S. (1985) Eur. J. Biochem., 146 15 - 22.

Smith, M. (1980) Methods in Enzymology, Eds. Grossman L. and Moldare, K. (New York, Academic Press), 65 560 - 580.

Steinberg, J.M. (1981) J. Physiol. (Lond.), 313 513 - 528.

Syapin, P.J., Salvaterra, P.M. and Engelhardt, J.K. (1982) Brain Res., 231 365 - 377.

Sumikawa, K., Houghton, M., Emtage, J.S., Richards, B.M. and Barnard, E.A. (1981), 292 862 - 864.

Sumikawa, K., Mehraban, F., Dolly, O. and Barnard, E.A. (1982a) Eur. J. Biochem., 126 465 - 472.

Sumikawa, K., Barnard, E.A. and Dolly, O. (1982b) Eur. J. Biochem., 126 473 - 479.

Sumikawa, K., Houghton, M., Smith, J.C., Bell, L., Richards, B.M. and Barnard, E.A. (1982c) Nucleic Acids Res., 10 5809 - 5822.

White, M.M., Mayne, K.M., Lester, H.A. and Davidson, N. (1985) Proc. Natl. Acad. Sci. USA, 82 4852 - 4856.

Witzemann, V., Borg, B., Nishikawa, Y., Sakmann, B. and Numa, S. (1987) FEBS Lett., 219 253 - 258.

Yaffe, D. and Saxel, O. (1977) Nature, 270 725 - 727.

Zing, H.H., LeFebure, D. and Almazan, G. (1987) J. Biol. Chem., 261 12956 - 12959.