Regulation of m4 muscarinic receptor gene expression.

by

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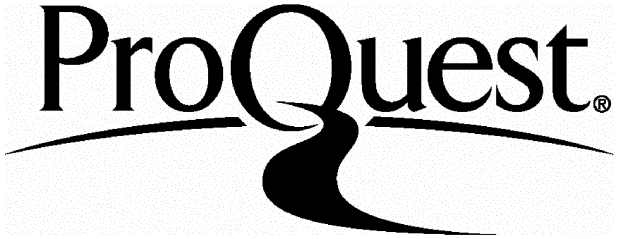

Abstract.

In order to identify the mechanisms that regulate the expression of the muscarinic receptors, the regulation of one of these receptors, the m4, was investigated. Four cell lines were identified that express the m4 gene and one of these, the NG108-15 cell line, was chosen as a model system for m4 expression. Pharmacological characterization of the m4 receptor expressed by the NG108-15 and the N18TG2 cell lines, was consistent with that previously reported for the M_4 receptor. DNA sequence analysis on the m4 gene expressed in the NG108-15 indicated that it is of mouse origin.

The levels of receptor expressed by the NG108-15 cell line varied greatly during routine culturing but removal of serum and inhibition of mitosis resulted in a reduction of receptor expression to a constant level. PGE_1 differentiation of the cells did not result in any further change in receptor levels. Activation and continued exposure of the muscarinic receptor with the muscarinic agonist, carbachol, resulted in a down-regulation of the receptor in a dose dependent manner.

In order to identify cis genomic regions responsible for the transcriptional regulation of the m4 gene, a genomic clone, R3-6, containing the coding region for the m4 gene and approximately 30 kb of 5' sequence was isolated.

Transfection of this genomic clone into the IMR32 and CHO cell lines demonstrated that this clone contains the m4 promoter and at least some tissue specific elements.

Because a full length cDNA for the m4 gene is not available to aid in locating the transcription initiation site for the m4 gene, several independent techniques were used to locate this sequence within the genomic clone. These included nuclear run on analysis, Northern blot analysis, PCR techniques, primer extension and nuclease protection assays. The data obtained suggests that the upstream sequence for the m4 gene is contained within a single exon of approximately 460 nucleotides and the transcription start site is located between 5.2 and 4.4 kb 5' from the initiating ATG.

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Contents.

List of figures and tables.

Glossary of Abbreviations.

AMV-RT	Avian myeloblatosis virus reverse transcriptase.
ATP	Adenosine 5'-triphosphate.
B_{max}	Total concentration of binding sites.
BSA	Bovine serum albumin.
cAMP	Adenosine 3':5'-cyclic monophosphate.
CAT	Chloramphenicol acetyltransferase.
Ci	Curie.
dATP	2'-Deoxyadenosine 5'-triphosphate.
dCTP	2'-Deoxycytidine 5'-triphosphate.
dGTP	2'-Deoxyguanosine 5'-triphosphate.
dTTP	Thymidine 5'-triphosphate.
DMEM	Dulbecco's Modified Eagle Medium.
DMSO	Dimethylsulphoxide.
DTT	Dithiothreitol.
EDTA	Ethylenediaminetetraacetic acid.
HEPES	N-[2-hydoxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IBMX	3-isobutyl-1-methylxanthine.
IC_{50}	Concentration of competing antagonist required to displace 50% of bound radioligand.
K_D	Dissociation binding constant.
MEM	Minimum essential media.
mRNA	Messenger RNA.
NGF	Nerve growth factor.
NMS	N-methylscopolamine.
OD	Optical density.
PCR	Polymerase chain reaction.
PDBu	4α-phorbol didecanoate.
PGE_1	Prostaglandin E_1.

PIPES	1,4-piperazinediethanesulfonic acid.
PK-A	Protein kinase A.
PK-C	Protein kinase C.
pK_I	Negative log of antagonist dissociation constant.
PMA	Phorbol 12-myristate 13-acetate.
RACE	Rapid amplification of cDNA ends.
RSV	Rous Sarcoma Virus.
SDS	Sodium dodecyl sulphate.
SV40	Simian virus 40.
TPA	12-O-tetradecanoyl phorbol 13-acetate.
tRNA	Transfer RNA.
UTP	Uridine 5'-triphosphate.

Chapter 1. Introduction.

Muscarinic receptors.

The mammalian brain is a highly complex organ containing more than 10^{11} neurons. A fundamental aspect of the brain is the capability to process an incoming signal by a mechanism of intercellular communication. The principal means of intercellular communication is by the release of chemical messengers such as neurotransmitters. These neurotransmitters interact with receptor molecules present on the cell surface and elicit responses within the cell which are dependent on the types or subtypes of receptor expressed.

One major excitatory neurotransmitter is acetylcholine, which exerts its effects via two main receptor types (Dale, 1914). These receptors have been classified into nicotinic and muscarinic receptors due to their selective activation by nicotine and muscarine respectively. The nicotinic acetylcholine receptor (nAChR) is a ligand gated ion channel, and interaction of acetylcholine with this receptor leads to an opening of its intrinsic cation channel and an influx of mainly Na^+ ions into the cell. The nicotinic receptor is responsible for the 'fast' effects of acetylcholine, such as muscle contraction, and is found in the peripheral and central nervous systems, most notably at the neuromuscular junction. Conversely, the muscarinic class of acetylcholine receptor (mAChR) is responsible for the 'slow' actions of acetylcholine, and is part of a receptor superfamily which is estimated to have several hundred members including rhodopsin, the α and ß-adrenergic receptors, the cannabinoid receptor, the vasopressin receptor and the olfactory receptors (which have been estimated to include more than 400 members (Buck and Axel, 1991; Parmentier et al. 1992)).

Each of these receptors contains seven putative transmembrane domains and a large intracellular loop between transmembrane segments 5 and 6. The effects of muscarinic receptors appear to be mediated via interactions of the receptor with guanine nucleotide regulatory proteins, (G-proteins). Some of the effects of muscarinic receptor activation are mediated directly by the G-protein, such as opening of potassium channels, inhibition of adenylyl cyclase and stimulation of phospholipase

C, whilst others are indirect, such as stimulation of arachidonic acid release and increase in cAMP.

Subtypes of muscarinic receptors.

Pharmacologically, three subtypes of muscarinic receptor have been identified (M_1, M_2 and M_3), largely by their affinities for pirenzepine (Hammer et al. 1980), AF-DX 116 (Hammer et al. 1986) and 4-DAMP (Barlow et al. 1976). It is likely that at least some of the pharmacological subtypes may be heterogeneous and include more than one molecular subtype but these cannot be differentiated due to lack of adequate selectivity of any single antagonist. The use of molecular biology has since led to the cloning of five genes encoding different muscarinic receptor subtypes (labelled m1-m5 (Kubo et al. 1986; Bonner et al. 1987)).

Receptor localization.

Each of the muscarinic receptors has been shown, by in situ hybridization studies, to have a unique pattern of mRNA tissue distribution within the CNS (Buckley et al. 1988; Vilaro et al. 1990). m1 has a predominantly telencephalic localization and is particularly abundant in the cerebral cortex, striatum and hippocampus. m2 transcripts are rare and only found in significant quantities in the medial septum and pons, with lower levels present in the thalamus. m3 is localized predominantly in the forebrain with expression also in some thalamic nuclei. The m3 transcripts in the cerebral cortex are found in the inner and outer layers, unlike m1 and m4 transcripts which are more evenly distributed throughout the cerebral cortex. The striatum contains the highest levels of m4 transcripts, whilst lower levels are present in the cortex and hippocampus. m5 transcripts are present at very low levels in the hippocampus, concentrated mainly in the CA1 pyramidal cells with some transcripts present in CA2, and there are also m5 transcripts present in some brain stem nuclei.

Recently subtype specific antibodies have been produced against muscarinic receptors and used in immunocytochemistry and immunoprecipitation studies to

localize the receptor proteins (Wall et al. 1991a; Li et al. 1991; Wall et al. 1991b; Dorje et al. 1991; Levey et al. 1991). These studies complement the *in situ* hybridization studies because mRNA is primarily localized in the cell bodies and proximal dendrites. Thus *in situ* hybridization indicates the distribution of neurons which synthesize the receptor subtypes. Immunocytochemistry, however, provides more information on the abundance and precise location of the receptor proteins themselves. The results of these studies show that the m1 subtype is present in the cortex and striatum, being localized in the cell bodies and neurites. The density of the m2 receptor protein appears relatively uniform throughout the brain regions in which it is expressed. Expression of the m2 receptor protein is found in the hindbrain, basal forebrain, striatal neurons, mesopontine tegmentum and cranial motor nuclei. This distribution is coincident with that of cholinergic neurons, and Vilaro et al. (1991) have found that cells in the caudate-putamen contain mRNA encoding both the m2 receptor and the enzyme choline acetyltransferase (a marker for cholinergic neurons), suggesting that the m2 receptor may act as an autoreceptor. The m2 receptor is also found in non-cholinergic neurons in cortical and subcortical structures, although whether it is located pre- or post-synaptically has not been determined. Immunoprecipiation studies by Wall et al. (1991b), using m3 specific antibodies, have identified m3 receptor protein in several areas of the brain. They found that the highest densities of the m3 receptor protein are in the cortex, hippocampus, striatum and olfactory tubercule, while lower receptor densities are found in the hindbrain regions. The m4 receptor protein is found localized in the neostriatum, olfactory tubercule and islands of Calleja, suggesting a role in extrapyramidal function. m5 receptor proteins could not be detected in the brain with this technique, perhaps because of low receptor protein levels.

Receptor effector coupling.

As well as containing a heterogeneity of subtypes, the muscarinic receptors can act through different effector mechanisms which include; inhibition of adenylyl cyclase (Jakobs et al. 1979), stimulation of phosphoinositide hydrolysis (Hokin and

Hokin, 1953), elevation of cGMP levels (Ferrendelli et al. 1970; George et al. 1970; Lee et al. 1972), increase of arachadonic acid release (Conklin et al. 1988), opening and closing of K^+ channels (North and Tokimasa, 1983; Fukuda et al. 1988; Yatani et al. 1988; Brown and Higashida, 1988) and closing of Ca^{2+} channels (Wanke et al. 1987). The coupling of the muscarinic receptor subtypes to various ion channels has been determined with the aid of individual muscarinic receptor genes transfected into various cell lines. This has shown that m1 and m3 receptors are capable of opening and closing K^+ channels (Fukuda et al. 1988), m1, m3 and m5 receptors can open Ca^{2+}-dependent K^+ channels (Jones et al. 1988; Jones et al. 1991) and m2 and m4 receptors can close Ca^{2+} channels (Higashida 1990).

The coupling of the muscarinic receptors to second messengers can be put into two main groups: m1, m3 and m5 preferentially couple to phosphoinositide hydrolysis via pertussis toxin insensitive G-proteins (Peralta et al. 1985), whilst m2 and m4 preferentially couple to inhibition of adenylyl cyclase via pertussis toxin sensitive G-proteins (Peralta et al. 1988). Although generally true, this scheme is a simplification as there have been reports that m1 and m3 can stimulate adenylyl cyclase, whilst m1, but not m3, transfected into RAT-1 cells couples to inhibition of adenylyl cyclase via a pertussis toxin sensitive G-protein (Pinkas-Karmarski et al. 1988; Stein et al. 1988). m2 and m4 have been shown to be capable of coupling to phosphoinositide hydrolysis albeit with low efficiency (Ashkenazi et al. 1987; Peralta et al. 1987). It would seem that at least some of the receptor-effector coupling constraints are a function of the cell type in which the receptor is expressed. This may be due to the repertoire of G-proteins and second messenger systems or ion channels expressed by the cell.

Functional studies.

The muscarinic receptors are widely distributed in central and peripheral neurons and autonomic effector tissues. In the autonomic nervous system they play an important role in mediating effects in target organs *e.g.* heart (inhibition of rate and force), exocrine glands (stimulation of secretion) and smooth muscle (contraction) (for

a review see Buckley and Caulfield, 1992). The role of the muscarinic receptors in the CNS is less well understood, and investigations into their functional significance has been hampered by a lack of chemical agents that can selectively damage cholinergic neurons. Several studies have implicated muscarinic receptors in arousal (Bradley and Elkes, 1957), the acquisition and maintenance of short term memory (Hagan and Morris, 1988) and in psychomotor control. Administration of physostigmine, an anticholinesterase, produces an electroencephalographic response associated with arousal, while the muscarinic antagonist atropine has the opposite effect. It has not been determined which receptor subtypes mediate this effect but it is presumed to involve the projection from the ventral forebrain to the cortex. Muscarinic receptor involvement in memory is thought to be mediated, at least in part, by the septo-hippocampal pathway (Hagan and Morris, 1988). The action of muscarinic agonists administered to trained rats produces an improved memory response whilst muscarinic antagonists, such as scopolamine, impairs memory function. Further studies using the two antagonists, pirenzepine and AFDX 116, have attempted to define which muscarinic receptor subtype is involved (Messer et al. 1990). When both of these antagonists are administered separately to the hippocampus, pirenzepine, which has a high affinity for M_1 and a low affinity for M_2, impairs memory to a greater extent than AFDX 116, which has the opposite selectivity. This suggests that the M_1 receptors have a greater involvement in learning than do the M_2 receptors but the extent of involvement of the other muscarinic receptors cannot be distinguished with these antagonists. Loss of M_2 receptors in the basal forebrain of patients with Alzheimers disease mimics the loss of cholinergic neurons as measured by the activity of the enzyme, choline acetyltransferase (Mash et al. 1985). This observation is consistent with the suggestion that at least some M_2 receptors act as autoreceptors, inhibiting the release of acetylcholine. Muscarinic receptors present in the striatum have been implicated in motor control. Patients with Parkinsons disease show degeneration of the inhibitory dopaminergic terminals in the striatum leading to hyperactivity of the cholinergic neurons. This hyperactivity leads to hypokinesia, rigidity and tremor, which can be reduced by the action of muscarinic

antagonists. The striatum predominantly expresses the two muscarinic receptor genes, m1 and m4. The m1 gene has been associated with the enhancement of dopamine release as this response is mediated by a receptor with a high affinity for pirenzepine. The m4 receptor is thought to be responsible for the inhibition of adenylyl cyclase in the striatum as m4 is seen to preferentially couple to adenylyl cyclase in some clonal cell lines.

Regulation of receptor activity and expression.

It can be seen from the studies previously described that acetylcholine can stimulate a wide range of second messenger systems by acting through each of the five muscarinic receptor subtypes. Therefore regulation of these receptors provides a mechanism by which the responses to acetylcholine are processed and determine what effects these have on the cell. Elucidation of the factors and mechanisms involved in the development, regulation and maintenance of expression of these neurotransmitter receptors will provide key insights into the control of signal processing in the nervous system. Regulation can occur at three basic levels; firstly at the level of transcription, which defines which cells can express receptor proteins and is probably the most important mechanism of regulation during development; secondly, by altering the levels of receptor that are expressed at the cell surface; and thirdly, by chemically modifying the receptor in order to alter its capability to interact with the neurotransmitter or to transduce a signal upon neurotransmitter interaction (*e.g.* desensitization).

Desensitization.

The cellular response to agonists acting on cell surface receptors diminishes rapidly with continued exposure of the cells to this stimulus. This phenomenon has been termed desensitization, and is the result of uncoupling of the receptor and effector mechanism. As a result, the receptor and the agonist binding site are still present on the cell surface but an agonist is unable to evoke a response upon binding

to the receptor. This desensitization requires agonist exposure to the receptor, antagonists being unable to elicit a desensitization, suggesting that the receptor has to be activated for desensitization to occur, and that occupation of the binding site alone is insufficient. The mechanism is also extremely rapid, desensitization reaching a maximum level within minutes in the presence of high concentrations of agonist. Despite some intense research efforts by a number of laboratories, the mechanism of desensitization has not been fully elucidated. Work by Kanba et al. (1990) has implicated a protein kinase C (PK-C) like protein in the mechanism of desensitization of the M_1 receptor in the N1E 115 cell line. They show that the action of phorbol 12-myristate 13-acetate (PMA - a potent PK-C activator), mimics the effect of muscarinic agonist desensitization in these cells. However it is unlikely that PK-C itself is involved in this desensitization, as cells depleted of PK-C activity by chronic stimulation with PMA still showed receptor desensitization with carbachol treatment. PK-C may be involved, at least in part, in muscarinic desensitization in the rat lacrimal gland (Tan and Marty, 1990). Activation of PK-C by 12-O-tetradecanoyl phorbol 13-acetate (TPA) enhances desensitization while down-regulation of PK-C by prolonged activation with TPA or inhibition of PK-C by staurosporine results in a decrease in desensitization. Lograno et al. (1991) have also provided evidence implicating PK-C modulation of muscarinic receptors. In their study desensitization of contraction of ciliary muscle in response to muscarinic activation by carbachol could be inhibited by staurosporine and enhanced by PK-C activation by 4α-phorbol didecanoate (PDBu).

It has been shown that not all muscarinic receptors desensitize on agonist exposure. For example, the SK-N-SH cell line expresses a muscarinic receptor that is coupled to phosphoinositide hydrolysis, and continued exposure of carbachol results in sequestration of the receptor from the surface of the cell, but no desensitization occurs (Baumgold et al. 1989). The lack of observable desensitization is not due to a population of spare receptors as no desensitization is seen to occur when 94% of the muscarinic receptors are alkylated with propilylbenzyl choline mustard. In contrast the muscarinic receptors expressed in the NG108-15 cell line, which are coupled to

inhibition of adenylyl cyclase and inhibition of Ca^{2+} current, are seen to become desensitized before sequestration (Baumgold et al. 1989).

Desensitization has been particularly well studied in another member of the G-protein coupled receptor superfamily, the ß-adrenergic receptor (ßAR). The ßAR is coupled via the stimulatory G-protein, G_s, to adenylyl cyclase and activation of the receptor results in increased levels of cAMP within the cell. The evidence suggests that desensitization of this receptor is the result of its phosphorylation by two different kinases; protein kinase A (PK-A) and ß-adrenergic receptor kinase (ßARK) (Sibley et al. 1987). PK-A phosphorylates the ßAR at low agonist concentrations and its effect is to decrease the potency of agonists, thus desensitizing the receptor to low, but not high, doses of agonist. At higher concentrations of agonist, ßARK is also active and phosphorylates different residues on the ßAR, leading to further desensitization, even at high concentrations of agonist. It has been thought that this dual desensitization may have a physiological role. *In vivo*, PK-A may mediate desensitization in situations where agonist concentrations are low, such as circulating catecholamines, and ßARK may mediate desensitization at high agonist concentrations, such as sympathetic synapses (Lefkowitz et al. 1990). It remains to be seen whether similar mechanisms exist for the muscarinic receptors, although evidence does suggest that phosphorylation may be involved in desensitization (Burgoyne, 1980; Kanba et al. 1990). *In vitro* studies have shown that purified muscarinic receptors from chick heart, (probably a mixture of m2 and m4), are phosphorylated by ßARK in an agonist dependent manner (Kwatra et al. 1989). The cardiac mAChR, (m2), has been phosporylated by PK-A but not by PK-C. PK-C has however been used to successfully phosphorylate purified m1 and m3 mAChRs (Haga et al. 1988).

From these studies it seems likely that phosphorylation plays a key part in muscarinic receptor desensitization. There also appears to be several pathways involved in desensitization and several protein kinases including, PK-C, ßARK, and PK-A may be involved but, as yet unidentified protein kinases may also play a role.

Down-regulation.

Continued exposure of cells to agonist results in a loss of cell surface receptors, a process known as down-regulation. Regulation of the numbers of receptors expressed at the cell surface provides a mechanism whereby the level of stimulus received by the cell, upon agonist exposure, can be regulated and it has been proposed that this down-regulation may play a physiological role in modulating synaptic activity (Shifrin and Klein, 1980).

Several studies on muscarinic receptor regulation in cultured heart cells and neuroblastoma cell lines, in response to carbachol, have suggested a model for this down-regulation. This model involves an initial and rapid sequestration of the cell surface receptors to a lighter membrane fraction that can be separated from the cell surface membrane by density gradient centrifugation. This sequestration begins within minutes of agonist exposure (Galper and Smith, 1980) and can be blocked by methylamine, an inhibitor of endocytosis, suggesting it is an internalization event (Ray et al. 1989). If agonist exposure is withdrawn within 30 minutes then there is a rapid recovery of cell surface receptors, which is complete within a further 30 minutes and does not require protein synthesis (Feigenbaum and El-Fakahany, 1985). If, however, the agonist is maintained for 6-24 hours, recovery after subsequent agonist withdrawal is much slower, taking up to 24 hours to reach pre-stimulation levels. This recovery can be blocked by cycloheximide, (a protein synthesis inhibitor), demonstrating that it requires protein synthesis (Shifrin and Klein, 1980). Nocadazole and colchicine are both antimicrotubular agents, and they are capable of preventing the agonist induced degradation, but not the sequestration, of mAChRs (Ray et al. 1989). It is thought therefore that activated mAChRs are initially internalized and then transported, via the microtubules, to the lysosomes where they are degraded.

The signalling mechanism for the agonist induced down regulation of mAChRs has not been determined, but a 6 hour incubation of N1E 115 cells with PMA and A23187 (a Ca^{2+} ionophore) is able to mimic the decrease in receptors

elicited by chronic stimulation with carbachol. Stimulation of these cells with either carbachol or PMA results in the translocation of PK-C activity from the cytosol to the membrane suggesting that this activation of PK-C may be involved in the regulation of the mAChR in response to agonist (Liles et al. 1986).

Most of the studies performed on the regulation of mAChR number involve long exposure times to agonist (up to 24 hr), a situation that is unlikely to occur *in vivo*. Although nothing is known about the levels of acetylcholine in a cholinergic synapse in the CNS, there are likely to be some similarities with cholinergic synapses in autonomic ganglia. In the autonomic ganglia high concentrations of acetylcholine are released. It has been estimated that the level of acetylcholine can be reduced about 3 fold before failure of synaptic transmission is seen (Sacchi *et al.* 1978). These levels of acetylcholine are probably only present for short periods of time before the agonist is rapidly hydrolyzed by acetylcholinesterase. In order to determine if short agonist exposure times are capable of regulating levels of mAChR, Shifrin and Klein (1980) incubated N1E 115 cells intermittently with carbachol for 15 minutes and without carbachol for 15 minutes. Although agonist stimulation for 15 minutes is much longer than *in vivo* stimulation times, withdrawal of agonist after 15 minutes results in a rapid return of receptors to control levels which does not require protein synthesis (Shifrin and Klein, 1980). After 6 hours of this cycling incubation the density of mAChR fell to 50% of the initial level suggesting that a series of short agonist exposure times is capable of influencing receptor number.

As has already been described, exposure of receptors to agonists results in desensitization and, on further exposure, down-regulation. Another long term effect of chronic agonist exposure is an alteration in the steady state level of mRNA encoding the receptor. Such a phenomenon has been observed for the mAChRs in cultured cerebellar granule cells (Fukamauchi et al.1991). These cells express transcripts for the m2 and m3 receptor subtypes, but transcripts for the other subtypes were not detectable. On chronic stimulation with carbachol differential regulation of the levels of these transcripts was seen to occur. The m3 mRNA levels steadily declined to about 50% of the control values after 8 hours, but by 24 hours had

returned almost to pre-stimulation levels. The m2 mRNA levels showed a much more rapid and transient reduction in levels. For this subtype, transcript levels fell to 50% of controls within 2 hr but then began to recover, so that after 8 hours the levels were at 85% of control levels and after 24 hr they had returned to normal. Whether these alterations in transcript levels were the result of a change in the rate of transcription or a change in the stability of the mRNAs was not determined. Habecker and Nathanson (1992) have also observed receptor activation induced reduction in mRNA levels for two muscarinic receptor genes, cm2 and cm4. Activation of the receptors in cultured chick heart cells with carbachol results in a reduction of the mRNA levels to approximately 60% of control levels within 24 hr. This reduction is not due to any alteration in the stability of the mRNA. In addition to this homologous regulation by muscarinic receptor stimulation, activation of angiotensin II and A_1 adenosine receptors also result in a reduction of muscarinic receptor levels in these cells. This heterologous down-regulation has been proposed to be due to the activation of phospholipase C and inhibition of adenylyl cyclase that occurs when these receptors are stimulated.

Several other systems have been studied and various types of effects on mRNA levels due to receptor stimulation have been observed. These include a rapid reduction in mRNA levels (Oron et al. 1987), an elevation of mRNA levels (McDonnell et al. 1988) and a transient increase in mRNA levels followed by a decrease in levels with time (Okret et al. 1986).

A great deal more data has been determined for the regulation of the $ß_2$AR by both homologous and heterologous stimulation. A 30 min incubation of the DDT_1 MF-2 hamster vas deferens cell line with adrenaline stimulates a transient increase in $ß_2$AR mRNA due to an increase in the rate of transcription (Collins et al. 1989). Prolonged exposure of these cells to adrenaline results in a decrease in $ß_2$AR mRNA which is thought to be due to a reduction in its stability (Collins et al. 1989; Hadcock et al. 1989a). The steroid, dexamethasone, causes an increase in the rate of transcription of the $ß_2$AR gene whilst $ß_2$AR agonists cause destabilization of the mRNA (Hadcock et al. 1989a). Opposing mechanisms such as this are one way in

which the level of cell surface receptors can be regulated *in vivo* by a combinatorial action of separate pathways.

By using mutant cell lines lacking either functional $G_{s\alpha}$ (through which the $ß_2$AR interacts to stimulate adenylyl cyclase and thereby increasing intracellular cAMP levels), or cAMP dependent protein kinase, insights into the pathway involved in mRNA regulation have been obtained (Hadcock et al. 1989b). It appears that for a $ß_2$AR agonist to cause a reduction in mRNA levels then the receptor must be coupled to $G_{s\alpha}$ but $G_{s\alpha}$ does not have to couple to adenylyl cyclase. Also cAMP dependent protein kinase is required but cAMP accumulation in the cell is not.

Transcriptional control.

Perhaps the most important aspect of mAChR regulation is that of transcriptional control because this is the first step along the pathway from DNA to functional cell surface receptors. Regulation of transcription has been ascribed to cis-acting genomic elements that are present in the vicinity of the coding region of the gene (Maniatis et al. 1987). These elements probably interact with the various transcription factor proteins that are present within the cell. There are basically two tiers of transcriptional regulation which have been noted for receptor genes. Firstly, there is the temporal and spatial regulation which controls where and when a gene is transcribed and thus expressed. On top of this a second, more subtle, tier of regulation has been observed, which determines the levels of a particular transcript that are expressed. This second level of regulation usually involves a feedback mechanism from the cell surface, (as mentioned in the previous section), and results in either a change in the rate of transcription or a change in the stability of the mRNA (Collins et al. 1989; Hadcock et al. 1989a).

For a cell to begin expressing a particular gene, the gene must first be transcribed. This is achieved by the action of RNA polymerases which synthesize an RNA copy of the DNA. The activity of the RNA polymerase is thought to be mediated by other DNA binding proteins, (known as transcription factors), which can have either a positive (activator) or negative (repressor) effect on transcription. The

transcription factors which are present within a cell will determine which genes are transcribed and which genes are repressed. The mechanism by which this regulation occurs is thought to involve the binding of the transcription factors to particular cis-acting genomic DNA sequences present in the vicinity of the coding region of the genes (Maniatis et al. 1987); however some distant regions of the genome can also influence transcriptional control as noted for the ß-globin gene (Grosveld et al. 1987). By a combinatorial action of transcriptional activators and repressors, each cell is able to express a particular repertoire of genes. Conversely, a gene is only expressed in those cells which contain transcription factors that bind to its genomic control elements and result in a permissive or positive action on transcription.

To date, no transcriptional control elements have been elucidated for any of the mAChR genes, but genomic elements responsible for controlling expression of a number of other genes have been isolated and characterized. Although the genomic regions controlling expression appear diverse for the genes that have been studied, many do have some similar motifs and some general control mechanisms appear to be emerging. Firstly, promoter elements (which are capable of driving transcription) are usually located 5' to the transcription start site. Many genes (Maniatis et al. 1987), but not all (Vidal et al. 1990; Minowa, M. et al. 1992), contain an AT rich TATA box motif about 30 nucleotides upstream from the transcription initiation site. This basic promoter element is thought to ensure correct initiation of transcription and further elements are required to increase the rate of transcription, as has been seen for the neurofilament light gene, (NF-L), (Nakahira et al. 1990) and the rod opsin gene (Lem et al. 1991). These enhancer elements, which alone are insufficient to drive transcription have been found in 5' flanking regions (Minowa, M. et al. 1992; Possenti et al. 1992), 3' flanking regions (Vidal et al. 1990; Puschel et al. 1991; Whiting et al. 1991) and in intronic sequences (Begemann et al. 1990; Vidal et al. 1990; Whiting et al. 1991). As well as enhancers, repressor motifs have also been described (Maue et al. 1990; Vandaele et al. 1991; Kraner et al. 1992; Mori et al. 1992; Possenti et al. 1992). The action of these repressor elements appears to be to restrict expression of a particular gene to specific cell types rather than to actually

lower the level of transcription. One interesting repressor, or as it has been termed, 'silencer', element that has been characterized in the SCG10 gene (Mori et al. 1990; Wuenschell et al. 1990; Mori et al. 1992), the type II sodium channel gene (Maue et al. 1990; Kraner et al. 1992) and the neuron specific enolase (NSE) gene (Sakimura et al. 1987; Foss-Petter et al. 1990) is thought to act to silence expression of these genes in non-neuronal cells. This 21 base pair silencer element has been shown to bind a factor which is present in non-neuronal but not neuronal cells (Mori et al. 1992), suggesting that this may act as a general mechanism for some of the specific expression of neuronal genes. A similar mechanism of general repression may also act in neuronal cells as suggested by Kemp et al.(1990). This group found that herpes simplex virus immediate early genes, which are active in non-neuronal but not in neuronal infected cells, contain a DNA motif in the promoters that is similar to a eukaryotic octamer motif. It was shown that this motif is bound by a repressor that is present in cells of neuronal origin but not present in cells of non-neuronal origin. Another gene that is regulated by selective repression is the α7 nicotinic acetylcholine receptor gene (Matter-Sadzinski et al. 1992). 1.4 kb of sequence 5' to the transcription initiation site correctly drives reporter gene expression in E13 retinal ganglion cells. This promoter is also active in proliferating neuroepithelial cells from the retina and tectum, where endogenous α7 has not been detected, but not in CHO cells. It appears that specific expression of the α7 gene may be acquired during development by selection rather than activation, and the stability of the mRNA prevents the α7 gene being detected earlier.

Usually the sequence of nucleotides in the promoter and enhancer elements for a particular gene appears to be sufficient to drive tissue specific expression of a reporter gene in both transgenic mice and eukaryotic cell lines. However, this is not always the case, as has been shown for the 68-KDa neurofilament (NF-L) gene. 1.7 kb of sequence 5' to the transcription initiation site is capable of driving expression of a reporter gene in a tissue specific manner in transgenic mice (Byrne and Ruddle, 1989). When this same fragment is used to drive expression of the ß-galactosidase gene in cell lines, it is seen to be active in cell lines that express endogenous NF-L

and in cells that do not express endogenous NF-L (Nakahira et al. 1990). This ectopic expression was found using a transient transfection assay on cell lines so may have been due to the promoter not being associated with chromatin. Another potential problem with transient transfection assays is the result of the high number of copies of cis elements introduced into the cell by transfection. Due to a high copy number of transfected elements, transcription factors may become limiting and result in a derepression, if the transcription factors acted to repress transcription. However, stable cell lines produced using the same construct still showed ectopic expression of the reporter gene (Nakahira et al. 1990). What is interesting about this study is that these stable cell lines contain two types of NF-L promoters which contain identical nucleotide sequences. The first is present in the endogenous NF-L gene and is inactive in these cell lines. The second is present as part of the reporter gene and is active in these cells. It is possible then that the expression of the NF-L gene may be controlled by a selective de-activation of its promoter during development. This switching off of the promoter may involve modification of the DNA such as methylation and occur only during a certain stage of development of any particular cell. Therefore introducing a promoter, that has not been de-activated, into cell lines which have already passed this stage in development, will allow the promoter to drive expression of its gene, or a reporter gene, in a non-specific manner.

Several motifs have been described in genetic control elements and the transcription factor proteins that interact with some of these motifs have been determined (for a review see Locker and Buzard, (1990)). Research into the interaction of transcription factors with DNA motifs has shown that a variety of mechanisms are used to control gene expression. Some transcription factors appear to be ubiquitously expressed, for example the OTF-1 protein, which recognises and binds to the octamer sequence ATTTGCAT and is involved in histone H2b transcription (Fletcher et al. 1987). Other DNA binding proteins are tissue specific such as the nuclear protein, APF, which is only found in liver cells and hepatoma lines and is responsible for the liver specific expression of the albumin gene (Cereghini et al. 1988). A third type of transcriptional control factors is inducible,

such as the products of the immediate early genes *fos* and *jun*. These two proteins recognise and bind to several DNA motifs including the AP-1 and AP-2 motifs (Angel et al. 1987; Angel et al. 1988). Motifs such as AP-1 and AP-2 may play an important role in the regulation of mAChR transcription as they have been found to mediate transcription levels which can be altered by protein kinase C and cAMP (Angel et al. 1987; Imagawa et al. 1987). Protein kinase C has itself been implicated in the regulation of mAChR transcription, (Kanba et al. 1990), and although no-one has looked at the effects of cAMP levels on mAChR transcript levels, the inhibition of adenylyl cyclase by m2 and m4 receptors suggests this second messenger would make a strong candidate for regulating the levels of these receptor transcripts in a feedback loop. Other elements that may be important in regulation of these genes are growth factor responsive elements as it has been shown that the levels of muscarinic receptors increases in PC12 cells on induction with nerve growth factor (Jumblatt and Tischler, 1982).

The size and position of genomic control elements with respect to the transcription initiation site appears to vary greatly. For instance, 212 base pairs of the upstream region of the tyrosine hydroxylase gene are sufficient for specific expression in cell lines and also include several transcription factor binding domains (Cambi et al. 1989). However 5.8 kb of the dopamine ß-hydroxylase gene promoter are required for expression in transgenic mice and even this results in some ectopic expression, suggesting further elements may be necessary. In a similar study, 7 kb of the *Hox-3.1* gene promoter were required to prevent ectopic expression of a reporter gene in transgenic mice when 5 kb of the gene promoter was used (Biebrich et al. 1990).

It would seem then, that the transcriptional regulation involves a complex interaction of negative (repressor) and positive (enhancer) transcription factors interacting with specific DNA elements. The DNA elements can be subdivided into three main functional categories; basal promoters, which are capable of driving constitutive transcription albeit at only a low level, enhancers, which increase the rate of transcription but are not alone capable of driving transcription and repressors, which decrease and usually silence transcription.

Summary.

At present there are 5 known genes encoding subtypes of muscarinic acetylcholine receptors, each of which has a unique pattern of expression in the CNS. Each of the receptors also has a unique pharmacological profile with respect to a range of antagonists and are coupled via G-proteins to a wide range of effector mechanisms.

Regulation of expression of these genes is obviously tightly controlled at the level of transcription. Transcriptional control of these genes is most likely to be conferred by cis acting genomic regions, within the vicinity of the coding region, as has been the case for a multitude of previously studied neuronal and non-neuronal genes. Regulation of the levels of receptor expressed on the cell surface has also been seen to occur. Regulation at this level will have functional consequences for the cell and has been proposed to be involved in modulating synaptic activity.

The elucidation of the mechanisms involved in the development, regulation and maintenance of expression of these neurotransmitter receptors should provide key insights into the control of signal processing in the CNS.

Aims of project.

The aims of this project are twofold:

Firstly to investigate the control of the muscarinic receptor levels expressed in a neuroblastoma, the NG108-15 cell line, which expresses the m4 muscarinic receptor gene and has been proposed to be a good model for neuronal cells.

Secondly to isolate and identify genomic sequences that are responsible for the transcriptional regulation for the m4 muscarinic receptor gene.

Chapter 2. Materials and Methods.

Experimental Methods.

Cell culture.

Cell lines were cultured in 5% CO_2 at 37°C in αMEM containing 6g/l penicillin, 10g/l streptomycin and 2mM L-glutamine, supplemented with either 10% foetal calf serum (FCS), 3μM hypoxanthine, 4.8μM thymidine and 0.12μM aminopterin (NG108-15 cells) or 5% FCS and 5% horse serum (PC12 cells) or 10% FCS and 10mM HEPES (IMR32 cells) or 10% newborn calf serum (CHO, 3T3 and 1321N1 cells). The N18TG2 cell line was cultured in DMEM supplemented with 10% FCS, 2mM L-glutamine, 60mg/l gentamycin and 30μM 6-thioguanine. NG108-15 cells were 'differentiated' as follows: at about 70% confluence growth medium was replaced with a similar medium that contained only 1% FCS and the cells left overnight. Prostaglandin E_1 (PGE_1, Sigma) and 3-isobutyl-1-methylxanthine (IBMX, Sigma) were added to final concentrations of 10μM and 50μM respectively. The cells were then cultured for 48 hr before experiments were begun.

Binding.

NG108-15 and N18TG2 cells were harvested and suspended in binding buffer (NaCl 120mM, KCl 3mM, $MgCl_2$ 1.2mM, HEPES 28mM, glucose 11.1mM and $CaCl_2$ 2.5mM (pH7.4)) at a density of $1x10^6$ cells/ml (NG108-15) or $5x10^6$ cells/ml (N18TG2). 200μl aliquots of this suspension were placed in Eppendorf tubes containing either ^{3}H-N-methylscopolamine (^{3}H-NMS) (total binding) or ^{3}H-NMS and 1μM atropine (non-specific binding). For competition binding analysis a range of concentrations of competing ligand were also included. The tubes were incubated at room temperature for 2 hr and the incubation terminated by centrifugation at 10,000g for 2 min. The pellets obtained were washed twice in normal saline and solubilized overnight using 100μl of Sol-u-sol (National Diagnostics). The solution was neutralised with HCl, scintillation fluid was added and the samples counted in a

scintillation counter.

Protein determination.

The protein concentration of samples was determined using a Bio-Rad protein assay kit. This kit is based on the Bradford method of protein determination (Bradford, 1976).

DNA Probe labelling.

Oligonucleotides were labelled by a 3' tailing method as follows: oligonucleotides (final concentration 100nM) were incubated with 20μCi of α^{32}P-dATP (6000Ci/mMol NEN), in 100mM potassium cacodylate (pH7.2), 20mM Tris pH7.2, 0.2mM magnesium chloride, 0.3mM cobalt chloride and 20 units of terminal deoxytransferase (BRL) in a total volume of 12.5μl for 1 hr at 37^{o}C. 10μl of 1M MOPS, 10μl of 2M sodium chloride and 167μl of water were added and the oligonucleotide purified using a Quiagencolumn. Specific activity of the probes was routinely greater than 10^9 dpm/μg of DNA.

DNA fragments were labelled using a random priming method. 25ng of DNA was denatured by boiling for 5 min and quenching on ice for 1 min. 2μl of each of 0.5mM dTTP, dGTP and dCTP, 15μl of random primers buffer (0.67M HEPES, 0.17M Tris, 17mM $MgCl_2$, 33mM ß-mercaptoethanol, 1.33mg/ml BSA and 18 OD_{260} units/ml oligonucleotide primers (hexamer fraction) pH 6.8), 50μCi α-^{32}P dATP (6000Ci/mMol, NEN) and water were added to give a total volume of 49μl. 1μl of Klenow fragment was added and the reaction incubated for 1 hr at room temperature. The reaction was stopped by addition of 50μl of G-50 buffer and unincorporated nucleotide removed by passing through a G-50 spun column at 3000 g for 5 min. Incorporated radioactivity was determined by Cerenkov counting and the specific activity of the probes obtained was routinely $>1x10^9$ dpm/μg of DNA. Before hybridization the probe was boiled for 5 min and quenched on ice for 1

min.

Library screening.

A rat genomic library constructed in the cosmid vector pTCF (Grosveld et al. 1987) was screened at high stringency with ^{32}P dATP tailed oligonucleotide probes and a 997bp internally labelled cDNA fragment obtained from a Pst I digest of the m4 coding region.

For screening with the oligonucleotide probes, a mixture of 3 oligonucleotides, Rm4A (5' CTCACCAGGCGCACAGACTGATTGGCTGAGCTGC CATTGACAGGCGTG 3'), Rm4B (5' GGTGTTCTGGGTGGCACTGCCTGAGCTG GACTCATTGGAAGTGTCCTT 3') and Rm4C (5' GCGCTCCCGGGCCGCCAT CTGCCGCTTCTTGCGCACCTGGTTGCGAGC 3') complementary to nucleotides 10-58, 866-914 and 1139-1187 of the m4 cDNA, were labelled by 3' tailing. The filters were prehybridized overnight at 37°C in buffer containing 4xSSPE (150mM NaCl, 10mM NaH_2PO_4, 1mM EDTA), 5x Denhardt's reagent (0.1% Ficoll, 0.1% BSA and 0.1% poly(vinylprrolidone)), 250μg/ml yeast tRNA, 500μg/ml denatured salmon sperm DNA and 0.1% SDS. After prehybridization, labelled probe was added ($1x10^6$ dpm/ml) and hybridization carried out at 37°C overnight. The filters were washed at 55°C in 1xSSPE, 0.1% SDS, four times for 15 min each.

For screening with a random primed probe, spanning the m4 cDNA between nucleotides 6-1003, the filters were prehybridized overnight at 55°C in buffer containing 50% formamide, 4xSSPE, 5x Denhardt's reagent, 250μg/ml yeast tRNA, 500μg/ml denatured salmon sperm DNA and 0.1% SDS. After prehybridization, labelled probe was denatured by boiling for 5 min, added to a concentration of $1x10^6$ dpm/ml and hybridization carried out overnight at 55°C. The filters were washed at 60°C in 0.1xSSPE, 0.1% SDS, four times for 15 min each. The nylon filters were blotted dry and exposed to X-ray film at -70°C for one week and colonies picked that gave hybridization signals on both replica filters. The filters were stripped in boiling 1% SDS before rehybridization.

Nuclear Run on Assay.

Nuclei from PC12 cells were isolated as follows. The cells were washed in PBS-A and harvested with trypsin. They were then spun at 200g and washed with ice-cold RSB (10mM Tris Cl (pH7.4), 10mM NaCl, 5mM $MgCl_2$ and 1mM DTT). The cells were resuspended in ice-cold RSB + 0.5% NP-40 (5ml/15cm dish) and incubated for 10 min on ice. The nuclei were spun at 200g, 0°C and washed by resuspending and centrifuging three times in ice-cold RSB. After the final wash the nuclei were resuspended at 10^8 nuclei/ml in freezing buffer (50mM Tris Cl (pH8.5), 5mM $MgCl_2$, 0.1mM EDTA and 50% w/v glycerol), and stored at -70°C in 200μl aliquots. For the transcription run-on a 200μl aliquot of frozen nuclei was thawed, 50μl ^{32}P UTP (3000 Ci/mMol, NEN) and 60μl x5 run on buffer (25mM Tris Cl (pH8), 12.5 mM $MgCl_2$, 750 mM KCl, 1.25mM rATP,rGTP and rCTP) was added. After a 10 min incubation at 30°C the DNA was digested by the addition of RNase-free DNase (Promega) for 45 min at 37°C. The protein was then digested by the addition of 75μl 5x SET (5% SDS, 25mM EDTA and 50mM Tris Cl (pH7.4)) and 5μl proteinase K (20mg/ml) and incubated for 45 min at 37°C. 90μl 2M sodium acetate (pH4), 500μl 4M guanidium thiocyanate, 25mM sodium citrate (pH7.0) and 0.5% Sarcosyl, 900μl water saturated phenol and 180μl chloroform isoamylalcohol were added. The mixture was vortexed and incubated on ice for 15 min before spinning in a microfuge at 4°C for 15 min. The aqueous phase was removed, the RNA precipitated with isopropanol, washed in 70% ethanol and dissolved in 100μl of 10mM Tris (pH7.2), 1mM EDTA and 0.1% SDS for 15 min at 65°C. Total and incorporated counts were determined and the probe hybridized to slot blots in a hybridization buffer containing 10mM Tris Cl (pH7.4), 0.2% SDS, 10mM EDTA, 300mM NaCl 1x Denhardt's reagent, and 200μg/ml tRNA at 65°C for 48 hr. The blots were washed 2x15 min in 1x SSC, 0.1% SDS at 65°C, 1x30 min in 0.1x SSC, 0.1% SDS at 65°C and excess RNA removed in 2x SSC, 10μg/ml RNAase at 37°C for 30 min. The blots were finally washed once in 2x SSC for 1 hr at 37°C before

exposing to X-ray film at -70°C for one week.

RNA isolation.

RNA was isolated using the method of Chomczynski and Saachi (1987). The cells were harvested and pelleted at 10 000g for 2 min. They were homogenized in 1ml of GNTC solution (4M guanidium thiocyanate, 25mM Na citrate (pH7), 0.5% sarcosyl and 0.72% ß-mercaptoethanol). Each sample was then split between two Eppendorf tubes and 50μl of 2M Na acetate (pH 4) was added. The RNA was phenol/chloroform extracted and precipitated with isopropanol. The pellet was dissolved in 0.3ml GNTC solution, precipitated with isopropanol and washed in 70% ethanol. The RNA was dissolved in TE (pH7.4) and the concentration determined by reading the absorbance at 260nm. Purity was assessed by the A_{260}/A_{280} ratio.

Northern blot screening.

Total RNA (15μg/lane) was precipitated with ethanol and dissolved in 14μl of denaturing buffer (50% formamide, 17.8% formaldehyde, 1xFRB (200mM MOPS, 50mM Na acetate, 5mM EDTA (pH7)) and bromophenol blue). This was denatured at 55°C for 5 min and quenched on ice. 1μl of ethidium bromide (5mg/ml) was added to each 14μl sample and these then run on a denaturing agarose gel (1% agarose, 1xFRB, 2% formaldehyde) and blotted onto a nylon membrane by electroblotting at 300mA for 4 hr. The filters were baked in a vacuum oven at 80°C for 2 hr. Prehybridization, hybridization and washes were carried out as described earlier for library screening.

Competent bacteria.

Competent *E.Coli* (DH5α) were prepared by a modified procedure of Dower et al. (1988). A 5 ml overnight culture was diluted into 500 ml of fresh L-broth and

the cells were grown at 37°C with vigorous shaking until they had reached an O.D. of 0.3-0.4 at 600nm. The cells were then harvested by chilling on ice and centrifuging at 4°C for 5 min at 4000xg. The pellet was resuspended in 500 ml of sterile, ice cold, water and respun at 4000xg for 5 min at 4°C. The cells were resuspended in 250 ml of ice cold sterile water and again centrifuged at 4°C for 5 min at 4000xg. The cells were then resuspended in 10 ml of ice cold 10% glycerol and respun as above. Finally the cells were resuspended in 750μl of ice cold 10% glycerol and 40μl aliquots were transferred to microfuge tubes pre-chilled on dry ice. The aliquots were stored at -70°C until use.

For transformations the cells were thawed at room temperature and placed on ice. 40μl of cell suspension was mixed with the DNA and transferred to a cold 0.2 cm electroporation cuvette. The suspension was pulsed once in a Bio-Rad Gene-Pulser^{TM} set at 2.5kV, 25μF and 200Ω and routinely gave a time constant of about 4.6ms. After electroporation, 1 ml of SOC medium (2% bactotryptone, 0.5% bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose) was immediately added and the cells transferred to polypropylene tubes. These were shaken at 37°C for 1 hr and 100μl of each sample spread onto an LB-agar plate, supplemented with 50μg/ml of ampicillin, and grown overnight at 37°C. The competency of the cells was tested by transforming with a control plasmid and was routinely found to be $>10^8$ transformants/μg.

DNA Transfections.

Caesium chloride banded DNA was transfected into eukaryotic cells using calcium phosphate precipitation as described by Chen and Okayama (1988). Test plasmids were co-transfected with a standard plasmid containing the luciferase gene under the control of the Rous sarcoma virus (RSV) promoter to allow corrections for tranfection efficiency to be made. For transient assays, cells were harvested 48 hr after transfection and cell extracts prepared as described below. To produce stable cell lines, cells were co-transfected with the cosmid clone, (R3-6), and a Simian virus 40

(SV40) neomycin resistance gene. 48 hr after transfection the cells were split 1:20 and cultured in the presence of G418 to select for those cells that expressed the neomycin gene. Single clones were selected after 10-14 days and RNA prepared from several lines to test for rat m4 expression.

ß-galactosidase staining.

Transfection efficiency for eukaryotic cell transfections was determined by transfecting cells plated onto a 3.5 cm culture dish with 4μg of an RSVß-Gal plasmid. After 48 hr the medium was removed and the cells washed 3 times with PBS-A. The cells were fixed for 5 min at room temperature in a solution containing 2% formaldehyde and 0.2% glutaraldehyde in PBS-A. The cells were then washed 3 times in PBS-A and overlaid with a solution containing 1mg/ml X-Gal, 5mM potassium ferricyanide, 5mM potassium ferrocyanide and 2mM $MgCl_2$ in PBS-A. The cultures were placed in the dark for the staining to develop which began to appear after approximately 1 hr. The cells were usually left staining overnight as no background staining developed over this period. Transfection efficiency was determined by the percentage of cells that stained blue indicating presence of the ß-galactosidase enzyme.

PCR analysis.

For PCR analysis 10μg of RNA was precipitated and resuspended in 10μl of water. 5μl 10x transcription buffer (400mM Tris-HCl (pH7.9), 100mM NaCl and 60mM $MgCl_2$), 29.5μl water, 0.5μl RNA guard (Pharmacia) and 5μl of RQ DNase I (Promega) was added and incubated at 37°C for 15 min. The RNA was phenol/chloroform extracted, precipitated with ethanol and resuspended in 5μl of water. 2μl of this DNased RNA was added to 10μl of oligo dT solution, denatured for 5 min at 65°C and quenched on ice. 5μl of 5mM dNTPs, 2μl of 10x RTase buffer (500mM Tris-HCl (pH8.3), 400mM KCl, 100mM $MgCl_2$ and 10mM DTT), 0.5μl

RNA guard and 0.5μl AMV-RT was added and incubation carried out at 37^{o}C for 1 hr. The reaction was stopped by heating to 80^{o}C for 10 min. The PCR reaction was carried out using 200ng, (2μl), of reverse transcribed RNA with 0.25mM dNTP, 1μM reverse primer, 1μM forward primer 5μl of 10x Taq polymerase buffer (500mM KCl, 100mM Tris (pH8.8), 15mM $MgCl_2$ and 1.0% Triton X-100) and 2 units of Taq polymerase (Promega). The reactions were run for 30 cycles. Products were visualised on a 2% agarose gel. The primers used for PCR analysis were: species degenerate primers that amplified rat, human and mice m4, Rm4 902s (5' ACCCAG AACACCAAGGAACGGCCA 3') and Rm4 1242a (5' TCACCAGGACCATGACAT TGTAGG 3'). To specifically amplify either rat or human m4 sequences a common sense primer, Hm4 1141s (5' GCTCGCAACCAGGTGCGCAAGAAG 3'), and specific antisense primers, Rm4 1559a (5' GGTACCTCACGGTGTCTGGGAGAC 3') and Hm4 1747a (5' TCGGCCCCCATCCAGATCTCC ATT 3') were used. Primers used to amplify hypoxanthine-guanine phosphoribosyl transferase (hprt) were: hprt 231s (5' CCTGCTGGATTACATTAAAGCACTG 3') and hprt 567a (5' CCTGAAGTACTCATTATAGTCAAGG 3').

Primer extension.

For the primer extension analysis the two m4 specific oligonucleotides Rm4 63aL (5' GAGGTACCCTGCAGGAATTCACTGTCTCCAGGTGGTTGTGGGCT 3') and Rm4 10a (5' GTCACCAGGCGCACAGACTGATTGGCTGAGCTGCCATTGA CAGGCGTG 3') were kinased. 10pmol of oligonucleotide was incubated with 2μl of 10x T4 polynucleotide kinase buffer, (0.5M Tris (pH7.6), 0.1M $MgCl_2$, 50mM DTT, 1mM spermidine and 1mM EDTA (pH8.0)), 20pmol of ^{32}P gamma-dATP (3000Ci/mMol, NEN) and 1μl of T4 polynucleotide kinase in a total volume of 20μl at 37^{o}C for 45 min. The kinase was inactivated by incubating the reaction at 68^{o}C for 10 min. 20μl of 80% formamide/10mM EDTA was added and the oligonucleotides ran on a 12% polyacrylamide gel. After running, the gel was exposed to X-ray film to indicate the positions of the radiolabelled oligonucloetides and a slice of the gel

containing the oligonucleotides was excised. The oligonucleotides were eluted from the gel by shaking at 37°C for 3 hr in 400μl of gel elution buffer, (0.5M NH_4 acetate, 10mM Mg acetate, 1mM EDTA (pH8.0) and 0.1% SDS). An aliquot of the eluted probe was counted to determine the incorporation of radiolabel and $1x10^5$ cpm were added to 2μg of poly A^+ RNA extracted from PC12, NG108-15 or CHO cells. The RNA-primer mixture was precipitated by the addition of 0.1 volumes of 3M Na acetate (pH5.2) and 2.5 volumes of 100% ethanol at -20°C for 30 min and spun at 4°C, 10 000g for 10 min. After washing with 70% ethanol the RNA-primer mixture was suspended in 8μl of water and denatured at 85°C for 10 min. 2μl of 5x annealing buffer, (50mM PIPES (pH6.4) and 2M NaCl), was added and annealing carried out at 52°C for 25 min. 5μl of 5mM dNTPs, 2μl of 10x RTase buffer (500mM Tris-HCl (pH8.3), 400mM KCl, 100mM $MgCl_2$ and 10mM DTT), 0.5μl RNA guard and 0.5μl AMV-RT was added and incubation carried out at 42°C for 1 hr. 0.5μl of AMV-RT was then added and the incubation continued at 52°C for 30 min in an attempt to overcome any secondary structure in the 5' end of the RNA. The cDNA was then phenol/chloroform extracted, precipitated with ethanol and dissolved in 10μl of gel loading buffer. Samples were then denatured for 5 min at 85°C and run on a 7% polyacrylamide gel. After fixing and drying the gel was exposed to X-ray film for up to 10 days.

RACE. - Rapid amplification of cDNA ends.

5' RACE was carried out using a modified procedure of Frohman et al. (1988). PC12 RNA was reverse transcribed using an m4 specific primer, 863a (5' GCTGGACTCATTGGAAGTGTCCT 3'). Reverse transcription was carried out in a total volume of 20μl, containing: 5μl of 5mM dNTPs, 2μl of 10x RTase buffer (500mM Tris-HCl (pH8.3), 400mM KCL, 100mM $MgCl_2$ and 10mM DTT), 0.5μl RNA Guard and 0.5μl AMV-RT, at 42°C for 1 hr and the temperature subsequently increased to 52°C for a further 30 min. The reverse transcription was terminated by incubation at 75° for 10 min. The cDNA was then purified and tailed with dATP as

described by Frohman et al. (1988). The tailed cDNA was subsequently amplified using a d17-adapter primer, RACE-1 (5' GACTCGAGTCGACATCGATTTTTTTTT TTTTTTTT 3'), and an m4 specific primer, Rm4 63a (5' ACTGTCTCCAGGTGGTT GTGGGCT 3'). The PCR amplification was performed in a volume of 50μl containing 1μM of each primer, 0.25mM dNTPs, 5μl of 10x Taq polymerase buffer (500mM KCl, 100mM Tris (pH8.8), 15mM $MgCl_2$ and 1.0% Triton X-100) and 2 units of Taq polymersase. The mixture was denatured at 95°C for 5 min and then put through 30 cycles of: 95°C for 30s, 58°C for 30s and 72°C for 90s. Finally the samples were incubated at 72°C for 5 min. 5μl (1/10) of the amplified product was used in a second amplification under the same conditions as above but using a set of primers internal to the m4 specific product amplified, in order to reduce the amount of non-specific amplification. This second set of primers consisted of an m4 specific primer, Rm4 10a (5' GTCACCAGGCGCACAGACTGATTGGCTGAGCTGCCATT GACAGGCGTG 3') and the adapter primer, RACE-2 (5' GACTCGAGTCGACAT CG 3'). ^{32}P-dATP (6000Ci/mMol), was also included to a final concentration of 10μM to internally label the amplified product. After 30 cycles of PCR the reaction mixture was added to 50μl of G-50 buffer and spun through a G-50 column to remove unincorporated nucleotides. Incorporated radioactivity was determined by Cerenkov counting. The RACE probe was denatured by boiling for 5 min and hybridized to Southern blots containg DNA fragments of the cosmid clone, R3-6. Hybridization and washing conditions were identical to those used for random primed probes (see library screening).

S1 Nuclease mapping.

S1 nuclease mapping was carried out by a slightly modified procedure of Sambrook et al. (1989). 5μg of plasmid DNA was cut with a suitable restriction enzyme which was then digested with proteinase K. The DNA was then extracted with phenol/chloroform and added to 100μg of either PC12 or CHO total RNA. The DNA and RNA were precipitated with ethanol, washed in 70% ethanol and dissolved

in 30μl of hybridization buffer (90% formamide, 400mM NaCl, 40mM PIPES (pH6.4) and 1 mM EDTA). The nucleic acids were denatured at 85^{o}C for 10 min and hybridized overnight at 50^{o}C. 300μl of ice cold nuclease buffer, (40mM K acetate (pH4.6), 340mM NaCl, 1.4mM $ZnSO_4$, 6.8% glycerol, 100 units/ml S1 nuclease and 20μg/ml ssDNA), was added to each tube and the tubes were quickly transferred to 37^{o}C and incubated for 90 min. The samples were then chilled on ice and 80μl of S1 nuclease stop (4M NH_4 acetate, 50mM EDTA (pH8.0) and 10μg/ml tRNA) was added. The samples were phenol/chloroform extracted and precipitated with ethanol at -20^{o}C for 1 hr. After centrifugation at 4^{o}C for 10 min the pellet was redissolved in 10μl of TE and 1μl of gel loading buffer added. The samples were run on a 2% agarose gel which was denatured, neutralized and blotted onto a nylon membrane using standard techniques. The blot was then screened with fragments of the R3-6 clone to determine which contained sequences that were protected and the size of the protected exonic material.

Nested Deletions.

The 5.0 kb Bam HI fragment believed to contain the transcription initiation site for the m4 gene was subcloned into the plasmid pGem 7Zf(+). After purification of this construct by caesium chloride banding, 10μg was digested with the enzymes Cla I and Kpn I to produce a linear DNA molecule containing one end that was resistant and one end that was sensitive to digestion by Exonuclease III. Nested deletions of this construct were then produced using the "Erase-a-base" kit from Promega. Single recombinant plasmids were isolated and the size of the DNA insert in each recombinant determined. A selection of the recombinants were then used in DNA sequencing to determine the sequence of this whole region.

DNA Sequencing.

DNA sequencing of PCR products was performed using the fmol DNA

sequencing kit (Promega). The PCR products were first purified using a Centricon 100 column and sequenced as per manufacturers' instructions.

DNA sequencing of plasmid DNA was performed with a Sequenase kit from United States Biochemicals using double stranded DNA. 4μg of plasmid DNA was diluted to 22μl with TE. RNAse A was added to a concentration of 20μg/ml and the DNA incubated at 37^{o}C for 30 min. 1 pmole of primer, (either T7 or SP6), was added and the DNA denatured by adding 4.8μl of 1M NaOH/10mM EDTA and incubating at 37^{o}C for 30 min. The samples were neutralized with 3.6μl of 2M Na acetate (pH4.5) and transferred to fresh tubes. The template/primer mix was then precipitated with ethanol, washed and dissolved in 8μl of water. Extension and termination reactions were then performed as per manufacturers' instructions using 0.5μl of ^{35}S-dATP (1000Ci/mMol, NEN) per set of sequencing reactions as the radiolabelled nucleotide.

Chapter 3. Results (part 1).

Introduction.

Neuroblastoma cells have been used in many studies as neuronal model systems. As such the neuroblastoma has several advantages over neurons as they provide a homogenous population of cells with which to work. Also, because neuroblastoma cells actively divide and proliferate, as many or as few cells as required can be obtained for each experiment. This lends itself well to a multidisciplinary study as some techniques, such as electrophysiology, require only a few cells whereas other techniques, such as radioligand binding, require substantially more. Neuroblastomas can also be treated in such a way so that they stop proliferating and appear to become more neuronal-like, often referred to as differentiation. Treatments used for this differentiation include: phorbol esters, dimethylsulphoxide (DMSO) (Kimhi et al. 1976), prostaglandin E_1 (PGE_1) (McFadzean et al. 1989), dibutyryl cyclic AMP (Mullaney et al. 1988) and nerve growth factor (NGF) (Jumblatt, and Tischler, 1982). Generally these treatments result in an inhibition of mitosis and the extension of neurites. Other properties of the cell may also be altered by this treatment. For instance the muscarinic receptors on PC12 cells (a rat phaeochromocytoma, included here because it differentiates into a neuronal-like cell on treatment with NGF) are seen to increase about 4 fold with NGF treatment (Jumblatt and Tischler, 1982). The muscarinic receptors present in the mouse neuroblastoma NIE 115 cell line are also seen to increase when these cells are treated with DMSO. However the extra muscarinic receptors are not expressed on the surface of the cell, which may be due to only a limited differentiation caused by DMSO treatment.

One of the best characterized neuroblastomas is the NG108-15 cell line (Hamprecht, 1977). This cell line is a hybrid cell line between the mouse neuroblastoma, N18TG2, and a rat glioma, C6BU-1 (Klee and Nirenberg, 1974) and the cells are comparable to sympathetic ganglia neurons to which the N18TG2 parent is related embryologically. The NG108-15 cell line also displays characteristics of neuronal cells when treated with prostaglandin E_1 (PGE_1) and IBMX or with

dibutyryl cAMP. This resemblance to neurons on PGE_1 treatment is manifest both morphologically and physiologically. The most marked morphological characteristic is the extension of processes which is also accompanied by a change in the appearance of the cell body which becomes more rounded and phase bright. Physiologically this differentiation is accompanied by the capability of the cell to generate trains of action potentials in response to depolarization and the appearance of several ion channels such as the nifedipine sensitive and conotoxin sensitive calcium channels, the calcium activated potassium channel and the potassium channel responsible for the M-current (Docherty et al. 1991). These channels are generally found only on neuronal cells suggesting the appearance of these channels in NG108-15 cells is an indication of a change in the cell line to a more neuronal phenotype. It has been reported that the NG108-15 neuroblastoma x glioma hybrid cell line expresses only one subtype of muscarinic receptor, that encoded for by the m4 gene (Peralta et al. 1987). The effect of PGE_1 or dibutyryl cAMP differentiation on the level of muscarinic receptors in these cells has not been determined; however, the levels of δ-opioid receptor has been reported to fall to approximately 50% of control levels after dibutyryl cAMP treatment (Mullaney et al. 1988). Work has also been carried out on two G-protein α subunits expressed in NG108-15 cells, G_o and G_i. It is thought that G_o is the G-protein responsible for the inhibition of Ca^{2+} currents seen on stimulation of α-adrenergic receptors (McFadzean et al. 1989) and opiod receptors (Hescheler et al. 1987). G_i has been implicated in inhibition of adenylyl cyclase observed by the activation of the m4 muscarinic receptor and the δ-opioid receptor (Mulaney et al. 1990; McKenzie et al. 1990). There is also evidence that the α-adrenergic receptor in NG108-15 cells can interact with G_i (McClue et al. 1990). Differentiation of these cells results in reciprocal regulation of these two G-protein subunits. The levels of G_o increase by approximately four fold while the levels of G_i decrease by two to three fold (Mullaney et al. 1988). None of these observations have been seen with NG108-15 cells that have not been treated with either PGE_1 or dibutyryl cAMP even under conditions of serum withdrawal. This cell line thus appears to be a good model system in which to study the neuronal regulation of the m4 muscarinic receptor gene and the

receptor for which it encodes.

Results.

Northern blot analysis on cell lines.

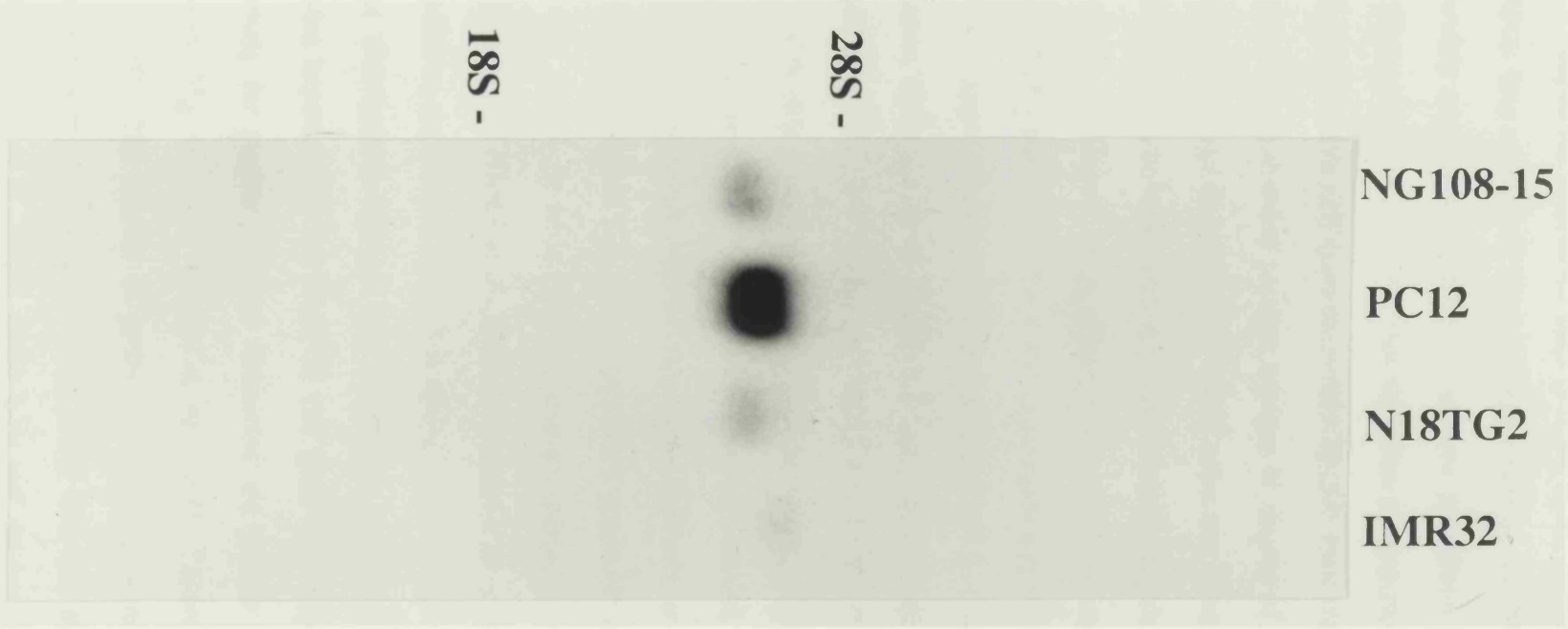
Initially a Northern analysis was performed on total RNA extracted from the four cell lines: PC12, NG108-15, N18TG2 and IMR32 to determine if these cell lines expressed m4 transcripts. The results of this analysis are shown in figure 3.1 where it can be seen that each of the cells do express the m4 transcript. The highest levels of m4 mRNA are expressed in PC12 cells with IMR32 cells expressing the lowest levels. NG108-15 and N18TG2 cells express similar levels of m4 transcripts which are intermediate between the levels expressed in PC12 and IMR32 cells. The IMR32 cell line is a human neuroblastoma and therefore expresses the human m4 transcript. It can be seen in figure 3.1 that this transcript is larger than either the rat m4 transcript, expressed in PC12 cells, or the mouse m4 transcript, expressed in N18TG2 cells. The mouse and the rat m4 transcripts appear to be of similar size and any slight size differences between the two are not resolved on this Northern blot. As the sizes of the rat and mouse m4 transcripts are very similar this does not distinguish whether the NG108-15 mouse/rat hybrid cell line expresses the rat or the mouse m4 transcript.

Pharmacological characterization.

The receptor present on the NG108-15 cell line was pharmacologically characterized using radioligand binding and antagonist competition studies. As the NG108-15 is a hybrid cell line between a mouse neuroblastoma and a rat glioma the m4 gene could be derived from either the mouse or rat parent. Therefore the mouse neuroblastoma parent of the NG108-15 cell line, the N18TG2 cell line, was also characterized as this also expresses the m4 gene (figure 3.1). Results of saturation binding experiments using ^{3}H-N methylscopolamine (^{3}H-NMS) are shown in figure 3.2. Binding to NG108-15 cells gave a dissociation constant (K_D) for NMS of 128 pM $\pm$ 9.9, n=4 whilst binding to N18 TG2 cells gave a K_D for NMS of 40 pM $\pm$

Figure 3.1 Northern blot analysis of cell lines.

This figure shows the results obtained from a Northern blot analysis on 15μg of total RNA from four cell lines (PC12, N18TG2, NG108-15 and IMR32) probed with a mixture of 3 tailed oligonucleotides (Rm4A, Rm4B and Rm4C - see Materials and Methods). After hybridization and washing, the gel was exposed to X-ray film for one week. The 3 oligonucleotides used are species degenerate between human and rat m4 sequences but are specific for the m4 subtype. Positive hybridization signals can be seen for all four cell lines with the highest levels of m4 being expressed in the PC12 cell line. The human cell line, IMR32, shows hybridization of a higher molecular weight mRNA because the human m4 mRNA is larger than the the rat m4 mRNA.

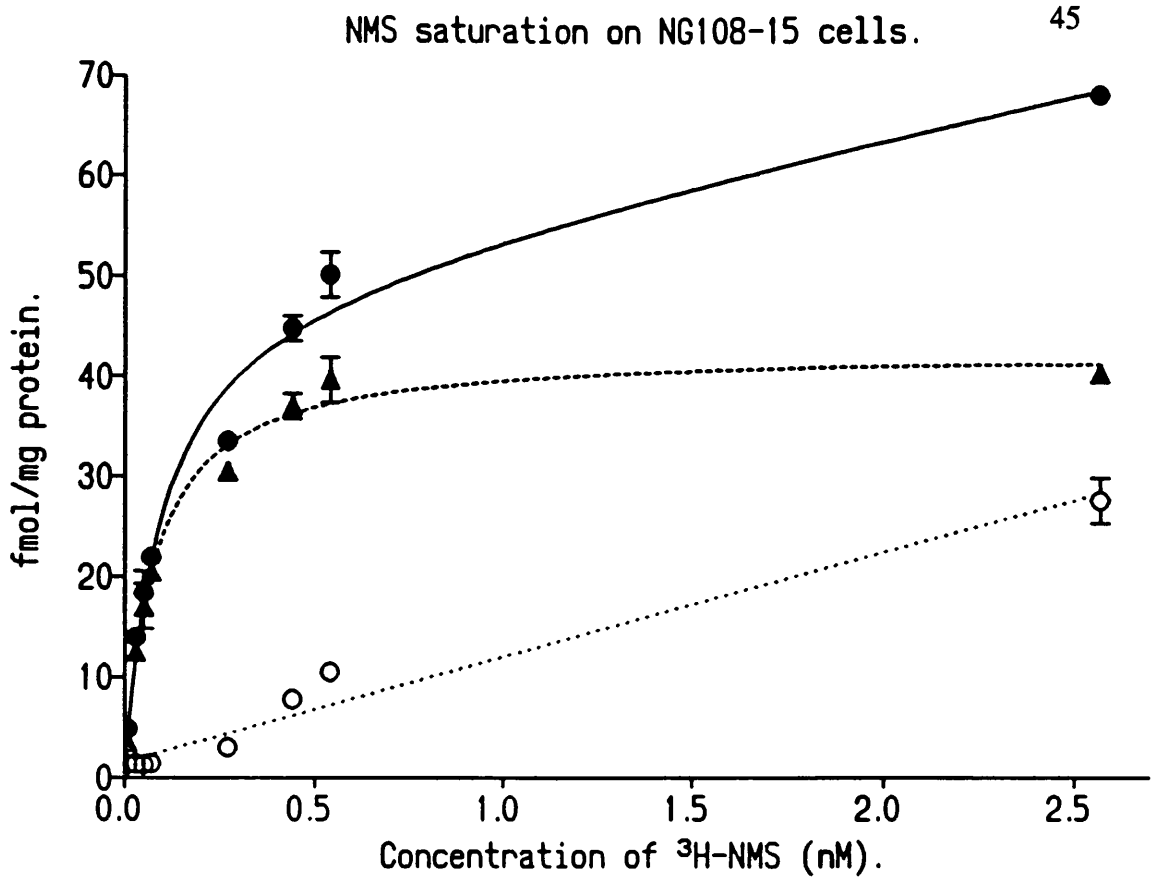

18S -
28S -
NG108-15
PC12
N18TG2
IMR32

6.2, n=4. Both of these values are consistent with previous published results, which range between 25 - 260pM for NG108-15 cells (Michel et al. 1989; Ray et al. 1989; Lazareno et al. 1990). The number of receptors expressed by the NG108-15 cell line was seen to vary widely on routine culturing as has been previously reported by Klein et al. (1979) and Baumgold et al. (1989). This variation ranged from 3000 receptors per cell to 30 000 receptors per cell (approximately 20-200 fmol/mg of protein), and did not appear to be a result of either passage number or degree of confluence of the cells when harvested. Different dishes of NG108-15 cells that were derived from the same passage and treated in the same way did show identical levels of receptor (data not shown) as has been previously noted (Klein et al. 1979; Baumgold et al. 1989). Upon treatment with PGE_1 and IBMX the levels of receptor expressed by the NG108-15 cells did stabilize at approximately 3000 receptors per cell (see later). Logarithmic plots of the saturation data, shown in figure 3.3, gave Hill coefficients of 0.97 $\pm$ 0.07, n=4 and 1.02 $\pm$ 0.06, n=4 for the NG108-15 and N18TG2 cells respectively which is consistent with only one subtype of receptor being present in these cell lines.

A further pharmacological characterization was carried out using three muscarinic antagonists (pirenzepine, himbacine and methoctramine) and the results of these studies are shown in figure 3.4. The dissociation constants obtained for these three antagonists are shown in table 3.1 and are compared to values previously reported for the M_4 receptor (Dorje et al. 1990; Lazareno et al. 1990; Waelbroeck et al. 1990). The inhibition curves and antagonist dissociation constants show that these antagonists do not discriminate between the muscarinic receptors expressed in the NG108-15 and those expressed in the N18TG2 cell line. The dissociation constants obtained in this study for pirenzepine and himbacine are similar to those reported previously for other M_4 receptors (Dorje et al. 1990; Lazareno et al. 1990; Waelbroeck et al. 1990). The affinity of methoctramine in this study for these two cell lines however is between three and eight fold lower than that previously reported for NG108-15 cells (Michel et al. 1989), rabbit lung and chicken heart (Lazareno et al. 1990), but is close to that reported for a cloned human m4 receptor gene expressed in CHO cells (Dorje et al. 1990). This difference in methoctramine affinity between

Figure 3.2. Saturation binding studies on NG108-15 and N18TG2 cells.

This figure shows the results obtained from a saturation binding study on NG108-15 cells (upper graph) and N18TG2 cells (lower graph) using the radioligand ^{3}H-N-methylscopolamine (3H-NMS). The binding studies were carried out as described in Materials and Methods. Each graph shows a representative experiment and the results obtained for total binding (●), non specific binding (in the presence of 1μM atropine (○)) and specific binding (▲) are plotted. Each data point was determined in triplicate, the error bars represent standard error of the mean and where not shown fall within the limits of the symbol. Data for total binding was fitted by the least squares procedure to $y = K_D \times [NMS](B_{max} + [NMS]) + E \times [NMS]$, where K_D represents the dissociation binding constant for NMS, B_{max} represents the total concentration of binding sites and E represents the gradient of the non specific plot. Non-specific data was fitted by linear regression. Specific data was fitted to the equation $y = K_D \times [NMS](B_{max} + [NMS])$.


NMS saturation on NG108-15 cells.
fmol/mg protein.
70
60
50
40
30
20
10
0
0.0
0.5
1.0
1.5
2.0
2.5
Concentration of ^{3}H-NMS (nM).

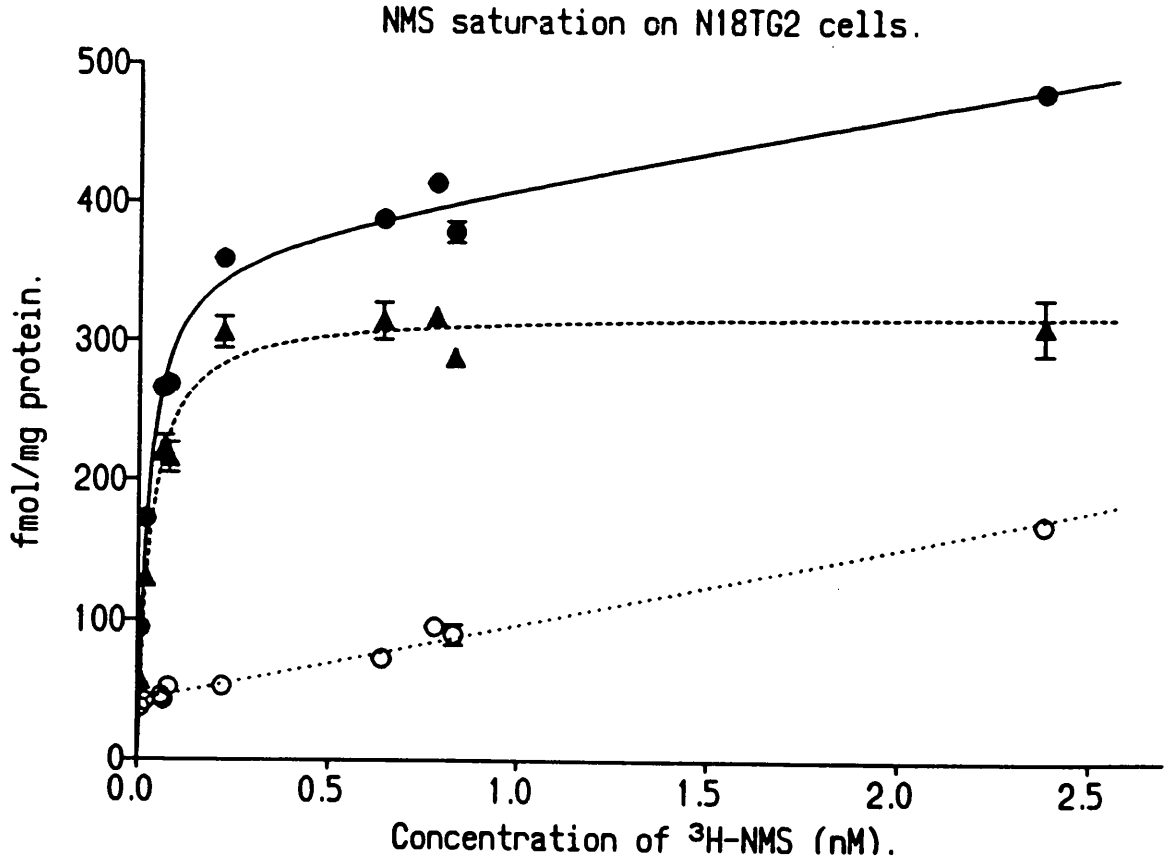
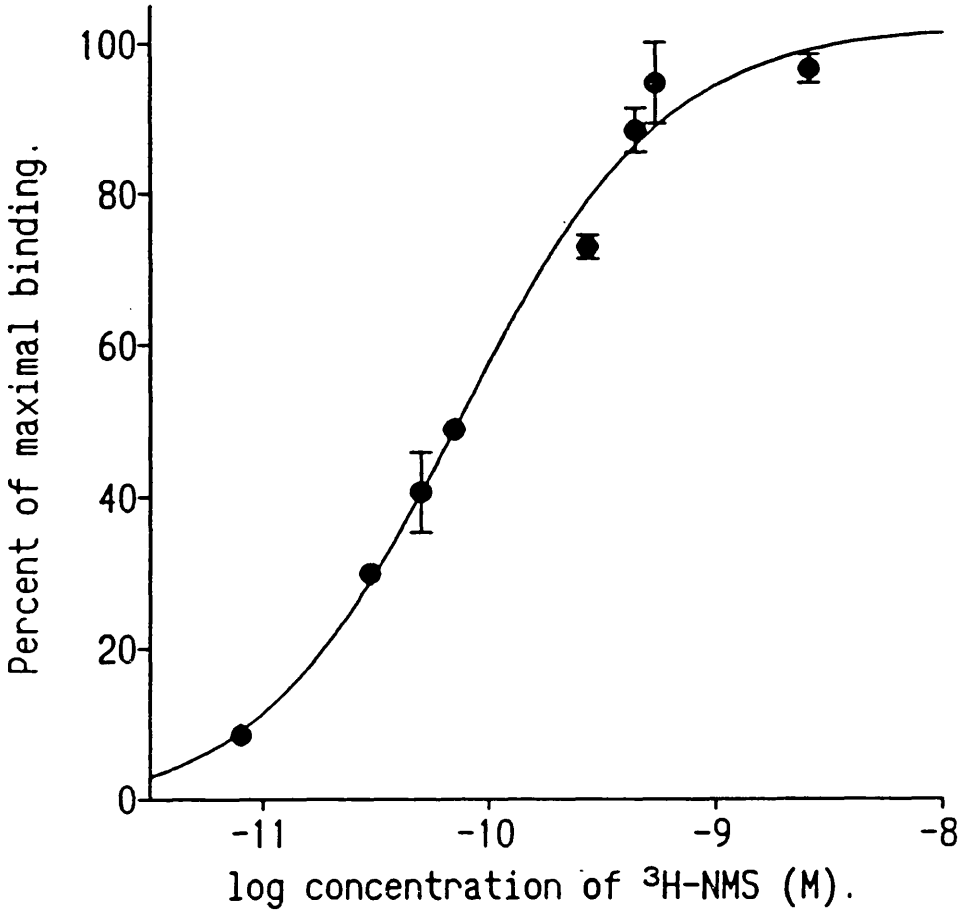

NMS saturation on N18TG2 cells.
fmol/mg protein.
500
400
300
200
100
0
0.0
0.5
1.0
1.5
2.0
2.5
Concentration of ^{3}H-NMS (nM).

Figure 3.3. Determination of Hill numbers for NMS saturation data.

Shows the results of saturation binding studies on NG108-15 cells (upper graph) and N18TG2 cells (lower graph). The results are plotted on a logarithmic scale against percentage of binding of B_{max}. Data was then fitted to the Hill equation. Data from one representative experiment is shown with each point being determined in triplicate. The error bars represent standard error of the mean and where not shown fall within the limits of the symbol. The Hill slopes for the NG108-15 and N18TG2 cells were 0.97 ± 0.07 and 1.02 ± 0.06 respectively which is consistent with one population of muscarinic receptor expressed in each cell line.

NMS saturation on NG108-15 cells.



NMS saturation on N18TG2 cells.

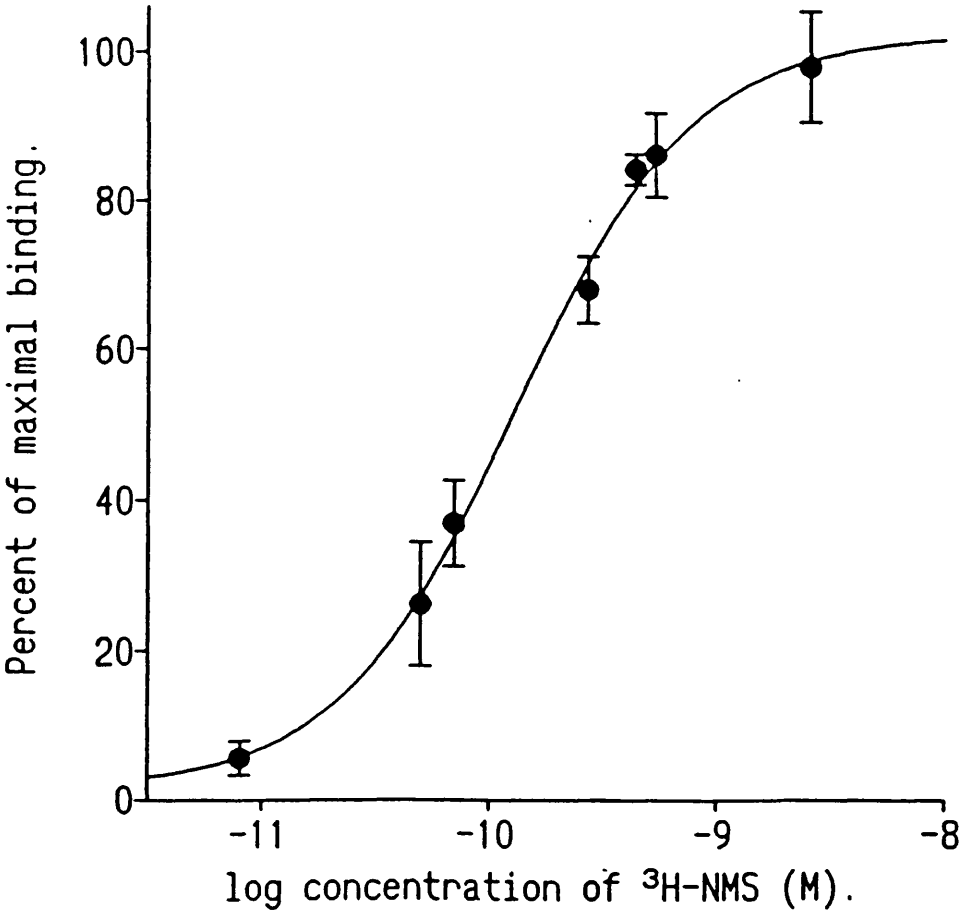
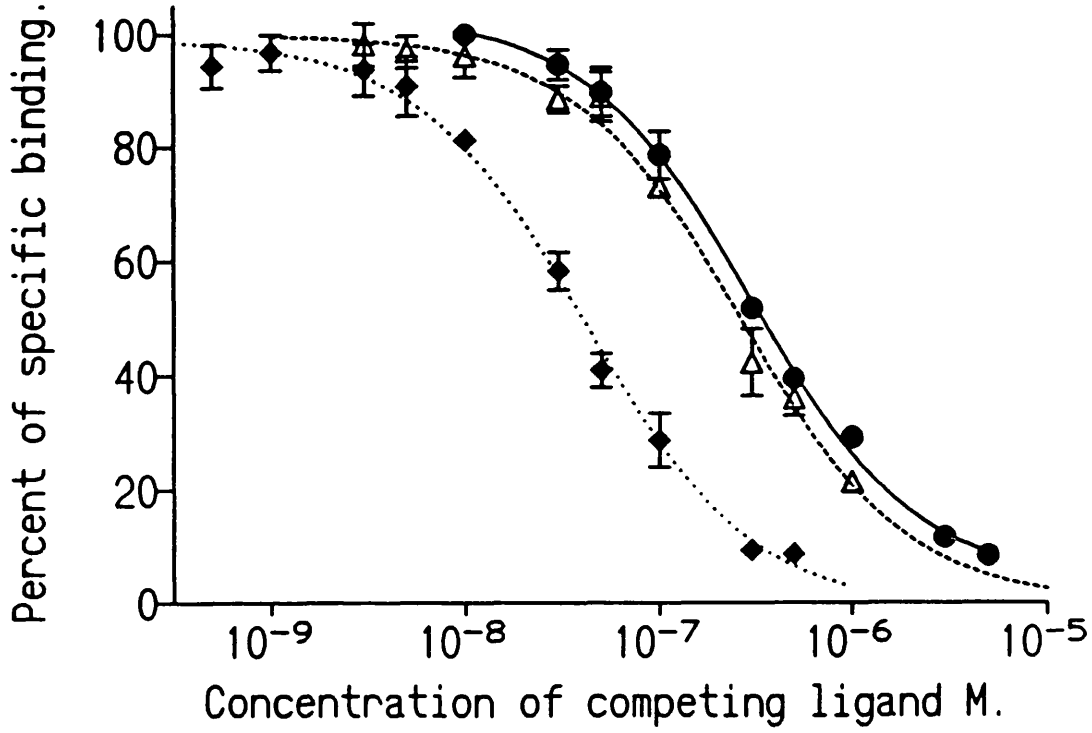


Figure 3.4. Results of competition binding studies with the three antagonists: pirenzepine, methoctramine and himbacine.

Shows the results obtained from antagonist competition binding studies using the antagonists: pirenzepine (●), methoctramine (△) and himbacine (◆). Non-specific binding was subtracted from total binding and the data expressed as a percentage of binding of ^{3}H-NMS in the absence of competing ligand. IC_{50} values were computed from the least squares fit to the expression $y = 100 - [\ 100xB/(A + IC_{50})\ ]$, where y = % specifically bound counts of ^{3}H-NMS and A = concentration of antagonist. ^{3}H-NMS occupancy was corrected for using the Cheng and Prussof equation (1973), $K_I = IC_{50}/(1 + B/K_B)$, where K_I = dissociation equilibrium constant for the competining antagonist, B = concentration of ^{3}H-NMS and K_B = dissociation equilibrium constant for ^{3}H-NMS.

Data for single representative experiments are shown in the figure, each data point represents the mean of triplicate samples, the error bars show the standard errors of the mean and where not shown fall within the limits of the symbol.

Competition binding results of the three ligands against ^{3}H-NMS on NG108-15 cell membranes.



Competition binding results of the three ligands against ^{3}H-NMS on N18TG2 cell membranes.

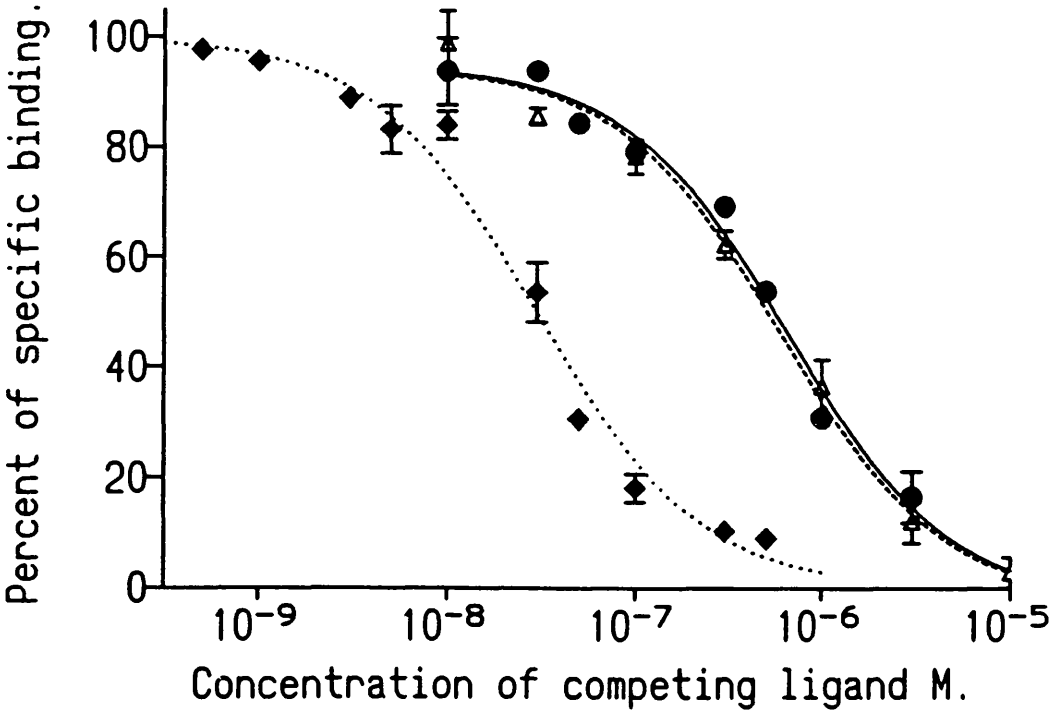


Table 3.1. Comparison of antagonist dissociation constants obtained in this study with previously reported values on M_4 receptors.

	N18TG2	NG108	NG108^o	Rabbit lung*	Chicken heart*	Human m4+
Pzp.	6.94 ±0.17 n=3	7.19 ±0.18 n=4	7.1 ±0.04	7.55 ±0.05	7.60 ±0.03	7.43 ±0.05
Himb.	8.43, 8.02 n=2	8.11 ±0.10 n=3	8.02 ±0.04	8.47 ±0.05	8.20 ±0.07	7.96 ±0.05
Meth.	7.18 ±0.18 n=4	7.21 ±0.15 n=4	7.65 ±0.11	7.86 ±0.11	7.86 ±0.05	7.50 ±0.04

The above table shows the values of dissociation constants (expressed as log dissociation constant, $pK_I \pm$ sem.) obtained from the competition binding studies. The first two columns show the values obtained in this study for N18TG2 and NG108-15 cells respectively for pirenzepine (pzp), himbacine (himb) and methoctramine (meth). The remaining four columns show values obtained for the three antagonists in NG108-15 cells (o) (Waelbroeck et al. 1990), rabbit lung, chicken heart (*) (Lazareno et al. 1990) and a cloned human m4 gene expressed in CHO cells (+) (Dorje et al. 1990). All of the previously reported values shown here have been assigned to the M_4 muscarinic receptor subtype.

different studies is unlikely to be due to the presence of other receptor subtypes because the inhibition curves were best fit to a one-site model in both cell lines. A possible explanation for the wide range in previously reported methoctramine dissociation constants may be allosteric interactions of this ligand with the receptor as has been proposed by Waelbroeck et al. (1990).

The NG108-15 cell line is a hybrid cell line between the mouse N18TG2 and the rat C6BU-1 and therefore contains both the rat and mouse m4 genes within its genome. Furthermore both the N18TG2 and C6BU-1 express m4 mRNA (figure 3.1 and N.J.Buckley unpublished observations). It is conceivable that the m4 mRNA expressed in the NG108-15 cell line could be the rat, the mouse or a mixture of both m4 mRNAs. To determine the species origin of the m4 receptor in the NG108-15 cell line two portions of the m4 gene were amplified using PCR and sequenced. The two parts of the m4 gene chosen for this were a section encoding part of the i3 loop of the receptor and a section of 3' untranslated sequence. These areas of the gene were chosen because they contain the most divergence between human and rat sequences. The amplified products were then sequenced as outlined in Material and Methods. Alignment of the rat nucleotide sequence with the mouse nucleotide sequence, obtained from the N18TG2 cells, showed 97% identity (figure 3.5). The differences between the sequences result in an amino acid substitution of a threonine in the mouse in place of an alanine in the rat. A threonine at this position, rather than an alanine, is also present in the human sequence. As well as the amino acid substitution the mouse m4 sequence also contains an extra alanine residue which is not present in either the rat or in the human m4 sequence. The sequence obtained from the NG108-15 cell line was identical to that obtained from the N18TG2 cell line indicating that the muscarinic receptor expressed in the NG108-15 cell line is of mouse origin.

PGE_1 stimulation.

Studies were next performed to determine if the effect of PGE_1 differentiation of the NG108-15 cells had any regulatory consequences for the expression of the m4 mAChR. PGE_1 activates the enzyme, adenylyl cyclase and

Figure 3.5 DNA sequence comparison between sequences in the rat and mouse m4 genes.

This shows a sequence comparison between DNA sequence obtained from the rat and the mouse m4 genes. The numbering shown at the right corresponds to the rat m4 sequence with the A nucleotide in the initiating ATG taken as 0. The upper sequence shown extends from nucleotide 895 to nucleotide 1019 of the rat m4. The nucleotides present in the mouse gene are only shown where they differ from the rat. The amino acids encoded for by the nucleotides are shown with the rat amino acids preceding the mouse where different.

Rat m4	AGT	GCC	ACC	CAG	AAC	ACC	AAG	GAA	917
Mouse m4									
	Ser	Ala	Thr	Gln	Asn	Thr	Lys	Glu	
	CGC	CCA	CCC	ACA	GAG	CTG	TCC	ACC	941
	Arg	Pro	Pro	Thr	Glu	Leu	Ser	Thr	
	GCA	GAG	GCC	- - -	ACC	ACT	CCA	GCG	962
	A			GCC		A			
	Ala/Thr	Glu	Ala	Ala	Thr	Thr	Pro	Ala	
	CTG	CCT	GCC	CCT	ACC	CTG	CAG	CCA	986
	Leu	Pro	Ala	Pro	Thr	Leu	Gln	Pro	
	CGA	ACC	CTC	AAC	CCA	GCC	TCC	AAA	1010
	Arg	Thr	Leu	Asn	Pro	Ala	Ser	Lys	
	TGG	TCC	AAG						1019
	Trp	Ser	Lys						

produces a rapid rise in the intracellular level of cAMP which then reaches a plateau after 10-15 minutes (Hamprecht, and Schultz, 1973; Sharma et al. 1975). It has been shown that the levels of muscarinic receptor in porcine thyroid cells increase when the cells are stimulated with a similar prostaglandin, PGE_2. The subtype of muscarinic receptor expressed in these cells has not been determined but the receptors are coupled to inhibition of adenylyl cyclase so it is likely that the subtype is either m2, m4 or a mixture of the two.

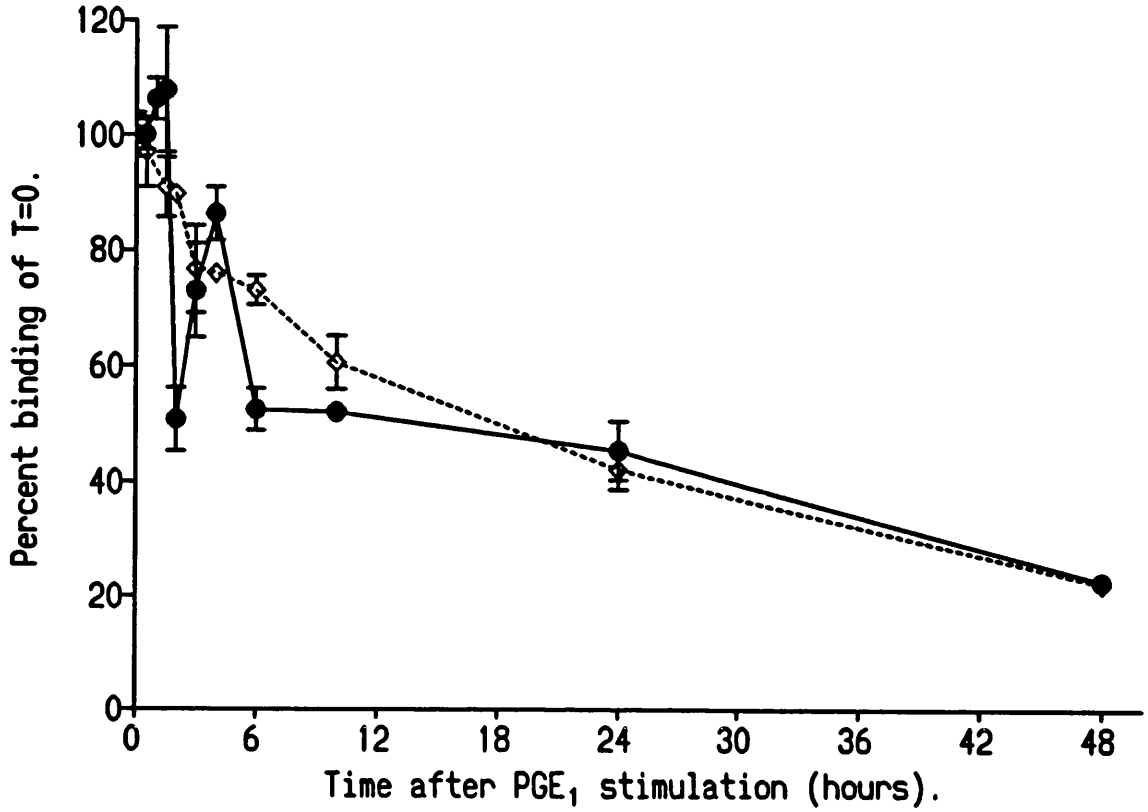
The results of these studies are shown in figures 3.6 and 3.7 where it can be seen that the levels of muscarinic receptor, on the NG108-15 cells, steadily falls to about 20% of control levels after 48 hours in both PGE_1 treated and control cells. This would tend to suggest that PGE_1 itself does not have any direct affect on mAChR number but by withdrawing serum and reducing mitosis the levels of mAChR stabilize at a basal level.

Normal cycling of the cells through the cell cycle during routine culture may lead to the huge variation of mAChR levels observed. When the cell cycle is stopped and the cells differentiated, mAChR reaches a stable base line that is reproducible between experiments. Indeed in a single experiment, stimulation of NG108-15 cells, that had been serum deprived for several hours, with FCS resulted in an increase in receptor number with time (data not shown). However this was not repeated as it did not appear worth pursuing.

Carbachol stimulation.

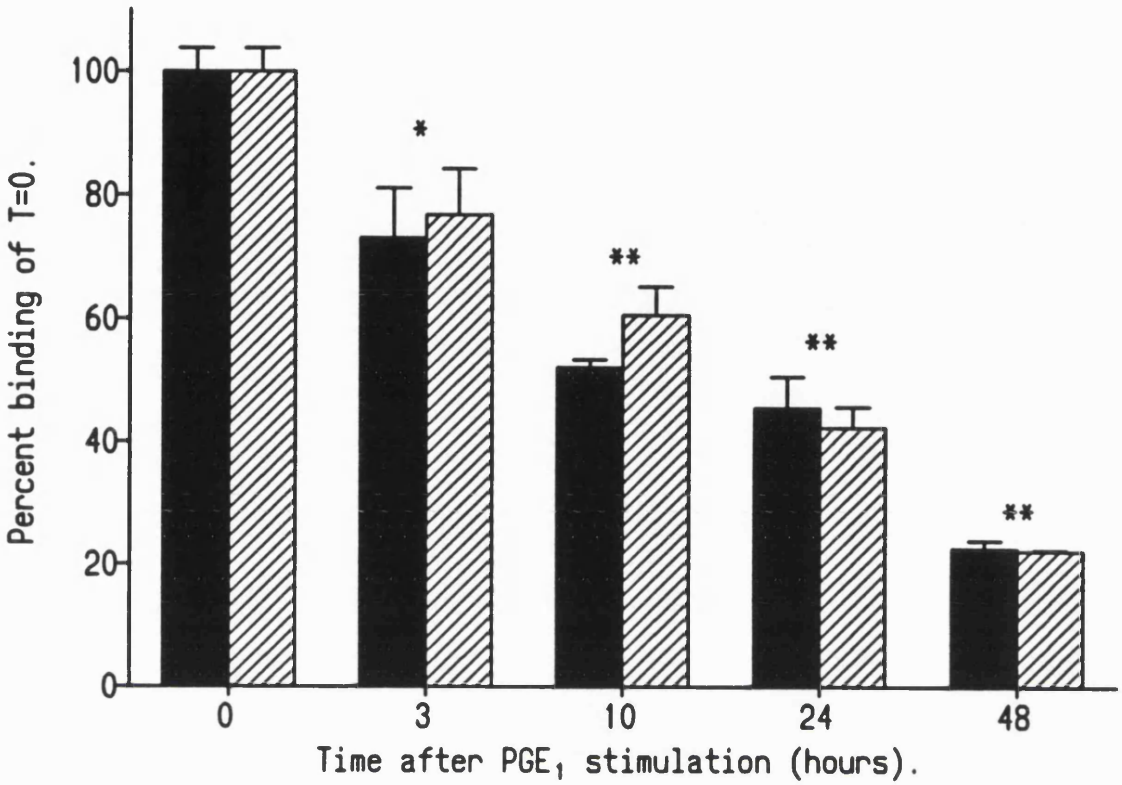
Several studies have shown that stimulation of the NG108-15 cell line with the muscarinic agonist carbachol results in a down-regulation of receptors with time to a basal level of approximately 20% (Maloteaux et al. 1983; Ray et al. 1989). All of these studies have been carried out on actively dividing, undifferentiated cells. Therefore this regulation may be due to an inhibition of mitosis and be mimicked by serum withdrawl and PGE_1 treatment. In order to determine if this regulation of receptor number was occuring via a convergent pathway or if different mechanisms were involved, NG108-15 cells that had been treated with PGE_1 for 48-72 hr were

Figure 3.6. Effect of PGE_1 stimulation on NG108-15 cells.



Shows the level of muscarinic receptors expressed by NG108-15 cells which have been stimulated with PGE_1 (◇), compared to control cells (●), over a period of 48 hr. All cells were cultured in normal medium containing 1% FCS. Cells were treated with PGE_1 and IBMX as described in Materials and Methods. The data points shown represent values obtained from 4 independent experiments each performed in triplicate. Error bars represent standard error of the mean and where not shown fall within the limits of the symbol.

Figure 3.7. Muscarinic receptor levels in control and PGE_1 stimulated NG108-15 cells.

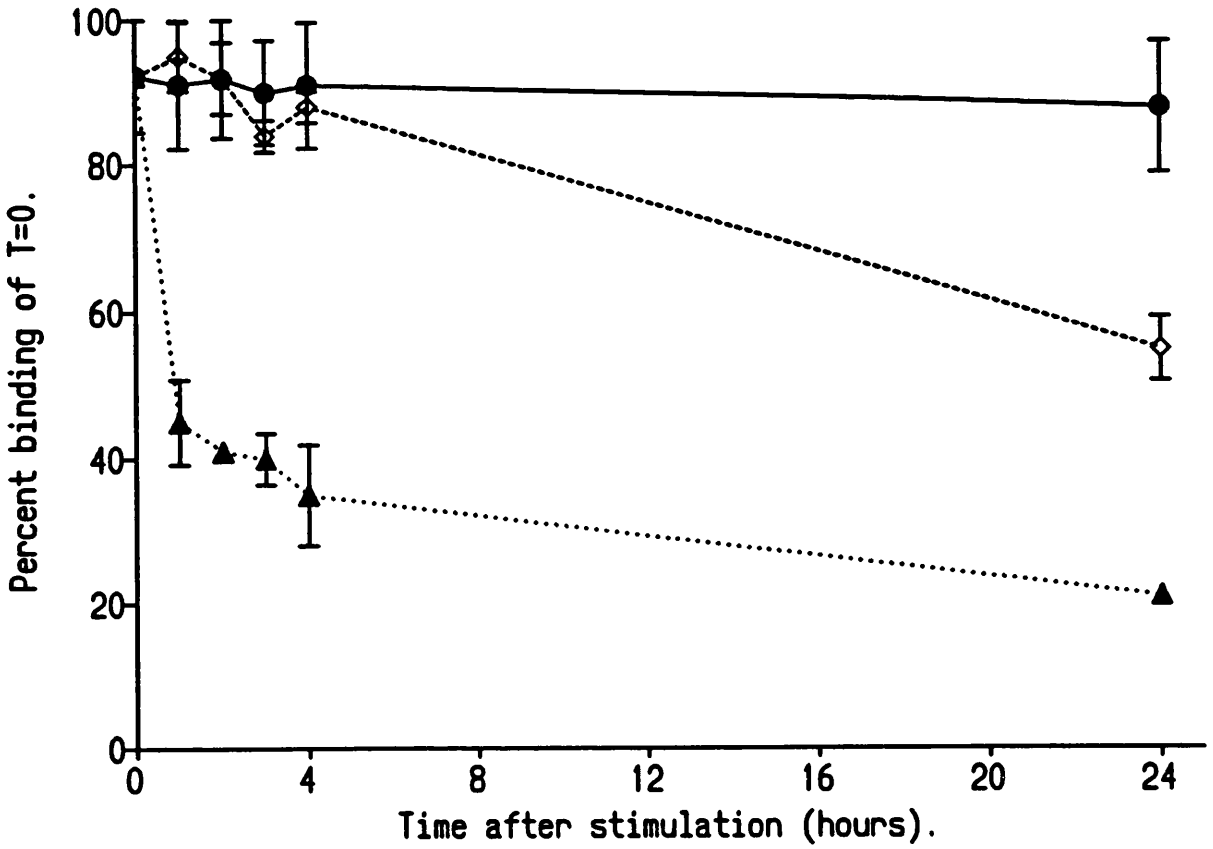


The above figure shows the level of muscarinic receptors on NG108-15 cells after stimulation with PGE_1 and IBMX at various time points. Data is expressed as a percentage of the binding of cells at T=0. Shaded bars represent control cells and hatched bars represent PGE_1 stimulated cells. For any time point the difference between the levels in untreated and PGE_1 treated cells was not significant as measured by an unpaired two tailed t-test. Levels of receptor at different time points were however found to be significantly different from the levels at T=0. (* $p < 0.05$ and ** $p < 0.01$).

subsequently cultured in the presence of carbachol and receptor number determined over time. Figure 3.8 shows the results obtained by incubating the NG108-15 cells in the presence of two concentrations of carbachol. 100μM of carbachol was used as this gave a maximal inhibition of adenylyl cyclase activity in NG108-15 cells in a study by Maloteaux et al. (1983). In the same study 1μM of carbachol gave a response that was approximately 20% of maximal and was used here to determine if the results obtained in this study were concentration dependent. Figure 3.8 shows that both 100μM and 1μM carbachol are capable of producing a loss of muscarinic receptors from the cell surface. This loss is concentration dependent as 100μM carbachol results in a rapid (< 1 hr) and maximal loss of muscarinic receptors to a basal level of approximately 20%. 1μM carbachol is also capable of reducing the level of muscarinic receptors but this occurs over a longer time course and the receptor level is still not reduced to basal level after 48 hr but the levels of receptor may still be falling.

The levels of ^{3}H-NMS binding to cells incubated in the presence of atropine, to block the effect of carbachol through the muscarinic receptors, were found to be no higher than non-specific binding. This was probably due to the continued presence of atropine in the samples even after extensive washing of the cells (6 washes at 10 min each wash). This may also have been a problem in other binding studies done on NG108-15 cells incubated in the presence of atropine (Maloteaux et al. 1983; Ray et al. 1989; Baumgold et al. 1989). Part of the problem with using atropine is that it is a weak base and will therefore tend to accumulate inside the cells during the time it is present in the culture. When the cells are harvested the atopine bound to the surface of the cells may be removed fairly quickly but the atropine present inside the cell is not so easily removed and thus presents a problem during the binding assay. This problem has been overcome in some studies where membrane homogenates are prepared and washed to eliminate atropine (Nathanson et al. 1978; Klein et al. 1979; Green and Clark, 1982). In each of these studies the effect of carbachol on NG108-15 cells was completely blocked by the presence of atropine indicating that these effects were mediated solely through the muscarinic receptors.

Figure 3.8. Effect of carbachol stimulation of NG108-15 cells.



Shows the levels of muscarinic receptors after carbachol stimulation over a 24 hour period. Cells were cultured in the presence of either 100μM carbachol (▲) or 1μM carbachol (◇) or without carbachol (●). After harvesting the cells were resuspended and washed three times in incubation buffer (see Materials and Methods), and finally suspended to a concentration of $1x10^6$ cells/ml. Data points shown represent the mean ± sem of triplicate samples and are expressed as a percentage of binding of cells at T=0.

Summary.

The data presented in this chapter show that a range of neuronal cell lines has been identified that express m4 mRNA. Three of these cell lines (NG108-15, N18TG2 and PC12) do not express any other muscarinic receptor subtypes. Binding studies on the NG108-15 and N18TG2 cell lines has shown that the pharmacology of the receptors expressed on these cell lines is similar to that previously reported for M_4 receptors. DNA sequence analysis has also shown that both of these cell lines express the m4 mRNA derived from the mouse m4 gene.

Further studies on the regulation of levels of muscarinic receptors expressed by NG108-15 cells has shown that levels of receptor vary during routine culturing but inhibition of mitosis results in a reduction and stabilization of receptor levels. The receptors which are expressed afterdifferentiationof the NG108-15 cells with PGE_1, which results in a more neuronal like morphology and physiology, are amenable to regulation by activation with the muscarinic agonist, carbachol.

Chapter 4. Results (part 2).

Introduction.

cDNAs encoding each of the 5 known muscarinic receptors were cloned several years ago, (Kubo et al. 1986; Bonner et al. 1987; Peralta et al. 1987a; Peralta et al. 1987b; Bonner et al. 1988), and shown to have distinct patterns of expression within the CNS (Buckley et al. 1988; Vilaro et al. 1990). However, no-one has yet reported the isolation or characterization of the genomic elements that are responsible for the regulation of the expression of these genes. In fact, of the several hundred members of the G-protein coupled receptor superfamily, only the regulatory elements of three genes, the ß2-adrenergic, the D_{1A} and the D_2 receptor have been identified (Emorine et al.1987; Minowa, M. et al.1992; Minowa, T. et al. 1992; Buck et al. 1992). Genomic regulatory sequences for many other different genes have been identified and several patterns have emerged through these analyses. Generally, the transcriptional control elements are located within several kb 5' to the transcription start site (Maniatis et al. 1987; Vidal et al. 1990; Minowa, M. et al. 1992), although further elements may also be located in the intronic regions (Vidal et al. 1990; Begemann et al. 1990; Whiting et al. 1991) or in the 3' untranscribed regions (Vidal et al. 1990; Puschel et al. 1991; Whiting et al. 1991). Also, the region immediately 5' (<100 bp) to the transcription start site appears to contain a basal promoter through which the level of transcription is modified by a combinatorial action of enhancer and repressor elements (Maniatis et al. 1987).

Identifying the genomic elements regulating the expression of a particular gene is greatly facilitated by first identifying the position of the transcription initiation site. Part of the problem in identifying genomic sequences that are responsible for the regulation of the muscarinic receptor genes is a lack of full length cDNA clones which would enable the position of the transcription start site of these genes to be determined. The coding region for each of the muscarinic receptors is contained within a single exon (Bonner et al. 1987; Peralta et al. 1987a; Peralta et al. 1987b), however evidence indicates that there are additional exons which contain 5' untranslated sequences for each of the genes (Bonner et al. 1987; Peralta et al. 1987b;

Bonner, 1989). Sequences present in these upstream exons have only been determined for one subtype, the m2 gene (Peralta et al. 1987b). cDNA clones obtained from a porcine atrium library showed that two alternative upstream exons, (1A and 1B), are used to generate two different m2 transcripts. cDNA clones obtained that contained sequence 5' to the splice site in the coding exon were seen to contain either exon 1A or exon 1B, but never both (Peralta et al. 1987b). The position of these exons within the genome in relation to the coding exon has not been reported. Sequence comparisons, between porcine m1 cDNA and rat genomic DNA containing the m1 gene, have led to suggestions that the m1 gene contains a cluster of 3 5' exons in a 2 kb region of DNA. Each of these exons are believed to be between 90-170 bp in size and they are separated from the coding exon by approximately 10 kb of DNA (Bonner, 1989). Cross hybridization studies between the human and rat m4 genes has indicated a region, about 10 kb from the coding exon, that shows some conservation, leading to suggestions that this region may contain the 5' untranslated sequences and the promoter region for this gene (Bonner, 1989).

To date only the coding exon for the m4 gene has been cloned which is 2.6 kb in size and extends from a putative splice acceptor site, 30 bp upstream of the translation initiation codon, to the polyadenylation site 2614 bp downstream from the initiation codon. Hybridization of Northern blots, with m4 specific probes, have indicated an m4 mRNA size of between 3.0-3.3 kb (Bonner et al. 1987; Peralta et al. 1987b; Bonner, 1989; Lazareno et al. 1990) indicating that there are approximately 300-600 bp of 5' sequence missing, depending on the exact length of the poly $(A)^+$ tail for these transcripts. It is not known however, if this sequence is encoded in one or in multiple exons.

Results.

Cloning of the m4 gene.

In order to isolate a clone containing the m4 gene, a rat genomic library constructed in the cosmid vector pTCF (Grosveld et al. 1982) was screened. The only

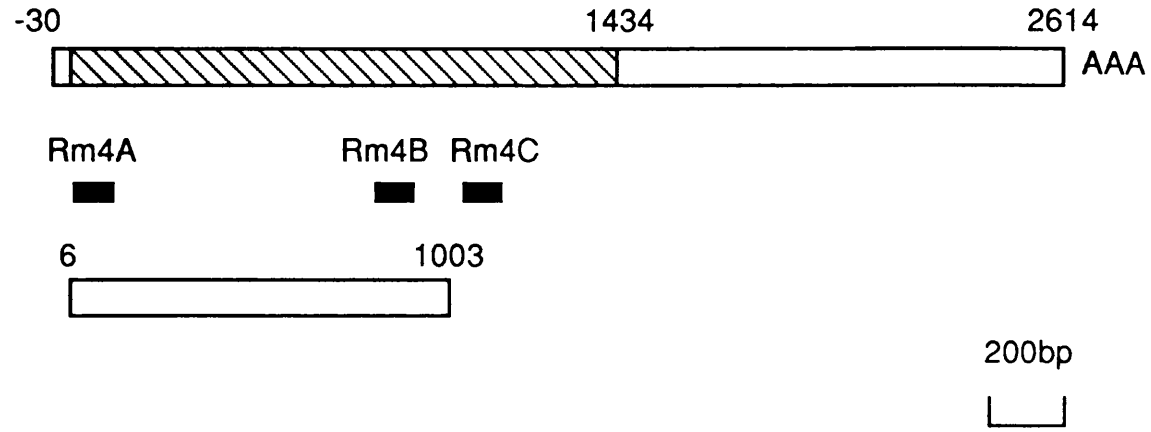
sequence known for the m4 gene, and thus the only sequence that could be used as probes for library screening, is that of the coding exon, which has been proposed to be approximately 10 kb downstream from the transcription start site (Bonner, 1989). A 13 kb genomic DNA fragment could therefore potentially contain both the coding exon and the transcription start site for the m4 gene. Sequences 5' to the transcription start site have usually been found to contain sufficient regulatory elements to drive cell and tissue specific expression and the size of these genomic elements has been seen to vary between 0.3-7.0 kb of DNA (Biebrich et al. 1990; Yoon and Chikaraishi, 1992). Therefore, it was thought that approximately 20 kb of sequence 5' to the coding exon of the m4 gene may be needed to obtain the coding region, the transcription start site and regulatory elements. A 20 kb DNA fragment can be cloned from a lambda library, but to obtain the transcription start site and promoter elements in a single recombinant would require the fortuitous positioning of the cDNA sequence in the 3' end of the fragment isolated. It was therefore decided to screen a genomic library constructed in a cosmid vector which can facilitate up to 50 kb of DNA, thus increasing the chances of isolating the full transcription unit for the m4 gene and its regulatory elements within a single recombinant. A total of approximately $1x10^6$ recombinants were screened with both a mixture of 3 oligonucleotide probes and also with a random primed DNA fragment isolated from the m4 cDNA (see figure 4.1a). Initially, 32 positive hybridization signals were observed and secondary screening was carried out resulting in the isolation of 23 independent single colonies which hybridized with the probes used. Preliminary mapping of some of the clones with the oligonucleotides Rm4A, Rm4B and Rm4C (figure 4.1a), indicated that all of the cosmid clones contained sequences complementary to Rm4B and Rm4C, but only some of the clones contained sequences complementary to Rm4A. This suggested that only some of the clones, those that hybridized with Rm4A, contained genomic DNA that was not truncated within the m4 cDNA. The genomic DNA used to produce this library was initially partially digested with the enzyme, Mbo I, and then ligated into the cosmid vector. There are 3 Mbo I restriction enzyme sites in the m4 cDNA between the two oligonucleotides, Rm4A

Figure 4.1 Organisation of the m4 cDNA and cosmid clones obtained from library screening.

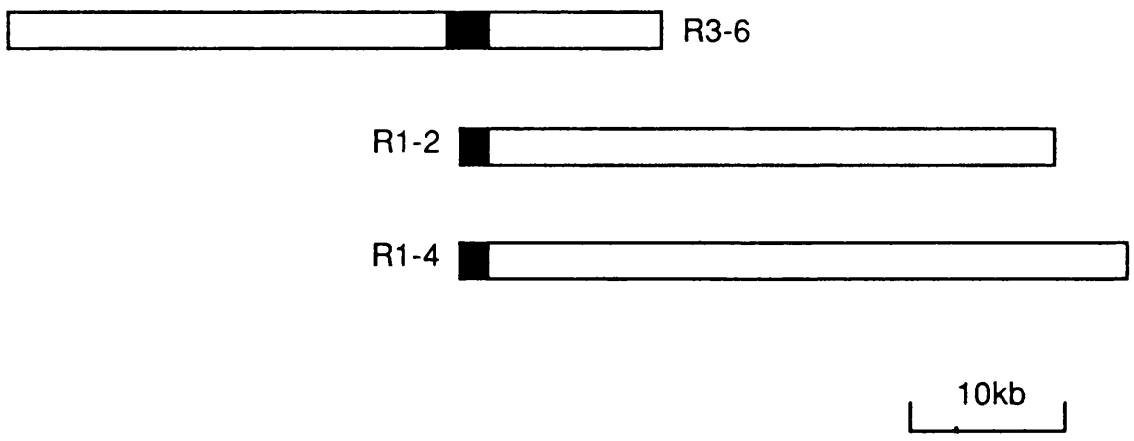
a) Shows the organization of the m4 cDNA extending from -30 to 2614 nucleotides with respect to the translation initiation site. The hatched box between nucleotides 0 to 1434 represents the coding region for the m4 gene. Below the m4 cDNA are shown the relative positions of the three oligonucleotides; Rm4A, Rm4B and Rm4C (shaded boxes) and the 997 bp Pst I fragment (open box) used in the library screening.

b) Shows the relationship between the three cosmid clones, R3-6, R1-2 and R1-4, obtained from the library screening using m4 specific probes. The shaded region represents the coding exon which is truncated at the 5' end in the two clones, R1-2 and R1-4.

a)



b)



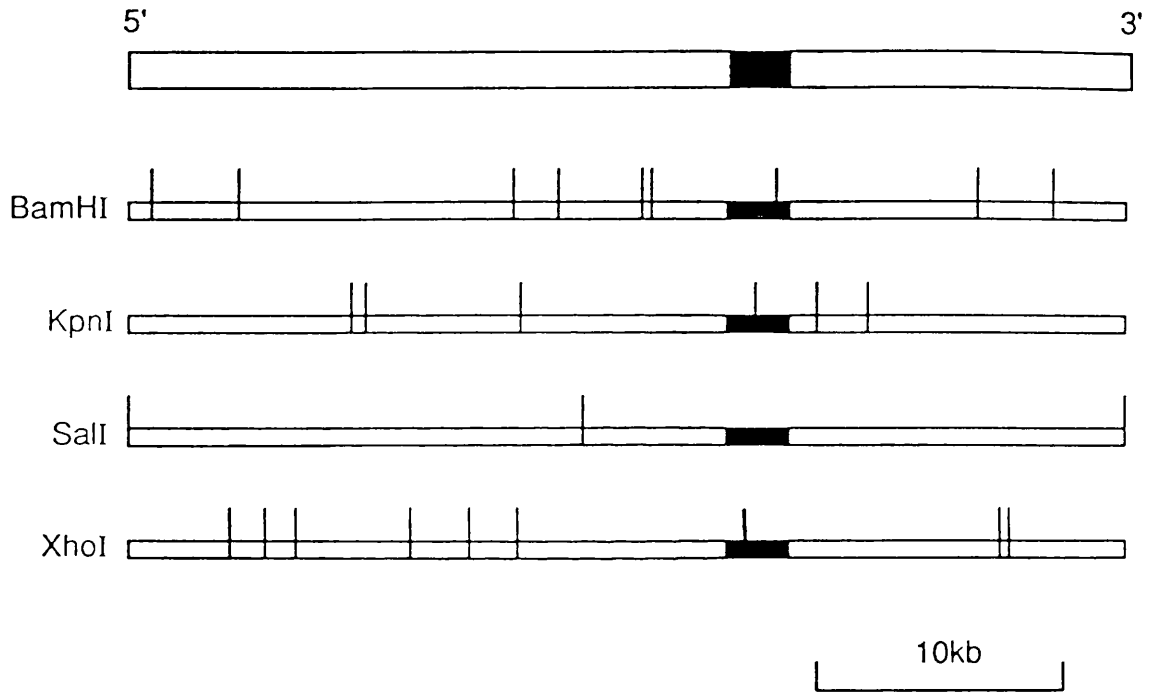
and Rm4B, suggesting that the clones containing truncated m4 coding exon result from cleavage at one of these sites. Further mapping of the position of the m4 cDNA within the clones confirmed that some of the clones did contain a truncated m4 coding exon and a total of 3 different types of clone were identified, (figure 4.1b). Only 3 clones were found to be of the R3-6 type and contain DNA sequences 5' to the m4 cDNA. 9 clones were found to be of the R1-2 type and 4 clones were of the R1-4 type. A further 7 clones were not characterized as they did not hybridize with the Rm4A oligonucleotide, so therefore contained a truncated m4 gene. As genomic elements are usually situated 5' to the transcription start site, further analysis of the clones obtained was restricted to the R3-6 clone as this is the only one containing sequences upsteam from the coding exon.

The R3-6 clone was restriction mapped using the enzymes; Bam HI, Kpn I, Sal I and Xho I, the results of which can be seen in figure 4.2. A portion of the clone, containing sequences that are present in the m4 mRNA and encode part of the i3 loop for the receptor, was amplified using PCR and the DNA obtained was sequenced. The nucleotide sequence obtained was found to be identical to the equivalent sequence in the rat m4 cDNA suggesting that this clone contains the true m4 gene and not a pseudo gene as has been previously identified for the dopamine receptor genes, $D_{1\beta}$ (Weinshank et al. 1991), D_5 (Nguyen et al. 1991; Grandy et al. 1991) and the 5-hydroxytryptamine receptor gene, $5HT_{1D}$ (Nguyen et al. 1992).

Functional analysis of R3-6.

Full length cDNAs for many genes, including the muscarinic receptors, have proved to be difficult to isolate. Such is the case with the m4 gene and various cloning strategies to isolate a full length cDNA have been attempted. Screening of 5' *STRETCH* cDNA libraries (Clontech), (in which, prior to cloning, the mRNA is completely denatured with methylmercuric hydroxide to reduce secondary structure that may result in premature termination of reverse transcription), PCR techniques, (such as amplification of cDNA libraries using a specific m4 antisense primer from near the 5' end of the coding region and a sense vector specific primer), and the 5'

Figure 4.2 Restiction enzyme map of the R3-6 cosmid clone.



Shows the relative positions of restriction enzyme sites within the R3-6 cosmid clone for the enzymes; Bam HI, Kpn I, Sal I and Xho I. The shaded area within the clone represents the coding exon.

RACE procedure designed by Frohman et al. (1988), have not provided any sequence further upstream from the ATG (Buckley, N.J. unpublished observations). The known sequence for the m4 cDNA is contained within one exon and extends from a putative splice acceptor site, 30 nucleotides 5' to the translation initiation codon, to the polyadenylation site at the 3' end. The size of the cloned cDNA is 2.6 kb whereas Northern blot analysis has indicated that the m4 mRNA is between 3.0-3.3 kb (Bonner et al. 1987; Nathanson, 1987; Peralta et al. 1987b; Bonner, 1989; Lazareno et al. 1990). Therefore there is approximately 300-600 nucleotides of the 5' end of the m4 mRNA missing from the m4 cDNA depending on the exact length of the poly $(A)^+$ tail for these transcripts. This missing sequence is present in a separate exon, although whether there is one or more exons is unknown. The cosmid clone, R3-6, contains approximately 30 kb of sequence 5' to the m4 coding region and thus should also include the transcription start site, (which has been suggested to be approximately 10 kb from the coding exon (Bonner, 1989)), and upstream regulatory regions. However the dopamine D_2 gene contains an intron between its putative 1^{st} and 2^{nd} exons of approximately 250 kb, (Buck et al. 1992), so the inclusion of the transcription start site for the m4 gene within the R3-6 clone could not be taken for granted. To test whether R3-6 contains a complete m4 transcription unit and is able to confer expression of the m4 gene, a functional assay was designed. For this assay the R3-6 clone was transfected into two cell lines, the IMR32 and the CHO cell lines. The IMR32 is a human neuroblastoma cell line that expresses the human m4 gene (figure 3.1), whilst the CHO cell line was chosen as it does not express the m4 gene. RNA from these transfected cell lines was isolated, reverse transcribed and the presence of m4 mRNA transcript from the rat clone, R3-6, determined using species specific primers in PCR amplification. Initially RNA from cells that had been transiently transfected with the cosmid clone was analysed by PCR. However, amplification products could be seen in these samples, without reverse transcription of the mRNA, suggesting that this signal was derived from the transfected R3-6 DNA. Isolation of RNA from cell lines usually results in some contaminating genomic DNA which can be detected in PCR analysis due to its inherent sensitivity. The lack of

sequence information of the upstream exons is also a problem here. If the upstream exon sequence was known then a set of primers could be designed that would span the intron. Amplified product from the genomic DNA would then also contain intronic sequences and thus be larger than amplified product from the mature transcript which would not contain any intronic sequences. However, the genomic DNA can be efficiently removed by a digestion with DNAse I (see figure 4.3.). Extensive digestions with DNAse I and restriction enzymes, however, was unable to remove the signal obtained in this PCR analysis due to R3-6 DNA, which is probably a result of the relatively large number of copies of cosmid DNA compared with copies of genomic DNA present. Because contaminating genomic DNA can be efficiently removed, it was decided to produce stable cell lines that contain R3-6 integrated into the genome of these cells. Cells were co-transfected with R3-6 DNA and a neomycin resistance gene under the control of the SV40 promoter. Stably transfected cells were then selected by their resistance to the neomycin analogue, G418. Single clones of cells were isolated and analysed for expression of the rat m4 gene. Figure 4.3 shows the result of an analysis of 6 independent clones of transfected IMR32 cells. PCR analysis on isolated RNA shows that at least 4 of the clones (figure 4.3 RNA samples 1, 2, 4 and 5) contain R3-6 integrated into the chromosome as they show amplified product without reverse transcription. When the samples were put through a DNAse I digestion no amplified products were seen showing that the genomic DNA has been removed (figure 4.3 DNAsed RNA lanes 1 to 6). Reverse transcription of the RNA and subsequent PCR results in amplification products in samples derived from two of the cell clones, (figure 4.3 reverse transcribed DNAsed RNA lanes 3 and 4), indicating that these two clones express the rat m4 mRNA. A total of 10 clones of IMR32 cells from 30 G418 resistant colonies were seen to express the rat m4 gene. Although the rat m4 gene was found to be expressed in 10 independant IMR32 clones a further 15 clones had the R3-6 cosmid integrated into the genome. One explanation for this may be that in these 15 clones the R3-6 cosmid has integrated into a transcriptionally silent part of the genome thus expression of the rat m4 gene is inhibited.

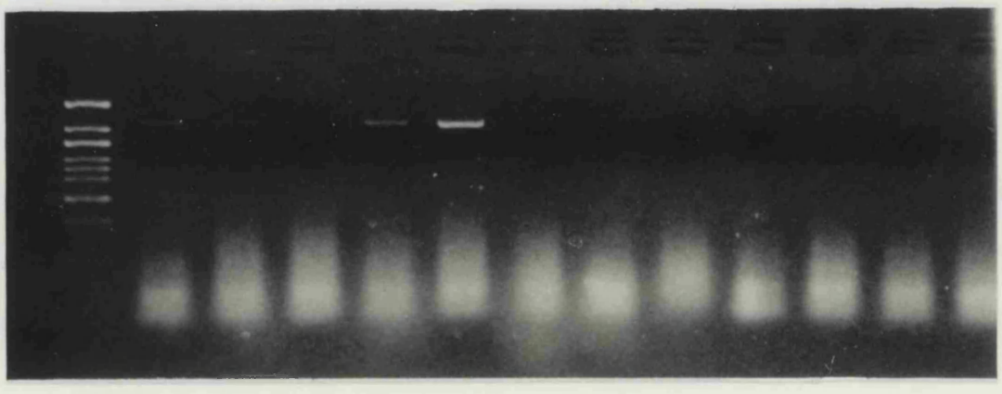
10 independent clones of CHO cells that were G418 resistant were screened

Figure 4.3 PCR analysis of R3-6 transfected IMR32 clones.

Shows the results obtained for PCR analysis on 6 G418 resistant, transfected IMR32 clones. Each of the clones, labelled 1-6, was analysed using the species degenerate m4 primer, HM4 1141s, and the m4 rat specific primer, Rm4 1559a (see Materials and Methods). PCR was performed on RNA, DNAsed RNA and reverse transcribed DNAsed RNA in order to show that; i) The clones contain rat m4 DNA integrated into the genome; ii) contaminating genomic DNA can be efficiently removed by DNAse I treatment; and iii) two of the clones, (3 and 4), express transcripts for the rat m4 gene. A total of 30 G418 resistant colonies were analysed and 10 of these clones expressed transcripts for the rat m4 gene. The lanes labelled - and + represent control PCR samples that contain no added template and added control template respectively.

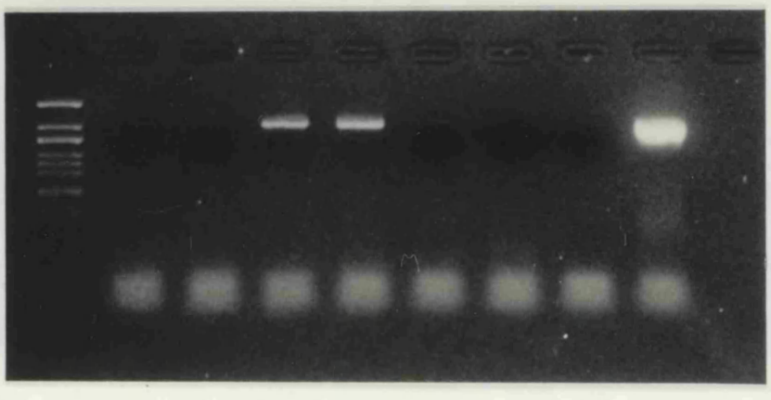
RNA DNAsed RNA

1 2 3 4 5 6 1 2 3 4 5 6



Reverse Transcribed
DNAsed RNA

1 2 3 4 5 6 - +



for the presence of the rat m4 transcript. Figure 4.4 shows the result of PCR analysis on 7 of these clones and it can be seen that none of the clones isolated expressed the rat m4 gene encoded in the cosmid, R3-6. Primers recognising the gene for the constitutively expressed enzyme, hypoxanthine-guanine phosphoribosyl transferase (hprt), were able to produce an amplification product in a PCR analysis (figure 4.4 hprt primers) of the same reverse transcribed RNA as that used for the m4 analysis, indicating that lack of amplification products for the m4 primers was not due to a problem with the reverse transcription. Analysis of the other 3 transfected CHO clones gave identical results. A PCR analysis of the genomic DNA obtained from each of the 10 CHO clones with the rat m4 specific primers showed that 9 of the clones contain the rat m4 DNA indicating that lack of expression is not due to a lack of transfection. Two conclusions can be drawn from this study. Firstly, the cosmid clone, R3-6, contains the complete transcription unit for the m4 gene, including a promoter region, as seen by its capability to drive expression of the rat m4 gene in the IMR32 neuroblastoma cell line. Secondly, this clone also contains at least some of the tissue specific elements for the m4 gene because no m4 mRNA is observed in the CHO cell line which does not endogenously express the m4 gene.

Primer extension.

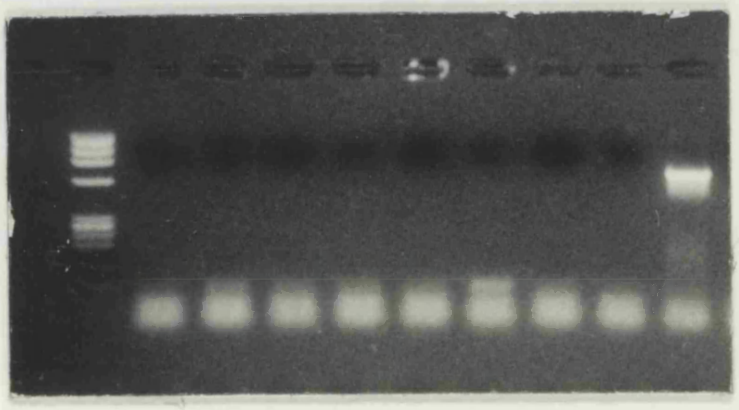
In order to try and accurately determine the size of 5' sequence that is missing from the m4 cDNA, primer extension analyses were performed. Two ^{32}P labelled m4 specific primers, 63a and 10a (see Materials and Methods), were hybridized to RNA extracted from PC12, a rat pheochromacytoma cell line which expresses the m4 gene (figure 3.1), NG108-15 and CHO cell lines. The hybridized primers were extended by reverse transcription and the products separated on a polyacrylamide gel. The use of the two primers, 63a and 10a, facilitates the identification of specific transcripts as each specific transcript generated from the 10a primer will be 53 nucleotides shorter than the equivalent transcript generated from the 63a primer. Therefore signals due to specific transcription can be identified by a comparison of transcripts derived from the two primers. Several attempts were made

Figure 4.4 PCR analysis of R3-6 transfected CHO clones.

Shows results obtained for PCR analysis on 7 G418 resistant, transfected CHO clones. The rat m4 transcript could not be detected in any of the clones analysed using the two primers, Hm4 1141s and Rm4 1559a. The hprt primers, Hprt 231s and Hprt 567a, were capable of producing an amplified product from the same samples indicating that there was no problem with the RNA isolation or reverse transcription. Also, the hprt primers are complementary to sequences from different exons thus the presence of an amplified product of 366 bp from these primers provides additional proof that the PCR products are derived from reverse transcribed mRNA rather than genomic DNA. The lanes labelled - and + contain control PCR samples with no added template and added control template respectively.

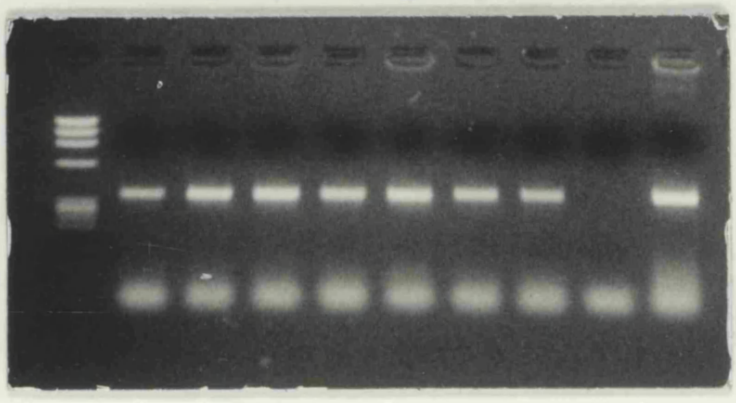
m4 primers

1 2 3 4 5 6 7 - +



hprt primers

1 2 3 4 5 6 7 - +



at this primer extension analysis but no specific products were ever observed. It was thought that this may be due to a GC rich region in the 5' end of the gene transcript resulting in a secondary structure that inhibits the reverse transcriptase enzyme. This is consistent with the absence of a full length m4 cDNA in cDNA libraries that have been screened, as the production of these libraries also require a reverse transcription step. Alternatively, lack of a primer extension signal may be a result of the low levels of m4 transcript observed in these cell lines. Difficulties with primer extension analysis has previously been noted for the D_{1A} gene (Minowa, T. et al. 1992).

Identification of transcribed regions.

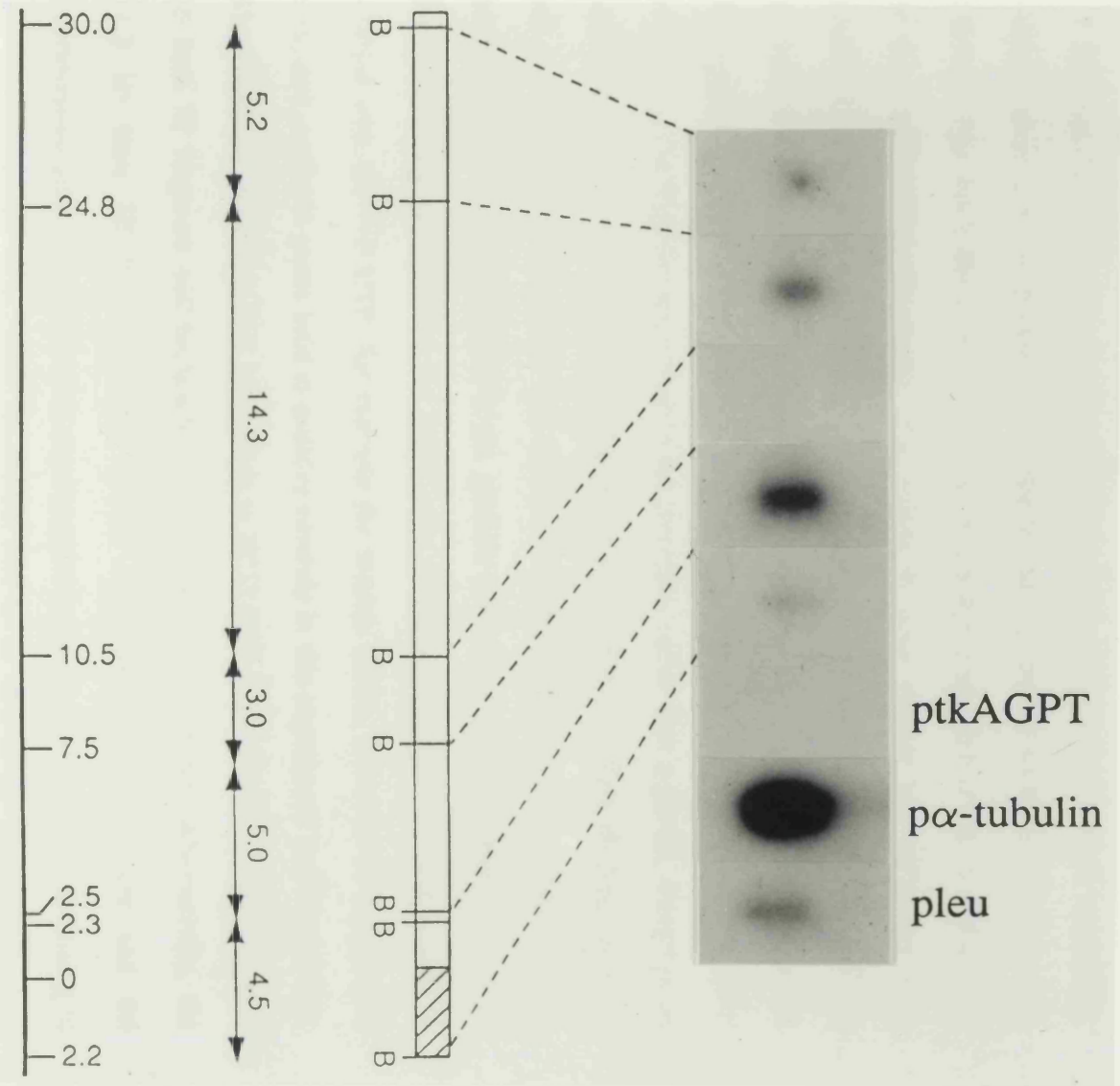
The next stage in the analysis of the R3-6 DNA was to determine which sequences within the clone are transcribed. This was done using a nuclear run on analysis. Briefly, the nuclei from PC12 cells were isolated and incubated in the presence of ^{32}P-UTP resulting in the production of radioactively labelled transcripts. These transcripts were then purified and hybridized to slot blots containing DNA fragments from the R3-6 clone. Hybridization signals should then be observed for those DNA fragments that contain sequences that are transcribed, whether they are intronic or exonic, and the results of this analysis are shown in figure 4.5. Hybridization signals are seen for the Bam HI fragment containing part of the coding exon for the m4 gene and also for the next upstream fragment to this, the 5.0 kb Bam HI fragment, demonstrating that both of these fragments contain sequences that are transcribed. The next upstream fragment, the 3.0 kb Bam HI fragment, does not show any hybridization with the labelled RNA transcripts, suggesting that this fragment does not contain any transcribed sequences. Repeated nuclear run on analyses failed to show any hybridization with this fragment, consistent with this conclusion. This data suggests that the m4 transcription begins within the 5.0 kb Bam HI fragment, between 2.5-7.5 kb upstream from the translation initiation codon.

Also noted in figure 4.5 is a hybridization signal from the 14.3 kb Bam HI fragment located further upstream in the R3-6 clone, suggesting that this fragment contains transcribed sequences. It is unlikely that transcription of the m4 gene begins

Figure 4.5 Nuclear run on analysis identifying transcribed regions of the R3-6 cosmid clone.

Shows the results obtained for a nuclear run on assay from PC12 nuclei. 500ng of each DNA fragment was immobilized on a nylon membrane, hybridized with the nuclear run on probe and exposed to X-ray film for one week. The results obtained for each of the fragments is shown in the top panel. Also shown is the hybridization obtained from a negative control plasmid, ptkAGPT (a CAT reporter plasmid in which some of the DNA fragments were subcloned), and two positive control plasmids, pα-tubulin (containing the coding region for the α-tubulin gene) and pleu (containing the cDNA for the tRNA that recognizes leucine).

The open box represents a portion of the R3-6 clone spanning a region between 30 kb 5' to 2.2 kb 3' from the initiating ATG. The hatched area represents sequences in the coding exon, and the position of the restriction enzyme sites for Bam HI (B) are shown. The limits of the DNA fragments used for each signal are represented by dashed lines and the size of each of these fragments in kb is shown between the arrowheads underneath the box representing R3-6 DNA. The scale at the bottom of the figure gives the distance, in kb, of each of the Bam HI sites from the initiating ATG codon.


30.0
24.8
10.5
7.5
2.5
2.3
0
2.2
5.2
14.3
3.0
5.0
4.5
B
B
B
B
B
B
B
ptkAGPT
pα-tubulin
pleu

within this fragment as the 3.0 kb Bam HI fragment would then also be transcribed and show a hybridization signal in this analysis. The most 5' fragment of R3-6, the 5.2 kb Bam HI fragment, does not appear to contain any transcribed sequences. Although there is signal present on the slot containing the 5.2 kb Bam HI fragment it is thought that this is due to background as it is a single spot rather than a slot as seen for the other positive fragments. The results of other nuclear run on assays were consistent with this interpretation. Two suggestions can be made about the hybridization seen with the 14.3 kb Bam HI fragment. Firstly, this Bam HI fragment may contain a complete transcription unit for a gene that is expressed in PC12 cells. In this case the fragment must contain the complete, rather than a partial, transcription unit as neighbouring fragments in R3-6, the 3.0 kb and 5.2 kb Bam HI fragments, do not give hybridization signals and so do not contain transcribed sequences. The nuclear run on assay performed is not specific for the m4 gene and transcipts of all the genes being expressed by the PC12 cells, at the time of cell harvesting, will be labelled with the ^{32}P UTP, for example the leucine tRNA, (encoded in the plasmid pleu) and α-tubulin genes used as positive controls in this experiment (see figure 4.5). Therefore any transcripts that are present in PC12 cells and are derived from the 14.3 kb Bam HI fragment will be labelled and hybridize to the DNA. Alternatively, the 14.3 kb Bam HI fragment may contain repetitive DNA sequences and the hybridization seen is due to similar sequences in the labelled transcripts hybridizing to this DNA fragment. It has been determined that up to 30% of the human genome is composed of sequences that are repeated at least 20 times and some highly repetitive sequences in the genome have copy numbers between 10^3-10^4 (Davidson and Britten, 1979). Complementary repetitive sequences have been identified in nuclear RNA, but not in mRNA, and it has been proposed that these repetitive sequences may be involved in gene regulation (Davidson and Britten, 1979).

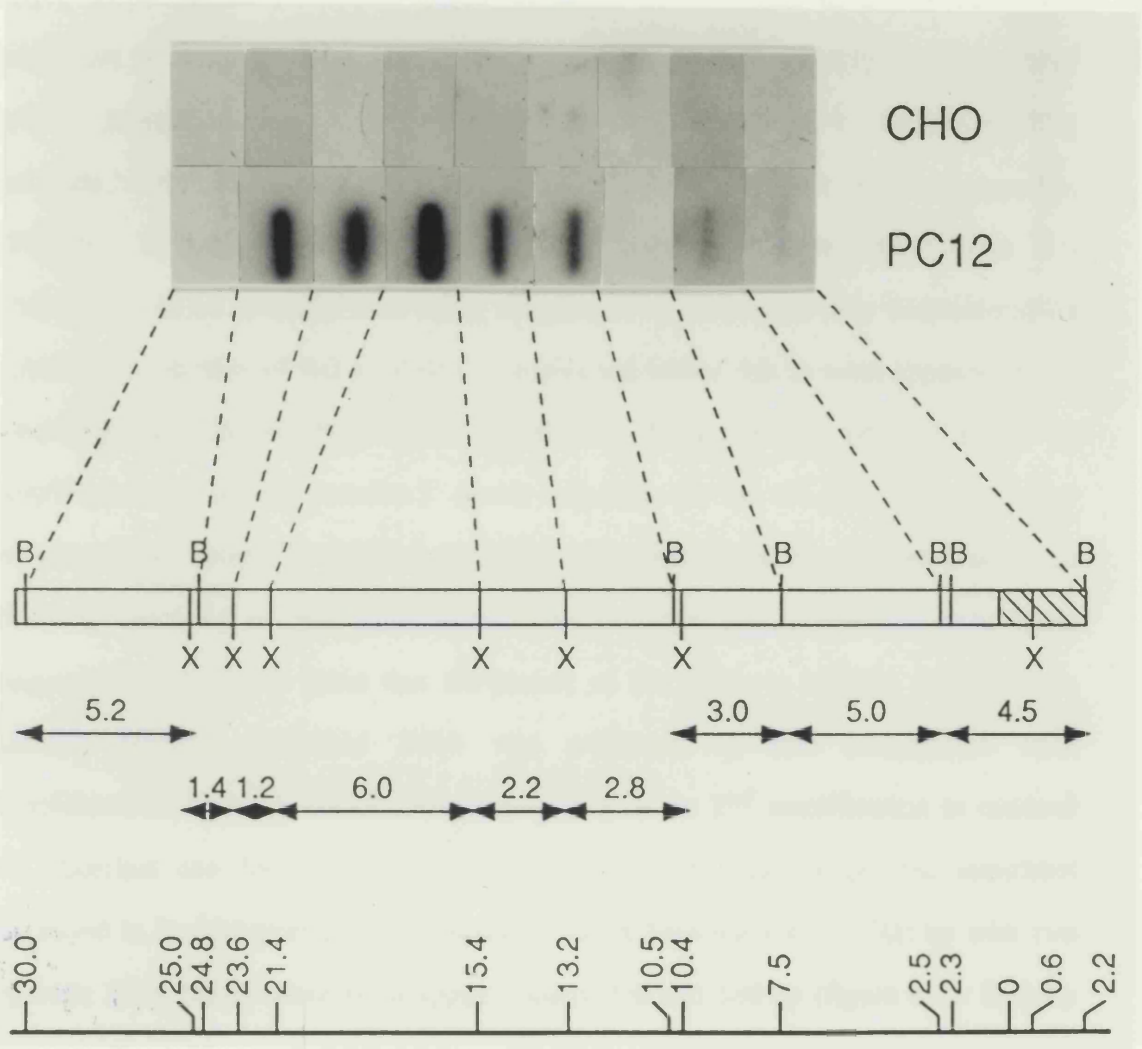
Northern blot analysis.

To complement the nuclear run on analysis, a Northern blot analysis was performed. This was carried out using radiolabelled DNA fragments from the R3-6

clone and hybridizing to slot blots of total RNA isolated from the PC12 and CHO cell lines. The information obtained from a Northern blot analysis is complementary to that obtained from a nuclear run on analysis because nuclear run on analyses indicate all of the DNA that is transcribed, whether it is intronic or exonic. Northern blot analysis, however, indicates only those DNA fragments that contain transcribed sequences that are exonic, and thus present in the processed mRNA. The use of two cell lines for this study enabled the identification of fragments that contain sequences that are transcribed in PC12 cells but not in CHO cells such as the m4 transcript. The results of the Northern blot analysis can be seen in figure 4.6. The 4.5 kb Bam HI fragment that contains most of the coding exon and the more 5' 5.0 kb Bam HI fragment both show hybridization signals, albeit very weak, with PC12 RNA but not with CHO RNA. This is consistent with m4 transcription initiating in the 5.0 kb Bam HI fragment as suggested by the nuclear run on analysis. The 3.0 kb Bam HI fragment, spanning the region 7.5-10.5 kb 5' from the ATG initiation codon, does not show any hybridization signal, indicating it does not contain exonic sequence for the m4 gene, which is also consistent with the nuclear run on analysis. The upstream fragments within the 14.3 kb Bam HI fragment, the 5 Xho I fragments spanning the region 10.4-25.0 kb 5' from the ATG initiation codon (figure 4.6), show a relatively intense signal when hybridized to PC12 RNA but do not show hybridization with CHO RNA. Because the intensity of the signals of the Xho I fragments is much greater than that seen for the 4.5 kb and 5.0 kb Bam HI fragments, it is unlikely that they are due to hybridization to the same transcript. This is consistent with the results of the nuclear run on analysis which suggests that there may be two transcription units present within the R3-6 clone. The 5 Xho I fragments, spanning the region 10.4-25.0 kb 5' from the ATG initiation codon (figure 4.6), also show no hybridization with CHO RNA which suggests that hybridization with this region of the clone is not due to the presence of repetitive sequences, but is due to a transcript that is expressed in PC12 cells at relatively high levels but is not expressed in CHO cells.

Figure 4.6 Northern blot analysis of cell line RNA with DNA fragments from the R3-6 cosmid clone.

Shows the results obtained for Northern blot analysis on PC12 and CHO total RNA immobilized onto a nylon membrane using a slot blot manifold. DNA fragments from the R3-6 clone were isolated and labelled by random priming as outlined in Materials and Methods. After hybridization and washing, Northern blots were exposed to X-ray film for one week. The results obtained for each of the fragments is shown in the top panel for both CHO and PC12 cell line RNA. The open box represents a section of the R3-6 clone extending from 30 kb 5' to 2.2 kb 3' from the translation initiation codon. The hatched area contains sequences in the coding exon and the restriction enzyme sites for Bam HI (B) and Xho I (X) are shown. The limits of each DNA fragment used is represented by dashed lines and the sizes of these fragments, in kb, is shown between the arrowheads below the open box representing R3-6 DNA. The scale at the bottom of the figure shows the distance, in kb, of the restriction enzyme sites from the translation initiation codon.


CHO
PC12
B
B
B
B
BB
B
X X X
X
X
X
X
5.2
3.0
5.0
4.5
1.4 1.2
6.0
2.2
2.8
30.0
25.0
24.8
23.6
21.4
15.4
13.2
10.5
10.4
7.5
2.5
2.3
0
0.6
2.2

5' RACE.

Both the nuclear run on analysis and the the Northern blot analysis suggest that the transcription start site for the m4 gene is situated within the 5.0 kb Bam HI fragment (figures 4.5 and 4.6). In order to map the position of exonic sequences within this fragment more closely it was decided to utilize the 5' RACE procedure, (rapid amplification of cDNA ends), designed by Frohman et al. (1988). This procedure is based on PCR and is specifically designed to amplify the 5' ends of mRNA molecules that are often difficult to obtain using more classical cloning methods. Although attempts to clone an m4 5' RACE product were unsuccessful, (Pepitoni, S. and Buckley, N.J. unpublished observations), it was obvious that specific amplified products were being obtained. Problems were only encountered on subsequent insertion of this product into a plasmid vector due to what appeared to be re-arrangement of the recombinant constructs. As a specific product could be amplified that contained putative 5' exonic sequences for the m4 gene, it was decided to produce an internally labelled RACE product that could be used as a probe in Southern analysis of the R3-6 clone. This analysis should identify only those sequences in the R3-6 clone that are exonic as the probe is initially derived from mature mRNA. Amplified DNA was produced by two consecutive PCR amplifications with ^{32}P-dATP incorporated only in the 2nd amplification as outlined in Materials and Methods. When analysed on a 2% agarose gel the radiolabel appeared to be incorporated into a smear of DNA between 100 to 1500 bp with two specific DNA bands observed at approximately 330 and 560 bp (figure 4.7). Initially the RACE probe was hybridized to a slot blot containing DNA fragments derived from the R3-6 clone, (figure 4.8). This data showed that two fragments, the 5.0 kb and 4.5 kb Bam HI fragments, hybridized with the RACE probe indicating that both of these fragments contain exonic sequences, consistent with the previous results discussed. The 4.5 kb Bam HI fragment hybridizes due to sequences from the coding exon that are contained within it. The 5.0 kb Bam HI fragment, 2.5-7.5 kb 5' from the ATG initiation codon, hybridizes because it must also contain exonic sequences for the m4 gene. The intensity of the hybridization signal seen with the 4.5 kb Bam

Figure 4.7 Analysis of the 5' RACE probe.

Shows an analysis of the radiolabelled 5' RACE probe produced by the 5' RACE procedure. Two probes are shown, A and B, which were synthesized using either the Rm4 63a primer (probe A) or the Rm4 10a primer (probe B) during the second round of amplification (see Materials and Methods). Approximately $5x10^4$ dpm of probe were loaded onto a 2% agarose gel and the DNA fragments size separated. After drying the gel at 80°C for 1 hr it was exposed to X-ray film for several hours. The numbers on the left of the gel represent the positions of the DNA size markers and their sizes in base pairs. Probe A contains radiolabel incorporated into a smear of DNA between 150-1500 bp with no recognisable individual bands even at shorter exposure times. Probe B also contains radiolabel incorporated into a smear of DNA but does show two distinct bands at approximately 330 bp and 560 bp.

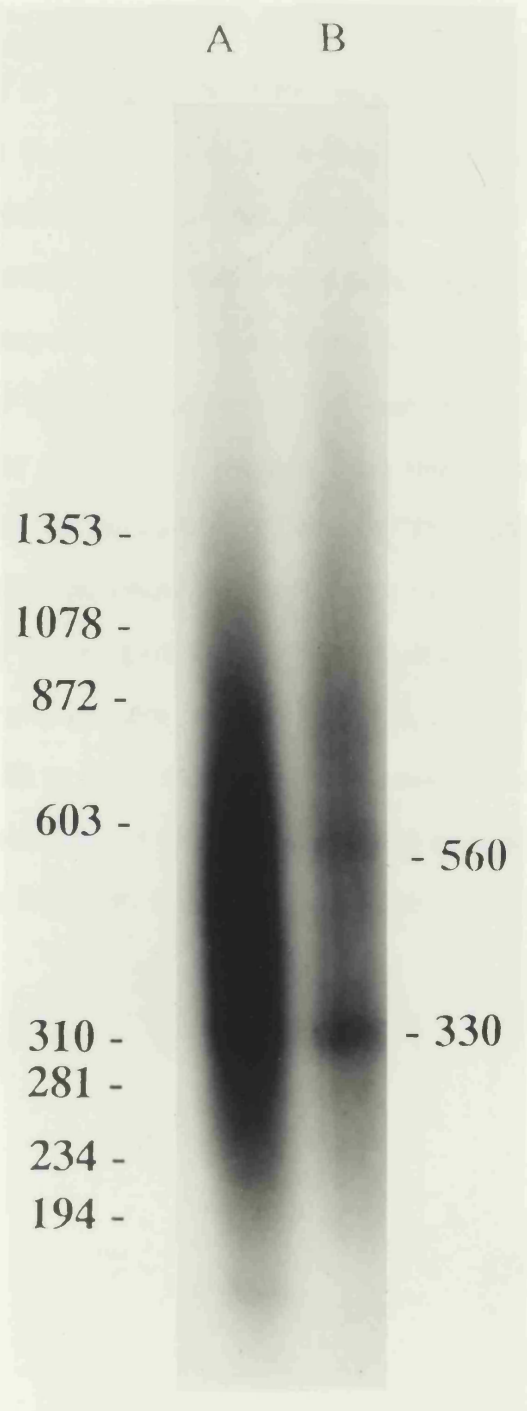

A
B
1353 -
1078 -
872 -
603 -
310 -
281 -
234 -
194 -
- 560
- 330

Figure 4.8 5' RACE analysis on the complete R3-6 cosmid clone.

a) The upper half of the figure shows the results obtained from hybridization of the 5' RACE product to a slot blot of DNA fragments isolated from the R3-6 clone. The open box represents R3-6 DNA extending from 30 kb 5' to 2.2 kb 3' from the translation initiation codon. The hatched area contains sequences from the coding exon and the restriction enzyme sites for the enzymes, Bam HI (B) and Xho I (X) are shown. The limits of the DNA fragments used for each slot are indicated by the dashed lines and the sizes of the DNA fragments are shown, in kb, between the arrowheads below the open box representing R3-6 DNA. The scale underneath shows the distance, in kb, of each of the restriction enzyme sites from the initiating ATG.

b) The lower half of the figure shows the results obtained from hybridization of the 5' RACE product to a Southern blot of R3-6 DNA. 5μg of cosmid DNA was digested with either Bam HI or Xho I and the fragments separated on a 0.6% agarose gel. After blotting, hybridization and washing the blot was exposed to X-ray film for one week. The size of the fragments that produce a hybridization signal are shown in kb.

5
10
Bam HI
Xho I

30.0
30.0
24.8
23.6
21.4
15.4
13.2
10.5
10.4
7.5
2.5
2.3
0
0.6
2.2
5.2
1.4
1.2
6.0
2.2
2.8
10.0
3.0
5.0
4.5
B
X
B
X
X
X
X
B
X
B
B
B
X
B

HI fragment is greater than that seen for the 5.0 kb Bam HI fragment. One reason for this apparent discrepancy in signal intensities may be due to non-specific amplification. Because α^{32}P-dATP was included in the second round of PCR amplification, extension of the primers will result in the incorporation of the radiolabel whether this is due to specific or non-specific extension. Non-specific extension of the Rm4 10a primer will result in a radiolabelled DNA fragment that will hybridize to the 4.5 kb Bam HI fragment, due to the original primer, but not to any upstream exonic fragments. However, only specific amplification products will contain sequences that will hybridize to upstream fragments, thus the signals resulting in this hybridization will be weaker. Further upstream fragments also appear to give weak hybridization signals in figure 4.8 but it was thought that these were due to background. Southern analysis of these fragments separated on an agarose gel is shown in figure 4.8 and is consistent with this suggestion as no hybridization signals with fragments between 30 kb and 7.5 kb 5' to the translation initiation site. Only one fragment appears to be hybridizing in the Bam HI lane because the intensity of the hybridization signal seen for the 4.5 kb Bam HI fragment masks that of the 5.0 kb Bam HI.

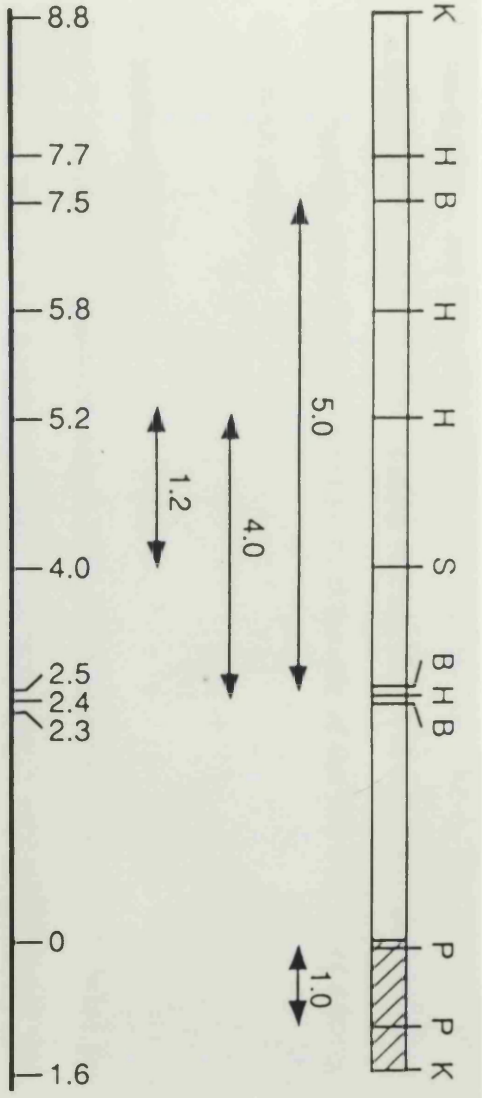
To identify more closely which sequences from the 5.0 kb Bam HI fragment were hybridizing and thus contained exonic material, a 10.4 kb Kpn I fragment, containing 1.6 kb of the coding exon at its 3' end and 8.8 kb of sequence 5' to the coding exon, was subcloned into pGem 7Zf(+) (figure 4.9). This construct was cut with various restriction enzymes and the DNA fragments were separated on an agarose gel and transferred to a nylon membrane. This Southern blot was then hybridized with the RACE probe, the results of which can be seen in figure 4.9. The DNA fragments that give hybridization signals with the RACE probe are shown in the lower half and the position of some of these fragments is illustrated in the upper half of figure 4.9. The 1 kb Pst I fragment and the larger fragments, (>5 kb), seen in the Hind III, Bam HI/Sal I and Hind III/Sal I lanes are all due to hybridization with sequences in the coding exon. The smallest upstream DNA fragment that hybridizes is the 1.2 kb Hind III/Sal I fragment suggesting that the upstream exon, or exons, are

Figure 4.9 5' RACE analysis on a 10.4 kb fragment of the R3-6 clone.

a) Shows a representation of the 10.4 kb Kpn I fragment extending from 8.8 kb 5' to 1.6 kb 3' from the initiating ATG. The hatched area contains sequences from the coding exon and the restriction enzyme sites for the enzymes Kpn I (K), Hind III (H), Bam HI (B), Sal I (S) and Pst I (P) are shown. The lines with arrowheads represent some of the DNA fragments that produced a hybridization signal and the figures represent the size of these fragments in kb. The scale underneath shows the distance, in kb, of each restriction enzyme site from the initiating ATG.

b) Shows the hybridization signals obtained from a one week exposure of a Southern blot containing restriction enzyme digests of the 10.4 kb Kpn I fragment subcloned into pGem 7Zf(+). The approximate size of each hybridizing fragment is shown at the side in kb.


4.0
3.5
1.2
1.0
Pst I
Hind III
Bam HI/Sal I
Hind III/Sal I


8.8
7.7
7.5
5.8
5.2
4.0
2.5
2.4
2.3
0
1.6
1.2
4.0
5.0
1.0
K
H
B
H
H
S
B H B
P
P
K

present within this fragment of DNA. However the possibility that further exonic sequences are present upstream of this cannot be eliminated because the RACE probe produced may not fully extend to the 5' end of the m4 mRNA. The presence of a specifically labelled RACE probe of about 560 bp, shown in figure 4.7, does however suggest that this probe should contain most, if not all, of the 300-600 bp of missing m4 mRNA sequence.

S1 nuclease analysis.

Ribonuclease protection assays have now become the method of choice for determining the size and position of exons. This technique is very sensitive and uses phage polymerase promoters such as T7 and SP6 to drive *in vitro* transcription of RNA complementary to that of the mRNA of interest. Radiolabelled ^{32}P-UTP can be incorporated into this synthesized cRNA which is hybridized to RNA isolated from cells that express the gene of interest. An RNAse digestion step is then employed to remove all of the cRNA sequences that are not hybridized to RNA. Analysis of the protected cRNA fragments on a denaturing polyacrylamide gel can then be used to determine the sizes of the exon or exons present. In an attempt to identify the number and size of exons present within the upstream regions of the m4 gene, several DNA fragments from the R3-6 clone were subcloned into pGem 7Zf(+) and complementary RNA was synthesized. Although several probes were produced that appeared to be full length, no information could be obtained concerning the number or sizes of exons present. It was not obvious why this method did not produce any results so an S1 nuclease analysis was then performed (as described in Materials and Methods) in an attempt to overcome any methodological problems.

Two fragments of DNA from the R3-6 clone were used for this analysis in separate hybridization mixes. Firstly the 10.4 kb Kpn I fragment (see figure 4.9), which contains both part of the coding exon and upstream sequences for the m4 gene, and the 5.0 kb Bam HI fragment (see figure 4.9) which is completely contained within the 10.4 kb Kpn I fragment and contains upstream sequences for the m4 gene but none of the coding exon. The use of two fragments for this analysis allows the

resolution of protected sequences due to hybridization with m4 mRNA from any artifacts, as the protected sequences due to upstream exons will be the same size for both of these fragments. In addition the use of RNA from PC12 cells (which express the m4 gene) and CHO cells (which do not express the m4 gene) aids in the identification of m4 specific protected fragments. Also, the 10.4 kb Kpn I fragment contains part of the m4 coding region and can be used as a positive control for this analysis.

The results are shown in figure 4.10 where it can be seen that protected fragments are present in the S1 nuclease analysis of PC12 RNA with both DNA fragments but no protected fragments are seen with CHO RNA. This is consistent with protected fragments resulting from hybridization of the DNA with m4 mRNA which is expressed in PC12 cells but not in CHO cells. Both DNA fragments show protected sequences of higher molecular weight than the 1.3 kb DNA size marker which are most likely the result of DNA:DNA hybidization between complementary strands of the plasmids. The 10.4 kb Kpn I fragment shows a protected sequence that is approximately 950 bases in length. This fragment is likely to be due to hybridization with the m4 coding exon as it is of the correct size and no equivalent protected fragment is seen for the 5.0 kb Bam HI fragment which does not contain any sequence from the m4 coding exon.

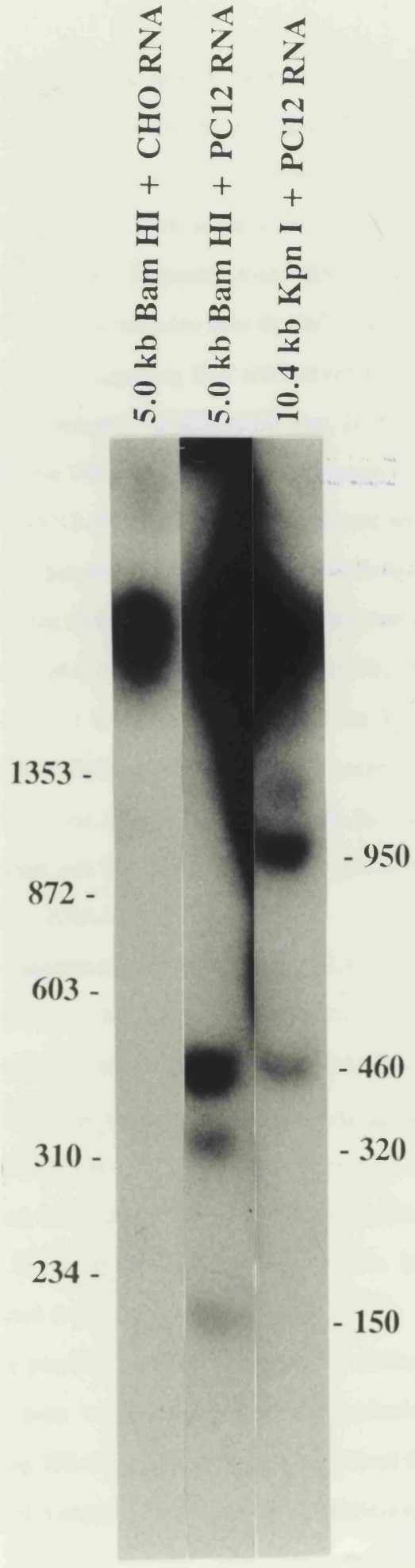
Both the 5.0 kb Bam HI and 10.4 kb Kpn I fragments produce protected fragments of 460 bases suggesting that this is the size of the upstream exonic material. This data, suggesting that the upstream exonic material is approximately 460 bases in length is also in good agreement with the labelled 5' RACE product of 560 bases obtained with the RACE amplification procedure (see figure 4.7). As well as containing exonic material the RACE product also contains approximately 80 bases of the coding exon and primer sequences. Therefore the 560 bp 5' RACE product contains approximately 480 bp of upstream exonic material.

It is not entirely clear why there are two further protected fragments of approximately 150 bases and 320 bases present in 5.0 kb Bam HI lane. These two bands are much less intense than the 460 base protected fragment and it cannot be

Figure 4.10 S1 nuclease analysis.

S1 nuclease analysis was performed as outlined in Materials and Methods. Two fragments of DNA from the R3-6 clone (the 10.4 kb Kpn I and 5.0 kb Bam HI fragments) were used in the analysis to aid in the identification of protected sequences due to specific hybridization with the m4 mRNA. One of the fragments, the 5.0 kb Bam HI fragment, was also hybridized with CHO RNA to act as a negative control. After hybridization and S1 nuclease digestion the protected fragments were run on a 2% agarose gel and blotted onto a nylon membrane using standard techniques. This blot was then probed with ^{32}P labelled DNA from the 5.0 kb Bam HI fragment and the Pst I fragment from the m4 cDNA to identify the protected sequences.

The figure shows the result of the three hybridizations. Each of the three lanes is labelled with the DNA fragment and cell line RNA used for the hybridization. The numbers on the left of the figure represent DNA size markers and the position of each of these and their size in nucleotides is indicated. The numbers on the right of the figure represent the approximate sizes of the protected fragments in nucleotides. The hybridization signals seen at the top of the figure are likely to be due to re-annealing of the plasmid DNA. Specific protected fragments are seen with both DNA fragments hybridized to PC12 RNA, but not with the 5.0 kb Bam HI fragment hybridized with CHO RNA, consistent with these being specific for the m4 gene.


5.0 kb Bam HI + CHO RNA
5.0 kb Bam HI + PC12 RNA
10.4 kb Kpn I + PC12 RNA
1353 -
872 -
603 -
310 -
234 -
- 950
- 460
- 320
- 150

determined if these fragments are also present for the 10.4 kb Kpn I fragment as the 460 base fragment is lower in intensity for the 10.4 kb Kpn I than for the 5.0 kb Bam HI fragment. It is tempting to speculate that these two protected sequences may be derived from the 460 base fragment because the sizes of these sequences adds up to approximately that of the 460 base fragment. Also, although the 150 base and 320 base sequences show a much lower intensity than the 460 base fragment they do show similar intensity to each other, suggesting they are derived from the same fragment. Two explanations that can account for this result are: i) The 460 base fragment encodes a single exon but the DNA used contains a mismatch in the sequence to the m4 mRNA obtained from PC12 cells. As such the S1 nuclease would be able to digest the exon at this missmatch but would cut at this point inefficiently. Therefore, three bands would be observed that corresponded to the complete exon and two lower bands resulting from the digestion at the mismatch. ii) Alternatively, the 460 base fragment contains two exons of 150 and 320 bases in length and the S1 nuclease digestion is incomplete in that the loop of DNA formed when the two exon sequences hybridize to the mRNA is excised but the mRNA itself may not be cut as efficiently. As denaturing conditions were not used for this gel it is possible for the two DNA fragments to run with the mRNA as a 460 base product.

The protected fragment of 950 bases for the 10.4 kb Kpn I fragment would suggest that the S1 nuclease is digesting the RNA efficiently and therefore the 460 base protected fragment is likely to be due to a single exon. This can be resolved with the use of a processive nuclease such as Exonuclease VII which only digests DNA from the ends and would not cut within sequences. If there is only one exon present then a 460 base protected fragment will be seen. If however there are multiple exons present the protected sequences would then also include intronic material and therefore a larger protected sequence would be observed.

There is also a possibility of the presence of a further small exon in this region. Any additional exon would be less than 140 nucleotides in length as 460 nucleotides of the missing 300-600 bases are already accounted for. Also a small exon may escape detection in this experiment due to a weak hybridization signal.

Summary.

A rat genomic clone (R3-6) containing the coding exon for the m4 gene and approximately 30 kb of 5' sequence has been isolated. It was thought that 30 kb of sequence 5' to the coding exon should be sufficient to contain the transcription start site for the m4 gene and at least some of its transcriptional regulatory regions. In order to test this, stable cell lines containing the entire cosmid clone were produced. The ability of the R3-6 clone to drive m4 expression in IMR32 cells indicated that the R3-6 clone contains the complete m4 transcription unit and also the promoter region. The lack of m4 expression in transfected CHO clones indicates that at least some tissue specific elements are also present within this clone.

As a full length cDNA for the m4 gene, or indeed any sequence for the upstream exonic material, is not available several independent studies were performed to identify these regions within the clone. Nuclear run on analysis and Northern blot analysis both suggest that there are two transcription units present within the R3-6 clone and that the m4 transcription start site is located between 2.5 kb and 7.5 kb 5' from the initiating ATG. The transcription start site was located to a region 4.0 kb to 5.2 kb from the initiating ATG using the 5' RACE procedure. The size of the upstream exonic material is approximately 460 bases as indicated by the S1 nuclease analysis and this is also in good agreement with the 5' RACE probe that was synthesized. It also appears likely that there is only one upstream exon but further experiments are needed to clarifiy this.

Chapter 5. General Discussion.

Discussion and Summary.

The principal means of intercellular communication within the mammalian brain is by the release of neurotransmitters from one neuron which then interact with receptor molecules present on other neurons and consequently elicit a response. Different types of receptors recognize different neurotransmitter molecules; however, different subtypes of receptor recognize the same neurotransmitter but may elicit different responses within the cell when they are activated. The repertoire of receptors expressed by a neuron will therefore determine which neurotransmitters will elicit a response in that neuron and how the response will be manifest. Each of the neurotransmitter receptors is expressed in a tightly controlled manner although the mechanisms that initiate and maintain this control have not been determined.

The work presented here has begun a study to identify the mechanisms and factors that control the expression of one receptor subtype, the m4 muscarinic acetylcholine receptor. By the use of a model neuronal system and the isolation of transcriptional control elements it should be possible to identify the mechanisms that initiate the expression of the m4 gene and also those that regulate the levels of receptors expressed by neurons.

Identification of cell lines that express the m4 gene.

Northern blot analysis has identified four cell lines that express the m4 transcript (figure 3.1). Three of these cell lines (N18TG2, NG108-15 and IMR32) are of neuronal origin whilst the fourth (PC12) can be differentiated into a neuronal-like cell by treatment with NGF (Jumblatt and Tischler, 1982). Three of the cell lines (NG108-15, N18TG2 and PC12) do not express any other muscarinic receptor subtype. The IMR32 cell line however, also expresses transcripts for the m1, m2 and m3 receptor subtypes (Buckley, N.J. unpublished observations). Therefore these cell lines should provide useful tools in the analysis of the regulation of expression of the m4 gene and the M_4 receptor for which it encodes. These cell lines will also be useful in determining the cis genomic sequences responsible for expression of the m4 gene.

If used in conjunction with cell lines that do not express the m4 gene e.g. CHO, 3T3 and 132N1 cell lines then the elements that confer tissue specificity of the m4 gene may be elucidated. These elements can then be tested in transgenic mice to see if they confer complete temporal and spatial expression for the m4 gene. Transgenic mice could then also be used to further dissect out which of the regulatory elements are responsible for each facet of the m4 expression.

PGE_1 stimulation.

As treatment of NG108-15 cells with PGE_1 and IBMX was found to induce phenotypic and physiological changes in the NG108-15 cell that resulted in a more neuronal-like character, it was thought that this treatment may also have an effect on the steady state levels of muscarinic receptor. However, this does not appear to be the case as is shown in figure 3.6 where muscarinic receptor levels of PGE_1 treated cells are identical to control cells that are cultured in 1% FCS. These cells show a reduction in the level of muscarinic receptors over time to a basal level of approximately 20%. This reduction in the levels of muscarinic receptors may be due to the cells leaving the cell cycle and the inhibition of mitogenesis seen on serum withdrawal. A preliminary experiment showed some evidence that this may be true as FCS added to cells that had been cultured in 1% FCS resulted in a rise in the levels of muscarinic receptors with time. This result is also different from the effect of differentiation of PC12 cells with NGF (Jumblatt and Tischler, 1982). The levels of muscarinic receptors on the PC12 cells are seen to increase approximately 4 fold over 15 days of NGF treatment (Jumblatt and Tischler, 1982). This increase in muscarinic receptors begins at around day 2 and precedes neurite outgrowth. DMSO differentiation of N1E 115 cells also results in a rise of muscarinic receptor levels in that cell line (Buyse et al. 1989). Differentiation of NG108-15 cells with PGE_1 appears to be faster than in PC12 cells with NGF and is more or less complete within 2 days of PGE_1 stimulation, but the levels of muscarinic receptors present remain at a basal level for up to 4 days.

Although the PGE_1 treatment does not seem to have any direct and obvious

effect on the level of muscarinic receptors expressed by the NG108-15 cells it does result in a differentiation of the NG108-15 cells to a more neuronal phenotype and does result in a change in the levels of G-proteins (Mullaney et al. 1988) and the emergence of electrophysiological properties characteristic of neuronal cells (Docherty et al. 1991). Therefore the treatment of NG108-15 cells with PGE_1 will be useful in that it provides a neuronal-like cell in which to study the regulation of the m4 muscarinic receptor.

Carbachol stimulation.

The effect of carbachol on the differentiated NG108-15 cells was to reduce the levels of muscarinic receptor expressed by the cells in a dose dependent manner (figure 3.8). This down regulation occurred rapidly in the presence of high concentrations of carbachol, 100μM, reaching a maximum response within hours of stimulation. A lower concentration of carbachol, 1μM, also caused a reduction in the levels of muscarinic receptors but did not reach a maximum level within 24 hours, although receptor levels may still have been falling.

It remains to be determined whether the levels of m4 mRNA are also down-regulated by this procedure. m2 and m3 mRNA in cultured cerebellar granular cells (Fukamauchi et al. 1991) and cm2 and cm4 mRNA in cultured chick heart cells (Habecker and Nathanson, 1992) all show a reduction in levels after stimulation of the receptors with carbachol. It would therefore seem likely that the m4 mRNA levels in NG108-15 cells would also show a reduction in levels with carbachol stimulation. This will be tested using solution hybridization techniques which are sensitive enough to allow quantitation of mRNA that is present at low levels, as is the case with the m4 mRNA in the NG108-15 cell time.

Gene cloning.

Screening of a genomic cosmid library with m4 specific probes resulted in the isolation of one clone, R3-6, that contained the complete m4 coding exon and approximately 30 kb of upstream sequence (figure 4.1b). Functional studies also

showed that this clone appears to contain the complete transcription unit for the m4 gene as it will drive expression of the rat m4 transcript when transfected into IMR32 cells (figure 4.3). This clone also contains at least some of the tissue specific elements of the m4 gene as the clone will not drive expression of the rat m4 gene in CHO cells (figure 4.4), which do not endogenously express the m4 gene.

Previous studies have shown that generally approximately 5 kb of DNA sequence 5' to the transcription start site is sufficient to drive tissue specific expression (Minowa, M. et al. 1992; Possenti et al. 1992). However some genes also require some intronic regions (Vidal et al. 1990; Begemann et al. 1990; Whiting et al. 1991) or some 3' untranscribed regions (Vidal et al. 1990; Puschel et al. 1991; Whiting et al. 1991). As the position of cis genomic sequences regulating any of the muscarinic receptor subtype genes has not been determined with respect to the transcribed regions no inference can be made about the position of these regulatory sequences within the R3-6 clone. Work carried out on two other G-protein coupled receptors, the D_{1A} and D_2 dopamine receptor genes (Minowa, M. et al 1992; Minowa, T. et al. 1992; Buck et al. 1992), has shown that regulatory elements located 5' to the transcription start site are sufficient for specific expression in cell lines; however, it remains to be determined whether these elements are sufficient for complete tissue specific expression in transgenic mice.

Identification of transcribed regions.

In order to identify the transcription start site within the R3-6 clone several complementary techniques were used. This was necessary because a full length cDNA is not available for the m4 gene to aid in the location of this site within the clone. It is known that there are in the region of 300 - 600 bp of sequence missing from the 5' end of the m4 cDNA but the exact length of this sequence and the number of upstream exons are unknown. Therefore the following methodologies were employed in order to accurately size the upstream exons and position them within the genomic clone.

Primer extension analysis did not give any results even after several attempts

under varying conditions of reverse transcription. This may be due to a highly structured GC rich region at the 5' end of the transcript prematurely terminating the reverse transcription. This may also explain the inability to synthesize a full length cDNA for the m4 even when conditions reducing secondary structure are used. The lack of reverse transcriptase to produce a primer extension product has also been reported for the D_{1A} gene (Minowa, M. et al 1992).

Nuclear run on analysis and Northern blot analysis both indicated that there appear to be two transcription units present within the cosmid clone, R3-6. The results obtained, (figures 4.5 and 4.6), suggest that the m4 transcription start site is located within the 5.0 kb Bam HI fragment that spans the genomic region 7.5 kb to 2.5 kb upstream of the initiating ATG. The hybridization of further upstream fragments suggests that there is another transcription unit in the clone which spans the region 24.8 kb to 10.5 kb upstream of the m4 ATG. The results from the Northern blot analysis also show that this second transcription unit is expressed in PC12 cells but is not expressed in CHO cells. The results do not indicate, however, if this second transcription unit is in the same or in a reverse orientation to the m4 gene.

5' RACE analysis.

In order to try and identify more closely the regions of R3-6 that contain exonic sequences the 5' RACE technique designed by Frohman et al. (1988) was employed. Initial attempts at cloning the 5' RACE product were unsuccesful even though it was obvious a specific product was being synthesized (Pepitoni, S. and Buckley, N.J. unpublished observations). Incorporation of ^{32}P-dATP into the synthesized product and analysis of the probe showed that two specific bands of approximately 330 bp and 560 bp were produced (figure 4.7). Radiolabel was also incorporated into non-specific products which can be seen as a smear of DNA between 150 bp - 1500 bp. It is not clear why two independent products are produced but the smaller band may be due to a truncated transcript; however, the use of alternative exons as seen for the m2 gene (Peralta et al. 1987) cannot be ruled out. Hybridization of this probe with DNA from the R3-6 clone showed that the smallest

fragment to hybridize was a 1.2 kb Hind III/Sal I fragment that spans the region 5.2 kb to 4.0 kb upstream of the initiating ATG (figures 4.8 and 4.9). Therefore the 5' sequences for the m4 gene are located in this region of the R3-6 clone.

The position of the transcription start site within the region 5.2 kb to 4.0 kb upstream of the initiating ATG is different from the position hypothesized by Bonner (1989) who has suggested that the 5' untranslated region is located approximately 10 kb from the coding exon. However this is based on cross hybridization studies between the human and rat m4 genes and although this technique may determine regions that contain conserved sequences it does not give any indication of what type of region this is. For example the conserved region may contain a similar promoter element that lies upstream of both the human and rat m4 transcription start sites.

S1 Nuclease analysis.

An S1 nuclease analysis was performed to further identify the size of upstream exonic sequences and to determine the number of exons. A protected fragment of 460 bases for the hybridization of the DNA with PC12 RNA but not with CHO RNA is consistent with this being due to m4 specific sequences. The size of 460 bases is also almost identical to the 480 bases of exonic material that is contained within the 5' RACE probe suggesting that this is the length of missing nucleotides from the m4 cDNA. It also appears likely that this upstream sequence is completely contained within one exon although further experiments will need to be done to clarify this. Digestion of the hybridization products with a processive nuclease, such as exonuclease VII, which would not digest intronic sequences will indicate whether this 460 base fragment is indeed one exon or if it is the product of incomplete digestion with the S1 nuclease. This method will also allow the identification of any other small exons that may be present and not identified in this experiment due to their small size. S1 nuclease analysis using DNA fragments that contain some, but not all, of the 1.2 kb Hind II/Sal I fragment, in which transcription of the m4 gene initiates, will allow the precise location of the transcription initiation site to be determined.

Future studies.

Identify transcriptional regulatory elements.

Identification of the transcription start site for the m4 gene and the functional demonstration that transcriptional control elements are present within the cosmid clone, R3-6, will now allow more precise mapping of these regulatory elements. Because it is not known if the regulatory elements are present in the 5' or 3' flanking regions or in intronic regions, a marker will be engineered into the m4 transcription unit so that any product produced from the transfected DNA can be identified. For this marker a c-myc epitope will be used and this can be identified by the use of readily available antibodies to the c-myc epitope. This c-myc epitope also has the advantage in that it has been successfully used before (Monro and Pelham 1987; Chidiac et al. 1992; Von Zastrow et al. 1992). A suitable site for this c-myc epitope within the sequences encoding the i3 loop of the m4 receptor has been identified and work is now progressing to engineer this epitope into a 12 kb Kpn I fragment that contains the entire m4 gene and 4 kb of 5' flanking sequence. Once this construct has been engineered, deletions of the 3' and 5' flanking regions of the m4 gene can be performed and the DNA transfected into cell lines that do and do not express the m4 gene, to determine which of the flanking sequences are important.

Although 3' flanking and intronic sequences may be important for gene regulation 5' flanking sequences have been found to be sufficient to control the regulation of many genes. Therefore complementary studies can also be performed using 5' flanking sequences in the m4 gene ligated in front of reporter genes such as the *chloroamphenicol acetyltransferase* (CAT) and the *luciferase* (luc) genes. These reporter genes have the advantage over a c-myc epitope in that detection of expression is more sensitive and that quantitative data can be obtained more easily.

The ß-galactosidase gene is another reporter gene that is useful particularly for cytological analysis of expression in transgenic mice. Here the ß-galactosidase gene has advantages in that the assay is very sensitive, relatively straightforward and

expression can be determined at the resolution of a single cell. As we will eventually want to test expression constructs in transgenic mice and that the expression we will be interested in is in neuronal cells, a ß-galactosidase reporter vector has been constructed which contains the ß-galactosidase gene with a nuclear localization signal attached. Therefore expression of the ß-galactosidase should be restricted to the nuclei of the neurons in which it is expressed and this will facilitate the identification of the neurons which are expressing the reporter gene as only cell bodies and not processes will be labelled.

Initially constructs will be transfected into cell lines which do and do not express the m4 gene in order to identify those elements that may drive tissue specific expression. Cell lines are easy to transfect and can provide information on a large number of constructs relatively quickly. However, several problems have been associated with the use of cell lines for this type of experiment. Because many copies of the same DNA construct will be introduced into the same cell it is possible to titrate out a DNA binding protein that recognizes and binds to sequences in the promoter region if the protein is only present in limited quantities. If this DNA binding protein acts to repress transcription then transcription will be driven in those constructs which have not been bound by the protein. This will result in apparent ectopic expression of the reporter construct even though the correct regulatory elements are present. DNA that is introduced into cell lines is also not associated with chromatin but is present in a 'naked' form which may alter the way in which transcription factors interact with the DNA. These two problems may be overcome by producing stable cell lines which will contain only a few copies of the construct integrated into the chromosomes although this does lose the advantage of allowing data to be obtained quickly. One problem that has been highlighted with cell lines that cannot be solved by producing stable cell lines is that seen for the NF-L gene (Nakahira et al. 1990). For this gene a construct containing the promoter region shows tissue specific expression in transgenic mice but ectopic expression in cell lines even when stable cell lines are produced. It is thought that the DNA in the NF-L gene is altered some way, e.g. by methylation, at some point during development, resulting

in a subsequent repression of expression in some cell types.

These problems can be overcome by the use of transgenic mice which provide exquisite information on both temporal and spatial aspects of reporter gene expression. Production of transgenic mice is however labour intensive and a great deal of time is required to screen only a few constructs. A compromise between these two systems which involves the transfection of primary neurons has recently been made possible and has been used successfully to test reporter gene expression (Bejanin et al. 1992; Matter-Sadzinski et al. 1992). The analysis of reporter gene expression in primary neurons allows the analysis of a large number of constructs in cells which do not have the transformed phenotype of immortal cell lines. The most efficient use of these three systems would be to screen a large number of constructs with cell lines and primary neurons and then to produce transgenic mice with several of the more interesting constructs.

One other approach to determining which regions of the m4 promoter are important for regulation and are involved in transcription factor interactions is by the use of footprint analyses. Determining which regions of the promoter region are protected from nuclease digestion, when the DNA from the m4 gene is incubated by proteins isolated from cell lines that do and do not express the m4 gene, would then implicate these regions as regulatory elements. This assay would be of further use once elements have been completely characterized to identify the transcription factors themselves that are important in controlling the expression of the m4 gene.

Once a promoter has been identified it can be further dissected by the use of nested deletions to discover the position of enhancers and the core promoter elements within for the m4 gene the 5' flanking regions.

Regulation of levels of m4 receptors and mRNA.

Initially studies would have to be performed to determine if the levels of m4 mRNA in NG108-15 cells are down-regulated by chronic exposure of the receptor to carbachol. This would be done using solution hybridization as this is a very sensitive technique which lends itself well to quantitation.

If it is assumed (as would appear likely from previous work on muscarinic and adrenergic receptors), that mRNA levels for the m4 are reduced on chronic exposure to agonist then it will be interesting to find out which signalling pathways are involved and which genetic regulatory elements are responsible for any alteration of mRNA levels. In order to determine the regulatory elements involved stable NG108-15 cell lines could be produced containing a selection of the regulatory elements driving expression of a reporter gene, e.g. CAT. If those elements responsive to chronic stimulation by carbachol are present in the m4 promoter-CAT constructs then the down-regulation of the m4 expression should be mimicked by a down-regulation of CAT expression. By using a series of different m4 promoter-CAT constructs, those elements responsible for this regulation could be determined.

The work presented here has laid a foundation to, and hopefully provided a doorway through which the control of expression of genes which encode molecules involved in neural communication can be investigated.

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