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CONTROL OF GENE EXPRESSION DURING DIFFERENTIATION OF MURINE EMBRYONAL CARCINOMA CELLS: INVOLVEMENT OF B2 SEQUENCES

by

Chantal J. Frégeau

A thesis presented to University of Ottawa in partial fulfillment of the requirements for the degree of Master of Science in Biology

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ABSTRACT

P19 is a pluripotent embryonal carcinoma (EC) cell line which can be specifically induced to differentiate in vitro, upon treatment with retinoic acid (RA), into neuroectodermal cell derivatives, neurons and glial cells. The molecular events concomitant to the commitment of these EC cells towards the neuronal lineage and the controls of gene expression in early neuronal differentiation remain largely unknown.

A cDNA library was constructed from messenger RNAs isolated from P19 neuronal derivatives and differential hybridization analysis was used to identify sequences specifically expressed in neurons. Eleven clones were selected for further analysis. No evidence for exclusive expression of their mRNAs was found in P19 neurons or brain tissue. A second screening using end-labeled mRNA revealed that the above eleven clones shared homology with parental EC cell sequences confirming the results of the RNA blot hybridization. This suggested 1) that the screening of a cDNA library may be more efficient and accurate using end-labeled mRNA and 2) that neuronal specific sequences may correspond to low abundance mRNAs. The RNA blot hybridization indicated that two classes of cDNA clones were isolated. Five clones each recognized one RNA species while six clones reacted with small RNAs ranging in size from 200 to 600 nucleotides.
A representative cDNA clone of the second class was sequenced and found to contain a 207 base-pair insert homologous to a short interspersed repeated element of the mouse genome called B2. Further analysis on the transcription of this repetitive B2 element during neuronal development indicated that whereas the steady state level of the small B2-homologous RNAs was initially very high in P19 EC cells, the addition of RA to the culture medium reduced this level significantly during the first hour of treatment. The steady state level dropped from 100% to 80% during the first hour then was reduced to 37% by 24 hours and reached 0% in adult brain tissue. Furthermore, we observed that this decrease in the steady state level of the small B2 RNAs was not restricted to the neuronal lineage but was a general phenomenon associated with differentiation events.

By using single-stranded probes, small B2 RNAs were identified as B2(+), the transcribed B2 strand featuring the RNA polymerase III promoter whereas large B2 transcripts were primarily B2(-). A good proportion of the small B2(+) RNAs (mainly the 600 nt-B2 RNAs) was located in the nuclear fraction where the large B2(-) transcripts were exclusively found. A small portion of the small B2(+) RNAs (primarily the 200 nt B2 RNAs) was detected in polysomes but a large population of 200 nt B2 RNAs seemed to be located in the cytoplasmic non-polysomal fraction of both P19 EC cells and P19 derived-neurons. Both small B2(+) and large B2(-) transcripts were associated with the poly(A+) RNA fraction. These results suggested that the B2 repetitive elements may play a role in gene regulation at the level of post-transcription (RNA processing/splicing).
RÉSUMÉ

P19 est une lignée cellulaire de carcinomes embryonnaires capable de se différencier in vitro, de façon spécifique suite à l'induction par l'acide rétinolique, en plusieurs dérivés neuroectodermiques tels que les cellules neuronales et gliales. Ces cellules s'avèrent un système expérimental unique pour l'étude des mécanismes de contrôle d'expression génétique impliqués lors de la différenciation des cellules à caractère pluripotentiel en neurones.

Afin d'isoler certains messages ou informations génétiques exprimés exclusivement chez les neurones, une librairie de séquences d'ADN complémentaires composée de 9,800 recombinants a été construite à l'aide d'ARN messagers provenant de neurones dérivées de P19. Par la méthode d'hybridation différentielle, onze recombinants ont été isolés. Cependant, après caractérisation partielle, aucun ne s'est avéré spécifique pour les neurones. Ces onze clones ont été classés en deux catégories: 1) ceux (cinq) qui ont chacun réagi avec une espèce d'ARN messager lors de transferts Northern 2) ceux (six) qui ont reconnu de petits ARN de 200 à 600 nucléotides. Le second tamisage à l'aide de sondes d'ARN messagers kinases a révélé que tous ces clones sélectionnés étaient homologues à des séquences retrouvées chez la lignée parentale non-différenciée P19.

La détermination de la séquence d'un clone représentant de la deuxième classe a indiqué qu'il contenait
une insertion de 207 paires de bases homologue à un élément répétitif très court du génome de souris appelé B2. Une analyse plus poussée du taux de transcription de cet élément lors du développement des neurones a indiqué qu'alentour que le niveau de transcription de base des petits transcrits d'ARN homologues à B2 est initialement très élevé chez les cellules parentales P19, la simple addition d'acide ropinoïque au milieu de culture réduit ce niveau de façon significative lors de la première heure de traitement. Ce niveau de petits ARN B2 baisse de 100 % à 80 % durant la première heure, est ensuite réduit à 37 % après 24 heures et tend progressivement vers 0 % dans le cerveau adulte. Nous avons aussi observé que cette diminution n'est pas limitée à la lignée neuronale mais représente un phénomène général relié plutôt à la différenciation cellulaire.

En utilisant des sondes à brin simple, nous avons observé que les petits transcrits d'ARN sont B2(+) (brin contenant les séquences du promoteur et transcrit par l'ARN polyénoase III) alors que les transcrits d'ARN à haut poids moléculaire sont B2(-). Les petits ARN B2(+) formés de 600 nucléotides sont localisés en majeure partie dans la fraction d'ARN nucéaire où se retrouvent exclusivement les grands ARN B2(-). La majorité des petits ARN B2(+) de 200 nucléotides semble se retrouver dans la fraction d'ARN cytoplasmique non-polysomique bien qu'une certaine portion ait été associée aux polysomes. Les petits ARN B2(+) ainsi que les grands B2(-) sont également associés aux ARN polyadénylés. Ces résultats suggèrent que les éléments de répétition B2 peuvent jouer un rôle dans la régulation post-transcriptionnelle de l'expression génétique.
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<td>avian myeloblastosis virus</td>
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<tr>
<td>ARA-C</td>
<td>cytosine arabinoside</td>
</tr>
<tr>
<td>BB</td>
<td>binding buffer</td>
</tr>
<tr>
<td>BRL</td>
<td>Bethesda Research Laboratories</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CIAP</td>
<td>calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>CRABP</td>
<td>cellular retinoic acid binding protein</td>
</tr>
<tr>
<td>CS</td>
<td>calf serum</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>ds-cDNA</td>
<td>double-stranded complementary DNA</td>
</tr>
<tr>
<td>EC</td>
<td>embryonal carcinoma</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EIA</td>
<td>early region-I of adenovirus</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>³H-GABA</td>
<td>tritiated gamma-aminobutyric acid</td>
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<tr>
<td>X-GAL</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's buffered salt solution</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HMW</td>
<td>high molecular weight</td>
</tr>
<tr>
<td>hn-RNA</td>
<td>heterogeneous RNA</td>
</tr>
<tr>
<td>ID</td>
<td>Identifier</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LINES</td>
<td>long interspersed repetitive sequences</td>
</tr>
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<td>LLC</td>
<td>Lewis lung carcinoma</td>
</tr>
<tr>
<td>LMW</td>
<td>low molecular weight</td>
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<tr>
<td>α-MEM</td>
<td>alpha minimal essential medium</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MIF</td>
<td>mouse interspersed fragment</td>
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<tr>
<td>MOPS</td>
<td>3-[(N-morpholino) propane-sulfonic acid</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>PIPES</td>
<td>piperazine-N,N'-bis[2-ethane sulfonic acid]</td>
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<tr>
<td>poly(A⁺)</td>
<td>polyadenylated RNA</td>
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<tr>
<td>pre-mRNA</td>
<td>precursor - messenger RNA</td>
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<tr>
<td>RA</td>
<td>retinoic acid</td>
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<td>RF</td>
<td>replicative form</td>
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<td>RNP</td>
<td>ribonucleoprotein particles</td>
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<td>rRNA</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>short interspersed repeated sequences</td>
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<td>ss-cDNA</td>
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<tr>
<td>TCA</td>
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<tr>
<td>TE</td>
<td>tris-EDTA [10 mM Tris-HCl, 1 mM EDTA, pH 8.0]</td>
</tr>
<tr>
<td>Tfb</td>
<td>transformation buffer</td>
</tr>
<tr>
<td>VRC</td>
<td>vanadyl-ribonuclease complex</td>
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CHAPTER I

INTRODUCTION

Embryonic development proceeds by way of biosynthetic and restructuring events that result in the continued molding of tissues and organs into highly restricted and specialized states required for adult function. In each of the various and complex steps of embryonic development, batteries of genes must be activated or repressed in response to various stimuli. Following the commitment of cells to overt differentiation into organs comprising multiple specific cell types, a spectrum of messenger RNA (mRNA) molecules are synthesized, processed and exported to the cytoplasm. This population of de novo synthesized mRNAs, encoding specific protein products, is responsible for the variety of particular cellular phenotypes observed (Hastle and Bishop, 1976).

Little is known about the mechanisms by which the pluripotent embryonic genome initiates expression of tissue-specific functions as differentiation progresses or how the expression of genes is restricted to those appropriate to a cell lineage. However, with recent advances in eukaryotic molecular genetics, new avenues for investigating structure, organization and expression of specific genes have resulted in a better understanding of the mechanisms of gene regulation during differentiation.

Differentiation is currently defined in terms of qualitative changes in gene expression. These changes are
elicited either by intrinsic or extrinsic factors whose effects are mediated by alterations in chromatin structure, modified deoxynucleotides, DNA rearrangements, transcriptional regulation, post-transcriptional processing, and the modulation of translational as well as post-translational events (Brown, 1981).

The purpose of this introduction is to outline the current state of knowledge pertaining to nucleotide sequences involved in the control of transcription and the processing of RNA messages.

1.1 Controlling transcriptional activity - Promoter and Enhancer Sequences

It has been generally accepted that the control of transcriptional initiation of mRNA is the most important step in determining phenotype. By altering the transcriptional states of sets of genes during differentiation, a spectrum of cellular phenotypes is obtained. This idea has been strongly supported by the observation that coordinate changes in the abundance of multiple RNA species occur during embryonic development in organisms such as sea urchins (Laskey et al, 1980), Xenopus laevis (Sargent and David, 1983), and the mouse (Vasseur et al, 1985b; Linzer and Nathans, 1983), as well as during the differentiation of Dictyostelium discoideum (Blumberg and Lodish, 1980; Barklis and Lodish, 1984), and of the mouse T lymphocyte subsets (Lobe et al, 1986). By comparing the sequences of genes that exhibit coordinate expression,
Investigators have been able to identify two highly conserved elements: the TATA and CCAAT boxes located approximately 30 base pairs (bp) and 80 bp upstream from the mRNA cap site. Deletion-mutation-linker scanning procedures followed by reintroduction experiments confirmed the role of the CCAAT box in the level of promotion and of the TATA box as a site for transcriptional initiation. The significance of these highly conserved sequence elements has been verified both in vitro and in vivo in several systems (Magram et al, 1985; Dierks et al, 1983; Charnay et al, 1985; Renkawitz et al, 1984; Searle et al, 1985; Chada et al, 1985; Dean et al, 1983; Kondoh et al, 1983; Zinn et al, 1983; Krümlauf et al, 1985).

A second class of regulatory elements—the enhancer elements—was identified at the same time. These elements can augment the transcriptional activity of different genes in the presence of promoter elements (Khoury and Gruss, 1983; Banerji et al, 1981; Laimins et al, 1982a; Moreau et al, 1981; Wasylyk et al, 1983; Treisman and Maniatis, 1985). Enhancer elements were first identified in the simian virus 40 (SV40) genome (Benoist and Chambon, 1981; Moreau et al, 1981) but have subsequently been found in other viruses (de Villiers et al, 1982) including retroviruses (Laimins et al, 1982a; Yoshimura et al, 1985; Mitsialis et al, 1983) and have recently been identified for cellular genes (Queen and Baltimore, 1983). These elements are usually located at the 5'-flanking region of genes (Fromm and Berg, 1982; Rosen et al, 1985; Gluzman and Shenk, 1983) but in some instances have been found within intervening sequences or at the 3'-end of a gene (Gillies et al, 1983; Queen and Baltimore, 1983; Picard
and Schaffner, 1984; Banerji et al, 1983; Laimins et al, 1984). Enhancer elements function in an orientation-independent manner and over relatively long distances (Bos, 1983). Enhancer activity can be tissue- or species-specific (de Villiers et al, 1982; Laimins et al, 1982b; Yoshimura et al, 1985; Berg et al, 1983) suggesting that their activity may be regulated by factors acting in trans. Recent studies have demonstrated that transcription of certain genes proceeds via the formation of a stable complex between the enhancer element and a trans-acting factor (Zaret and Yamamoto, 1984; Mercola et al, 1985; Ephrussi et al, 1985). Such a factor could then activate the promoter either by releasing another factor specific for the promoter (direct action) or by modifying the chromatin structure which would allow RNA polymerase II to gain access and bind to the promoter (indirect action) (Zaret and Yamamoto, 1984). Some enhancers may even require more than one factor to become activated and the same factor may activate more than one enhancer (Yamamoto, 1984; Mercola et al, 1985). Although the mechanism of action of enhancers is still speculative, such a model allows one to imagine how enhancers could act as dynamic modulators of gene transcription.

The debate as to which of these regulatory elements (promoter or enhancer) is more important in conferring tissue-specificity still prevails. For example, the immunoglobulin enhancer functions only in lymphoid cells but the promoter sequence of an immunoglobulin \(k\) light-chain gene also shows specificity to lymphoid cells regardless of the presence of the enhancer sequence (Foster et al, 1985).
1.2 Control at the Post-Transcriptional Level

RNA processing (splicing, translational or post-translational processing) may represent important sites for the regulation of gene expression. There are examples in which two tissues express different mRNAs transcribed from the same gene by differentially processing the same primary transcript (Amara et al, 1982; Henikoff et al, 1983; Kress et al, 1984; Capetanaki et al, 1983; Schwarzbauer et al, 1983) or by using different promoters (Darnell, 1982; Carlson and Botstein, 1982; Schibler et al, 1983; Nabeshima et al, 1984; Kitamura et al, 1983). Alternative RNA processing has been shown to be a major way of regulating genes in neural tissues (Amara et al, 1984; Rosenfeld et al, 1983; Rosenfeld et al, 1984). This mode of RNA processing occurs in a tissue-specific fashion to produce alternative polypeptide products and therefore, serves to increase the diversity, in this case, of neuropeptides generated from a single locus.

During development of B cells, it appears that alternative poly(A) site selection, in association with alternative RNA splicing dictate production of mRNAs encoding the membrane form (um) or secreted forms (us), respectively, of immunoglobulin M (Rogers et al, 1980; Alt et al, 1980; Early et al, 1980) or immunoglobulin D (Cheng et al, 1982). Similar events may occur in expression of several developmental genomic loci in Drosophila (Rozek and Davidson, 1983).

What determines poly(A) site selection is still to be understood but as in the cases of the brain calcitonin and
thyroid C cell-calcitonin gene related peptide genes, the mechanism may resemble that regulating the formation of the 3' termini of sea urchin histone H3 mRNAs (Stunnenberg and Birnstiel, 1982) which involves the action of a 60 nucleotide RNA species present as a small ribonucleoprotein particle (RNP) (Calli et al, 1983).

Vannice et al (1984) reported that glucocorticoids stimulated the accumulation of α-acid glycoprotein mRNA in rat hepatoma cells by inducing an RNA processing factor that allowed production of stable transcripts. This suggested that RNA processing factors or enzymes involved in processing may influence gene expression. Liston et al (1984) studied the processing of the precursor protein, proenkephalin, in two different bovine tissues and showed that two different processing pathways existed for this neuropeptide precursor.

Palatnik et al (1984) presented a model in which transcriptional and translational controls could be coupled by altering the state of adenylation of the pre-existing mRNA population. In Dictyostelium discoideum, a critical poly(A) length regulated the pattern of protein synthesis by affecting the efficiency with which mRNAs can interact with the translational machinery. The model allows radical changes in the pattern of protein synthesis without wholesale destruction of preexisting mRNAs.
1.3 \textbf{Families of genomic repetitive sequences: possible regulatory elements}

1.3.1 \textit{Low abundance repetitive sequences}

Large fractions of the genomes of many eukaryotes are composed of short, repeated segments of DNA which are interspersed with single-copy sequences. These short repeats can be found within introns, within either terminal of genes or within transcribed regions and they are present in moderately low abundance (100 to 300 copies) in the genome of the host organism. Their constant occurrence in coordinately expressed mRNAs suggests a role in regulation of gene expression but the mechanism of action is still obscure.

Examples of such repetitive elements are the rat identifier (ID) sequence (82 bp) (Sutcliffe et al., 1984a; Sutcliffe et al., 1984b), the Dictyostelium discoideum sequence (35-150 bp) (Zuker and Lodish, 1981; Kimmel and Fortel, 1985), the repeat in the Notch locus of Drosophila melanogaster (93 bp) (Wharton et al., 1985), the element in yeast genes (9 bp) (Donahue et al., 1983), the short repeat shared among the Drosophila heat-shock genes (15 bp) (Pelham, 1982), the sequence of 150-200 bp in sea urchin (Constantini et al., 1980), the repeat in Xenopus laevis (Davidson and Posakony, 1982) and the sequence shared by homeotic genes in Drosophila melanogaster (Scott and Weiner, 1984).
1.3.2 **High abundance repetitive sequences**

Highly repetitive sequences comprise between 15% and 80% of the total genomic DNA in certain organisms (Britten and Davidson, 1969). Several of these highly repeated non-satellite families of DNA sequences have been reported in the mouse (Bennett et al., 1984; Vizard and Yarsa, 1984) and human (Schmid and Jelinek, 1982; Singer and Skowronski, 1985; Sharp, 1983). These highly repeated sequences have been classified into two groups: the short interspersed repeated sequences (SINES) and the long interspersed repeated sequences (LINES). These sequences range from a few hundred to several thousand nucleotides in length and are interspersed throughout the genomic DNA around and sometimes within structural genes. They are present at about 10,000 to 300,000 copies per haploid genome.

The LINES family of repeated sequences comprises the primate Kpn I block repeats (Singer and Skowronski, 1985; DiGiovanni et al., 1983; Grimaldi et al., 1984; Singer et al., 1983) and the mouse MIF-1/BamHI family (Bennett et al., 1984; Bennett and Hastie, 1984; Vizard and Yarsa, 1984). Family members are very heterogeneous in size and only a fraction are over 6 Kb in length. Sequence analysis has revealed that LINE-1 elements are processed pseudogenes (Martin et al., 1984; Singer and Skowronski, 1985). Recently, cytoplasmic polyadenylated RNA (approx. 6.5 Kb) isolated from the human teratocarcinoma cell line Ntera-2 clone D1 was found to hybridize to cloned LINE-1 DNA probes. This mRNA thus represents a transcript originating from one or more functional genes in the LINE-1 family. The authors have
Further suggested that functional LINE-1 genes may be specifically active in cleavage-stage primate embryos as well as in other mammals (Skowronski and Singer, 1985). In fact, a similar situation has recently been reported during mouse development (Vasseur et al., 1985a). The above observations are strongly suggestive of the involvement of LINE-1 repeat sequences in the process of cell division.

The SINES family includes the Alu I family in primates (Schmid and Jelinek, 1982; Sharp, 1983) and the murine BI (Alu I equivalent) (Krayev et al., 1980) and B2 families (Krayev et al., 1982). These repeats are usually less than 500 bp in length.

A. The Alu I family

The Alu I elements range in length from 135 bp in the mouse to 300 bp in man and have long consecutive runs of deoxyadenosine poly(dA) at their 3' ends. They share sequence homology to the small cytoplasmic 7S and 4.5S RNAs and most are flanked by direct repeats of sequences 7-20 nucleotides long. Flanking direct repeats are characteristic of mobile elements that have been integrated in the genome; the repeats are formed by replication from staggered nicks at the site of insertion. The origin of the integrated Alu-like elements is suggested by the high sequence homology and structure that exists between these elements and 7SL RNA genes, RNA polymerase III genes of known function. Many Alu members could be amplified 7SL RNA pseudogenes (Daniels and Deininger, 1985). The intragenic RNA polymerase III promoter that most Alu members contain, initiates transcription at the 5' end of the
repeated DNA sequence and has been proposed to facilitate the transposition and amplification of these sequences by an RNA-intermediate mechanism (Sharp, 1983). Many or all of these AluI elements have or once had the capacity to transpose during evolution. The diffusion throughout the genome of transposable cis-regulatory sequences and their occasional insertion at productive locations in the vicinity of structural genes might have provided a convenient means of creating novel coordinately induced structural gene networks. Furthermore, as only 1% of mammalian genomic DNA is thought to encode sequences expressed as protein, most of the remaining 99% may be functionally neutral for insertions and deletions and may be derived and maintained by a continuous flux of such events (Davidson and Britten, 1979).

The very high degree of homology (80%) between Alu I fragments and small RNA species (7S) suggested that these repeats may be involved during the processing of mRNA by promoting the binding of 7S RNA to one or more of the six RNP proteins comprising the hnRNP particle (Blin et al., 1983; Walter and Blobel, 1982). It is also possible that similar complexes are formed in the nucleus between proteins and the 1-5% of hnRNA sequences that contain Alu elements derived from introns and untranslated regions. These complexes are required for proper splicing and subsequent transport of mRNA to the cytoplasm (Pederson, 1983; Padgett et al., 1983).

The significance of Alu I sequences remains controversial. Some reports suggested that the Alu I repeats may represent processed 7S RNA pseudogenes (Ullu and Tschudi,
1984). This hypothesis cannot be excluded since only indirect evidence supported the former hypothesis in which the repeats were thought to be involved in the processing of primary transcripts.

B. **The B2 family**

1. **Structural characteristics of the B2 elements**

The other SINES family of highly repetitive sequences is the rodent B2 family (Krayev et al, 1982). These short repeats of about 190 bp in length share many characteristics with the B1 members. They contain sequences homologous to the split RNA polymerase III promoter and its termination signal, they are usually flanked by short direct repeats typical of mobile genetic elements and, as opposed to B1 elements, they contain a polyadenylation signal followed by the A-rich region of about 8 to 20 nucleotides. B2 sequences are often found within introns of protein-coding genes such as the rat growth hormone gene (Page et al, 1981) and the mouse cytokeratin endo A gene (Vasseur et al, 1985b). They have also been detected at the 5' end termini of the murine \( \alpha \)-fetoprotein gene (Kioussis et al, 1981) and within the 3' untranslated region of the murine major histocompatibility complex (MHC) class I genes (Kress et al, 1984; Lalanne et al, 1982). As a result, these repeats are very numerous in the nuclear heterogeneous RNA (pre-mRNA) but rare in the mature mRNA (Kramerov et al, 1979; Ryskov et al, 1983).

The strong homology (64 %) observed between the B2 elements and serine-transfer RNA genes suggested that B2
members may have evolved from tRNA genes (Daniels and Deininger, 1985). Transposition and amplification of these B2 repeats may be mediated by the intragenic RNA polymerase III promoter found in most of these B2 copies. As suggested by Daniels and Deininger (1985), the finding that mouse B2 elements may have evolved from RNA polymerase III genes of known function could imply that most of the members of these families are simply pseudogenes with no physiological function.

2. **B2 transcription and post-transcriptional processing**

B2 small RNAs are not formed in the course of pre-mRNA processing but they are transcribed independently by RNA polymerase III from many different B2 sequences in the genome (Kramerov et al, 1985b). B2 RNA polyadenylation occurs post-transcriptionally and the size of the poly(A) tails may be as long as 100-120 nucleotides whereas in genomic copies of B2, the oligo(dA) length does not exceed 7-15 nucleotides. This difference in poly(A) length gives rise to the size variations usually observed in B2 transcripts. Size heterogeneity of the small B2 RNAs also results from the extension of transcription beyond the usual 3' -terminus due to the loss of one T from the RNA polymerase III termination sequence TCTTT. Sequence TCTTT does not terminate transcription and RNA polymerase III moves along until it encounters another oligo(dT) block (Kramerov et al, 1985b). Haynes and Jelinek (1981) showed the formation of extended RNA polymerase III transcripts *in vitro* when cloned hamster B2-like sequences were used as templates.
Kramerov and his colleagues (1985b) showed that a similar phenomenon occurs *in vivo* by isolating a 49 nucleotide extended transcript.

3. **Elevated levels of B2 RNAs associated with cell proliferation**

The small B2 RNAs were initially observed in Ehrlich carcinoma and MOPC plasmacytoma cells where their levels were higher than in mouse liver cells (Kramerov et al., 1982; Kramerov et al., 1985a). Reports by Scott et al. (1983) and Singh et al. (1985) added significance to this initial observation by showing that small RNAs are induced in various transformed mouse cells (NIH3T3 transformed with SV40 and mouse SV3T3 cells transformed with polyoma virus). The mechanism of control leading to this amplification is not known. In the case of mouse cells transformed with adenovirus, it has been shown that a product from the EIA region is produced and acts as an enhancer of RNA polymerase III transcription (Rosenthal, 1985; Svensson and Akusjarvi, 1985). Elevated levels of B2 sequences have also been observed in cytoplasmic RNA from serum-stimulated mouse embryo fibroblasts (Edwards et al., 1985). This again implies that there may be a factor(s) in the serum involved in enhancing the transcription of RNA polymerase III. These factors are somehow different from epidermal growth factor, fibroblast growth factor, insulin or 12-tetradecanoylphorbol-13-acetate, which have been shown to be poor inducers of B2 RNAs. Small B2 RNAs have also been detected in very high amounts in mouse embryonal carcinoma cell lines (Bennett
et al., 1984; Murphy et al., 1983). In contrast to the high level of B2 RNAs found in undifferentiated and transformed cells, reduced levels of B2 RNAs have been documented in some normal tissues such as heart and liver (Gregoryan et al., 1985) and in some EC differentiated derivatives such as F9 endoderm (Murphy et al., 1983).

The abundance of B2 small RNAs in tumor cells, transformed cells or serum-stimulated cells suggested that B2 repeats may be involved in cell proliferation. The accumulation of B2 RNAs in the very first events of embryogenesis (cleavages) also suggested that regulation of B2 transcription may be related to cell division (Vasseur et al., 1985a). However, the demonstration that B2 sequences were restricted to the ectoderm and mesoderm in 7.5 day embryos also implies that B2 transcription may be associated with particular programs of differentiation.

The time of activation and the role(s) played by the small B2 RNAs in the murine cell are two major issues which remain obscure. Many suggestions have been made to the effect that B2 repeats may function at the transcriptional, post-transcriptional and/or translational level.

4. Involvement of B2 repeats in transcription

The evidence that B2 repeats are acting at the transcriptional level is indirect. Sutcliffe et al. (1982) found a series of pre-mRNA molecules in rat nerve cells which share the same 82-nucleotide sequence in the introns,
which they designated as ID-sequence. Similar ID sequences have now been identified in mouse and hamster genomic DNA (Sapienza and St-Jacques, 1986). It was suggested that ID sequences may be involved in the regulation of brain-specific gene expression (Sutcliffe et al, 1984b). These ID sequences possess 65% homology with the 5' part of B2 sequence (homology reaches 78% in a 35 bp stretch of these repeats). In cells in which these brain-specific genes are expressed, the ID elements themselves are also transcribed into small poly(A⁺) RNA by RNA polymerase III (Milner et al, 1984; Sutcliffe et al, 1984b). However, as opposed to B2 RNAs, these ID RNAs are detectable only in post-natal samples, no transcripts are detectable in early embryos (Sutcliffe et al, 1984b). Thus, the ID RNA seems to be associated with differentiation events whereas B2 RNA seems to relate to proliferation or the transformed state. In addition, the ID sequences are more numerous in small hnRNAs and mRNAs isolated from rat brain than from rat liver or kidney, showing a tissue-specificity (Sutcliffe et al, 1984a; Sapienza and St-Jacques, 1986). The same situation seems to prevail in the mouse and hamster total RNA samples where ID-homologous transcripts appear more restricted to brain than liver or kidney (Sapienza and St-Jacques, 1986). As shown in this report and by other studies, this does not seem to be the case for B2 RNAs; no tissue- nor lineage-specificity has been found.
5. Involvement of B2 repeats in post-transcriptional events

Evidence for the involvement of B2 elements in post-transcription (RNA processing) and translation regulation is also indirect. The B2 sequence shows little homology to 4.5S RNA (Krayev et al., 1982) which interacts with high molecular weight RNA in a sequence-specific manner in the nucleus and allows for proper processing and transport of mRNA across the nuclear envelope (Harada et al., 1979; Zieve, 1981). A recent report demonstrated that in the cell, B2 RNA is associated with proteins as a constituent of light RNP (ribonucleoprotein) particles as well as of free informosomes (Kramerov et al., 1985a). It was further shown that roughly a quarter of B2 RNA molecules are bound to heavy cytoplasmic RNP and seem to be associated with the mRNA of the RNP complex. By analogy with other small RNA, this favours the involvement of B2 RNA in the regulation of mRNA transport or translation (Zieve, 1981).

Other provocative observations have recently been made. It has been known that B2 carries a functional polyadenylation signal (Krayev et al., 1982). This signal can be used by the B2 gene itself (Kramerov et al., 1985b) or by any other gene upon transposition of a B2 element into that particular gene. The only example known to date of such an occurrence is for the mouse MHC class I genes in which a B2 element integrated into the 3' untranslated region of an H-2 gene thereby producing another polyadenylation site (Kress et al., 1984). As a result, the same antigen (H-2D) is produced from two different mRNAs utilizing one or the other polyadenylation
signal. The second polyadenylation signal donated by B2 RNA is efficiently used and demonstrates a biologically important function for B2 repeats. These reports are provocative since many neuronal genes are regulated by different modes of RNA processing (Rosenfeld et al., 1984) and it would be of interest to analyse their 3' untranslated regions to detect remaining B2 sequences that could act as a second polyadenylation signal.

Thus, although the significance of the B2 elements remains obscure, the possibility remains that B2 may play a role at more than one level and that some copies play functional roles while yet others represent "junk DNA" or pseudogenes and are thus non-functional.

1.4 Embryonal carcinoma cells as a model system for the study of gene expression during differentiation

Despite the recent tremendous increases in our knowledge of eukaryotic genes, we have only just scratched the surface in our understanding of their regulation. Sequences involved in the initiation of transcription have been identified along with enhancer elements and repetitive sequence families. Yet, their role in determining the transcriptional potential of the genes to which they are associated during the course of embryogenesis and cytodifferentiation remains elusive.

The establishment of embryonal carcinoma (EC) cell lines has greatly facilitated the study of the mechanisms of
control underlying tissue-specific gene expression in early stages of mouse embryogenesis. EC cells are derived from the pluripotent stem cells of teratocarcinomas and share antigenic, biochemical and morphological characteristics with early undifferentiated embryonic cells (Martin and Evans, 1975; Jones-Villeneuve et al, 1983; McBurney et al, 1982). Upon variations in culture conditions, many EC lines undergo differentiation into derivatives of all three germ layers in vitro. Following chemical induction with dimethyl sulfoxide (DMSO), P19 EC cells differentiate into the mesodermal derivatives of skeletal and cardiac muscle whereas retinoic acid (RA) induction will cause the same cells to develop into the neuroectodermal cell derivatives of neurons, glial cells and fibroblast-like cells (McBurney et al, 1982; Jones-Villeneuve et al, 1983).

The validity of EC cells as a model system has been substantiated by the ability of some EC lines to participate in the development of normal chimeric mice after injection into blastocysts (Rossant and McBurney, 1982). As opposed to intact embryos, the EC cell system can be easily manipulated and can provide large amounts of material. EC cells can be grown easily in large numbers and as pure cultures and their ability to differentiate into mature cell types can be used as a convenient model for normal embryonic development to study early controls of tissue-specific gene expression. We therefore proposed to study control mechanisms underlying neuronal differentiation in P19 EC cells by identifying genetic information expressed preferentially in either undifferentiated or differentiated cells.
Our approach in investigating the regulation of tissue-specific gene expression during early murine development via mRNA population analysis is based primarily on the hypothesis of Hastie and Bishop (1976). They postulated that since the phenotype of any given cell is operationally governed by the proteins it expresses, each histological cell type comprised within the organism would in turn be characterized by a restricted set of abundant mRNAs encoding distinct proteins, vitally important to the end-stage function(s) of the cell. This hypothesis has now been verified. It further suggests that the early stages of development or cyto-differentiation would yield a different mRNA population, specifying protein effectors involved in the initial process of "determination".

This experimental strategy (i.e. to isolate and characterize those mRNAs expressed at elevated levels in the cells or tissues of interest) has proven successful in several systems including the study of the heat-shock response in various species (Findly and Pederson, 1981; Bensaude and Morange, 1983), the molecular cloning of the gene specifying $\beta$-interferon (Gray and Goeddel, 1982) and the isolation of a number of myoblast-specific genes including those of six contractile proteins (Hastings and Emerson, 1982). More recently, the use of a "differential hybridization" scheme has allowed the molecular cloning of several brain-specific genes (Milner and Sutcliffe, 1983) and the isolation of the T-cell antigen receptor gene (Hedrick et al, 1984; Yanagi et al, 1984) as well as having facilitated efforts to characterize sequences involved in cell-cycle progression (Hirschhorn et al, 1984) and gastrulation (Sargent and David, 1983; Jonas et al, 1985).
1.5 **Thesis project**

Some of the RNA transcripts synthesized during the course of early neuronal differentiation should be different from those expressed in the undifferentiated parental or progenitor cells. Substantiation of this working hypothesis is provided by evidence that the artificial induction of neuronal differentiation in vitro of several embryonal carcinoma cell lines (including P19) is accompanied by noticeable alterations in the profile of proteins expressed. Eddé and his colleagues (1983) have thus identified through the use of two-dimensional gel electrophoresis two proteins present only in cells with the neuronal phenotype. One of these appeared to be synthesized de novo whereas the other (whose presence was detected in the parental EC cells) appears to be amplified upon differentiation. Furthermore, Jones-Villeneuve et al. (1983) reported that P19 derived-neurons were characterized by the presence of such markers as neurofilaments, acetylcholine esterase, and the receptors for both γ-amino-butyric acid and acetylcholine. These observations led us to believe that by constructing a cDNA library from P19 derived neurons and screening this library through the use of a differential hybridization scheme, coding sequences corresponding to these and other as yet unidentified gene products specifying the neuronal phenotype could be readily identified, isolated and their structure analysed.

Murine embryonal carcinoma cells provide a model system with which to study the mechanisms of control underlying tissue-specific gene expression in the early events of development. Following induction with retinoic acid
(5 x 10^{-7} M), the P19 EC cell line can differentiate specifically into the neuroectodermal cell derivatives neurons and glial cells found in the intact embryo. To isolate genomic information expressed exclusively in such embryonic neurons, I constructed a complementary DNA library from P19 differentiated cells and used the differential hybridization procedure to detect neuronal-specific sequences that could be analysed and lead to a better understanding of early tissuespecific regulation.

The thesis takes the following form: Chapter II contains the details of the experimental procedures which were used; Chapter III focuses on the results obtained for the screening of the P19 neuron-enriched cDNA library and the reasons why the results came as a surprise; Chapter IV documents the full characterization of one abundant sequence detected in the library (mouse B2 repetitive element) and the novel data on the transcription rate of the small B2 RNAs during neuronal development. Finally, Chapter V describes a model for the mechanism of action of retinoic acid on B2 transcription and gives suggestions to define a role for B2 repeats in controlling gene expression during early murine differentiation.
CHAPTER II

MATERIALS AND METHODS

2.1 Cell Lines and Culture Techniques

The P19 line of embryonal carcinoma (EC) cells was derived from a primary teratocarcinoma induced by grafting a 7.5 day C3H embryo into an adult testis (McBurney and Rogers, 1982). These pluripotent cells are euploid, present a normal male karyotype and can grow rapidly in culture in the absence of feeders. They can form a variety of normal embryonic tissues when injected into blastocysts (Rossant and McBurney, 1982) or when treated with various drugs in vitro (McBurney et al, 1982; Jones-Villeneuve et al, 1982; 1983). Upon induction of differentiation with various concentrations of retinoic acid (RA), P19 EC cells can either form skeletal and cardiac muscle ($10^{-8}$ M) or neurons, glial cells and fibroblast-like cells ($5 \times 10^{-7}$ M). When dimethyl sulfoxide (DMSO) (1%) is used as a chemical inducer however, the spectrum of cell types is mainly restricted to muscle cells.

P19S18 is a cell line derived from a single P19 cell. P19S1801Al is a 6-thioguanine and ouabain resistant subclone of the P19 parental cell line (McBurney et al, 1982). The P19S18RAC65 (RAC65) and P19S1801AID3 (D3) are mutant cell lines selected for their ability to grow continuously in the presence of $10^{-5}$ M RA (Jones-Villeneuve et al, 1983) and 1% DMSO (Edwards and McBurney, 1983). RAC65 cells do not differentiate into neurons at concentration of RA as high as $10^{-5}$ M and D3 cells do not form muscle at a concentration of
1 % DMSO whereas the parental cell line does. The RAC65 D+ cell line is a subclone of RAC65 which forms more muscle cells than the parental cell line at the usual concentration of DMSO (obtained from Steven Smith). The P19 (ras\(^+\))-1 cell line is a subclone of P19 EC cell line which has been transfected with the human EJ ras oncogene (Shih and Weinberg, 1982). This particular cell line expresses the ras product (Bell et al, 1986). The P19 (ras\(^+\))-1 fibroblast cell line is a line of transformed fibroblasts derived from RA-treated P19(ras\(^+\))-1 cells. These cells are immortal and anchorage independent (Bell et al, 1986). The P19(ras\(^-\))-1 cell line is a subclone of P19 EC cell line which has been also transfected with the human EJ ras oncogene but does not express the ras product. An immortal but anchorage dependent cell line derived from P19 (ras\(^-\))-1 is 3RAP1 (Bell et al, 1986). NIH3T3 is an immortal mouse fibroblast anchorage dependent cell line which was given by Cliff Stanners (Jaichelli et al, 1969). F9 clone 9 is an endodermal-like cell line derived from RA treatment of the nullpotent F9 EC cell line (given by David Solter) (Strickland and Mahdavi, 1978). The L6 cell line (Yaffe, 1968) is a rat myoblast cell line which was given by Bill Sanwal. The human neuroblastoma cell line GM3320C (Tymilowicz et al, 1970) was obtained from the N.I.G.M.S. Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, N.Y.

All cell lines except for the human neuroblastoma cells were cultured in alpha minimal essential medium (\(\alpha\)-MEM) (Stanners et al, 1971) (Gibco Laboratories, Grand Island, N.Y.) supplemented with 2.5 % (v/v) fetal calf serum (FCS) (Gibco Laboratories, Chagrin Falls, Ohio) or Clex serum.
(Dextran Products Limited, Scarborough, Ontario) and 7.5 % (v/v) calf serum (CS) (Animal Health Laboratories Inc., Toronto, Ontario) and 500 µg/ml penicillin and 100 µg/ml streptomycin. They were maintained at 37°C in a 5 % CO₂ atmosphere. The human neuroblastoma cells were grown in α-MEM supplemented with 10 % (v/v) fetal calf serum along with both antibiotics. They were allowed to grow on gelatin coated (0.1 %) tissue culture dishes.

For the large scale preparation of P19 undifferentiated EC cells, roller bottles were used (Canlab, Toronto, Ontario). Empty roller bottles were kept at 37°C with cap loosened for 30 minutes to one hour in 5 % CO₂ atmosphere. Approximately 250 ml of α-MEM supplemented with 2.5 % FCS/7.5 % CS were added along with four confluent 100mm plates of P19 EC cells (3 X 10⁷ cells per bottle). The bottles were placed on the roller machine and the speed was first set at low for a few hours to allow the cells to stick to the inner surface of the bottles. It was then increased to a higher level. These cells were grown for one to two days or until a high density was reached (5 X 10⁸ cells per bottle.)

Differentiation of P19 EC cells into neuroectodermal cell derivatives was carried out using two different procedures:

A. Cells in exponential growth were treated with Ca⁺⁺ and Mg⁺⁺ free phosphate buffered saline (PBS) containing 0.025 % trypsin and 1 mM EDTA to remove them from the surface of the tissue culture dish (Canlab, Toronto, Ontario). They were plated at a concentration of 10⁵ cells per ml into a bac-
teriological grade Petri dish (Canlab, Toronto, Ontario) where they aggregated spontaneously (Martin and Evans, 1975). The medium supplemented with RA at a concentration of $5 \times 10^{-7}$ M was replaced every two days. After four days in suspension, the aggregates were washed three times with PBS and then placed into tissue culture dishes into medium without drug. Twenty-four hours after plating, the second drug, cytosine arabinoside (ARA-C) (5ug/ml) was added to the medium and aggregates containing neurons (90%) were harvested for RNA isolation two days later, i.e., day 8 from the initiation of the aggregation. In some instances, the P19 aggregates were left ten days in medium containing $5 \times 10^{-7}$ M RA then were washed three times with PBS and transferred to medium depleted of the drug for a further seventeen days. They were then plated on fibronectin (10 ug/ml) (Sigma Chemical Co.) and collagen (Vitrogen 100; Flow Laboratories, Mississauga, Ontario) coated dishes. ARA-C was added to the medium the next day and aggregates were scored for neurons two days later, i.e., 30 days after the initiation of aggregation.

B. Cells in exponential growth were treated with Ca$^{++}$ and Mg$^{++}$-free phosphate buffered saline (PBS) containing 0.025% trypsin and 1 mM EDTA to remove them from the surface of the tissue culture dish. They were plated at a concentration of $10^5$ cells per ml into a tissue culture dish, allowed to grow in monolayer in the presence of RA ($5 \times 10^{-7}$ M) until they reached confluency ($2 \times 10^6$ cells per ml); usually two days and then washed twice with 5 ml of PBS. They were trypsinized and plated at the usual concentration
of $10^5$ cells per ml into a bacteriological grade Petri dish to initiate aggregation (Martin and Evans, 1975). After two days in suspension in medium depleted of the drug, the cells were plated into tissue culture dishes. Twenty-four hours after plating, the second drug ARA-C (5 μg/ml) was added to the medium and aggregated containing neurons (90%) were harvested for RNA isolation two days later, i.e. at day 8.

Differentiation of P19 EC cells into fibroblasts was performed as follows: cells in exponential growth ($1 \times 10^6$ cells per ml) were treated as previously described with trypsin/EDTA and were plated at a concentration of $10^5$ cells per ml into tissue culture dishes in α-MEM supplemented with RA at a concentration of $5 \times 10^{-7}$ M. They were grown until subconfluency was reached and were then passaged at low density in the same culture medium and dishes. This last step was repeated until a pure fibroblast culture was obtained; usually eight to ten passages were required.

Differentiation of P19S1801A1 and RAG65 D+ cell lines into muscle-containing cultures was performed by aggregating the cells as previously described in method A except that DMSO was used at a final concentration of 1% in both cases and that ARA-C was omitted.

2.2 Preparation of drugs

Retinoic acid (all-trans-retinoic acid; Eastman Kodak Co., Rochester, N.Y.) was prepared as a stock solution of $10^{-2}$ M in DMSO and stored at 4°C in the dark. The stock
solution was diluted to a concentration of $10^{-4}$ M in $\alpha$-MEM supplemented with serum and added to each individual culture dish to obtain the final concentration of $5 \times 10^{-7}$ M (Jones-Villeneuve et al., 1982, 1983).

Cytosine arabinoside (cytosine-1-β-D-arabinofuranoside; Sigma Chemicals Cie., St-Louis, Mo.) was prepared as a stock solution of 5 mg/ml in PBS and stored at 4°C. The stock solution was diluted directly into the culture medium to obtain a final concentration of 5 ug/ml (Seil et al., 1980).

Dimethyl sulfoxide (Sigma Chemicals Cie., St-Louis, Mo.) was used directly into the culture medium to get a final concentration of 1 % (v/v) (McBurney et al., 1982).

2.3 Preparation of coating agents and coated dishes

Gelatin (Fisher Scientific Co., Nepean, Ont.) was prepared as a stock solution of 10 % (v/v) in water but it was used as a solution of 0.1 % on the tissue culture dishes. The bottom of each dish was covered with gelatin, the excess was removed with a sterile pasteur pipette and the plates were put in a 37°C incubator without CO$_2$ atmosphere to dry.

Fibronectin (Sigma Chemicals Cie., St-Louis, Mo.) was prepared as a stock of 1 mg/ml in PBS and was diluted to give a final concentration of 10 μg/ml. Tissue culture dishes were treated the same way as gelatin-coated ones.
Collagen (Vitrogen 100; Flow Laboratories, Mississauga, Ont.) was bought as a solution of 3 mg/ml (3%) and used as is. The coating was done as previously described. For the preparation of collagen-fibronectin-coated dishes, fibronectin coating always followed collagen.

2.4 **3H-Gamma aminobutyric acid uptake experiments**

The aggregates were scored for neurons using the procedure of determination of 3H-GABA uptake described by Yamamoto et al., 1981. Aggregates of RA-treated P19 cells were plated directly onto coverslips and the cultures were allowed to develop undisturbed for four days (day 8 after the initiation of aggregation) in the presence of ARA-C (5 ug/ml). The coverslips were washed twice in prewarmed (37°C) Hank's buffered saline solution (HBSS) (KCl 0.40 g/l, KH₂PO₄ 0.06 g/l, MgCl₂.6H₂O 0.10 g/l, MgSO₄.7H₂O 0.10 g/l, NaCl 8.0 g/l, NaHCO₃ 0.35 g/l, Na₂HPO₄ 0.09 g/l, D-glucose 1.0 g/l pH 6.2 with NaOH) and were placed in a humidifying chamber (Petri dish with a wet kleenex) for the incubation of 30 minutes at 37°C with 2,3-3H)γ-aminobutyric acid (5 X 10⁻⁸ M) (spec. act. 74.5 Ci/m mole, New-England Nuclear, Boston, Ms.). For each coverslip, 1.2 ul of 3H-GABA was diluted into 100 ul of prewarmed HBSS and only then was the coverslip added. The cells were then washed six times in cold PBS and fixed in cold (4°C) 2.5% (v/v) glutaraldehyde in PBS with gradual warming to room temperature over 15 minutes. Following a wash for 30 seconds with double distilled water, cells were dehydrated with ethanol as follows: 25% ethanol for 30 sec., 50% ethanol for 30 sec., 75%
ethanol for 2 minutes, 85% ethanol for 5 minutes, 95% ethanol for 5 minutes and 100% ethanol for 15 minutes. The fixed and dehydrated cells were prepared for autoradiography. The coverslips were placed cells face up on slides and edges were sealed with Permount (Fisher Scientific Co., Fairlawn, N.J.) or transparent nail polish (Cutex) avoiding formation of air bubbles. In the darkroom, slides were dipped very quickly (2 sec.) into prewarmed (40°C) Ilford L4 emulsion (Ilford Photo Canada, Don Mills, Ont.) (10 ml aliquots stored at 4°C wrapped in aluminum foil) diluted 1:1 with warm distilled water, were allowed to drain on paper towel and were placed in a black slide box containing drierite wrapped in gauze to remove humidity from the chamber. The slide box was covered with foil and stored at 4°C for two to four days. Autoradiographs were developed at room temperature as follows: the slides were placed in a plastic slide holder, developed for 2 minutes in a Kodak Dektol/water 1:1, stopped in 1.4% CH₃COOH acetic acid solution for 15 seconds, fixed in Kodak rapid fix for 4 minutes and washed in water for 30 minutes. Cells were then stained quickly with methylene blue and mounted for microscopy. The number of nucleated cells and number of stained cell were scored. This method gives a good estimation of the proportion of the population represented by neuroectodermal cell derivatives.

2.5 **Construction of a P19 neuron-enriched cDNA library**

2.5.1 **First and second-strand cDNA synthesis**

Total RNA was extracted from fifteen 100 mm confluent plates of P19 neurons (2 X 10⁷ cells total) by a modifi-
cation of the lithium chloride-urea procedure described by Auffray and Rougeon (1980). Cells were washed once with ice cold PBS and were put immediately at 4°C. Approximately 2 ml's of 3M LiCl/8M urea (solutions prepared with diethylpyrocarbonate water) were added to each plate to lyse the cells. The lysed cells were then pooled in a plastic tube using a rubber policeman and shearing of the DNA was performed on ice using a polytron (PT 10/35 generator PTA 10S; Brinkmann Instruments Division, Rexdale, Ont.) set at 8 for 3 to 4 minutes. Samples were left on ice overnight to allow for the precipitation of RNA then were centrifuged at 4°C for 10 minutes in a microfuge. The white resultant pellet was rinsed twice with 3 M LiCl and resuspended in binding buffer (BB) (0.4M LiCl, 10 mM Tris-HCl pH 7.6) supplemented with 0.1 % SDS and 10 mM EDTA. From 12 to 15 plates, one to two milligrams of total RNA were obtained. This RNA was either stored at -70°C or used for the preparation of poly(A⁺) RNA.

Poly(A⁺) RNA was isolated by two passages of adsorption and elution from oligo(dT) cellulose (Type 3; Collaborative Research Inc., Lexington, Ma.) following the procedure of Aviv and Leder (1972). 0.2 g of oligo(dT) cellulose was suspended in elution buffer (10 mM Tris-HCl pH 7.5) and poured in a 3 ml syringe barrel previously rinsed with 0.1 % SDS and plugged with siliconized autoclaved glass wool. The column was washed with ten bed volumes of binding buffer. The RNA sample in BB/SDS was heated to 65°C for 10 minutes then quick cooled on ice 3 minutes prior to its loading on the column. The eluate was heated again, quick cooled and run through the column again to ensure that all poly(A⁺) RNA had bound to the oligo (dT). The previous step
was repeated one more time. The final eluate containing poly(A⁺) RNA was saved for further analysis. The column was then washed with BB/SDS until the ratio 260/280 equals zero (usually 10 mls) followed by a wash with 10 mls of BB to get rid of SDS in the preparation. The poly(A⁺) RNA was then eluted from the column using diethylpyrocarbonate-treated water (DEPC) (0.1 %, BDH Chemicals Ltd., Poole, England) Fedorcsak and Ehrenberg, 1966). The column was reconstituted as follows: it was washed with 10 bed volumes of elution buffer followed by 5 mls of 0.1 N NaOH to remove all traces of bound material then reequilibrated with elution buffer followed by BB/SDS. The poly(A⁺) enriched fraction collected previously was heated at 65°C for 5 minutes, quick cooled on ice and reloaded on the reconstituted column. Washes were done as described above. The poly(A⁺) fraction was eluted with DEPC water and fractions of approximately 500 uls were collected (poly(A⁺) RNA was usually found in the first mls.). The optical density was measured for each fraction and those containing poly(A⁺) RNA were pooled and precipitated with 0.3 M NaOAc pH 5.5 and 2.5 volumes of cold 95 % ethanol at -20°C, overnight. Ammonium acetate cannot be used because it interferes with the reverse transcriptase reaction. Centrifugation was done at 10,000 rpm for one hour at 4°C in a RC-5 Sorvall (HB-4 swinging rotor). The resultant pellet was rinsed with 70 % cold ethanol, vacuum dried and resuspended in DEPC water or water containing vanadyl ribonuclease complex (VRC) 10 mM (Bethesda Research Laboratories, Gaithersburg, Md.) (Berger and Birkenmeier, 1979). The sample was stored at -70°C. From 1 mg of total RNA, approximately 50 ug of poly(A⁺) RNA (1 to 5 %) was obtained after two passages on oligo(dT) cellulose column, with 2-5 % contamination with ribosomal RNAs.
15 µg of mRNA was used as a template for the oligo(dT) primed cDNA synthesis. First and second strand cDNA synthesis was performed by using avian myeloblastosis virus (AMV) reverse transcriptase (Life Sciences, St-Petersburg, Fl.) following a procedure given by Martin Tenniswood based on Efstratiadis and Villa-Komaroff (1979). 50 µCi of [³H]α-dCTP (Amersham Co. Oakville, Ont.) (spec. act. 50 Ci/mmol, 1 µCi/µl) and 0.5 mM dCTP were mixed in an Eppendorf tube and vacuum dried. The following components were added at the final concentration specified in a final volume of 100 µl: 0.1 M Tris-HCl pH 8.3, 0.14 M KCl, 10 mM MgCl₂, 100 µg/ml of oligo(dT)₁₂-₁₈, 1 mM dNTPs (dA, dT, dG), 20 mM DTT and 15 µg of poly(A⁺) RNA. The reaction mixture was prewarmed at 42°C for 5 minutes then the reverse transcriptase was added at a concentration of 10 units/µg of mRNA and incubation was set at 42°C for two hours. The reaction was stopped with 10 mM EDTA pH 8.0 and NaOH at a final concentration of 0.075 N was added to hydrolyse the mRNA strand by incubating one hour at 65°C. To neutralize, HCl to a same final concentration of 0.075 N was added to the mixture along with 250 mM Tris-HCl pH 8.0. The sample was extracted once with phenol saturated with TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA)/chloroform (1:1 v/v), back extracted with TE buffer and passed through a Q-100 column (Pharmacia Fine Chemicals, Dorval, Quebec). Ten fractions of approximately 500 µls were collected, counted using the liquid scintillation counter and the ones containing radioactivity were pooled, precipitated with 0.1 volume of 3M NaCl and 2.5 volumes of cold 95% ethanol at -20°C overnight. The RNA was centrifuged at 10,000 rpm, 60 minutes at 4°C in a Sorvall RC-5B (HB-4 swinging rotor). The pellet was rinsed with 70%
cold ethanol, vacuum dried and resuspended in 20 ul of autoclaved water. The synthesis of the first strand was repeated once more to increase the amount of single-stranded cDNA. Both preparations were pooled. From 30 ug of mRNA (two reactions), 2.45 ug of [3H] single-stranded cDNA was synthesized.

The second strand of the cDNA was obtained as follows: 2.15 ug of [3H] ss-cDNA (about 40 ul) and a same volume of 2X second strand buffer (0.2 M HEPES pH 6.9, 20 mM MgCl₂, 5 mM DTT, 0.14 M KCl, 1 mM dNTPs) were mixed together in an Eppendorf tube. Approximately 20 units of E.coli Klenow fragment (New England Nuclear, Boston, Ms.) were added for every microgram of single-stranded cDNA used and the incubation was set at 15°C for 18 hours. The reaction was stopped with 10 mM EDTA and a phenol/chloroform extraction (1:1 v/v) was performed before the purification through the G-100 column. Again, fractions of 500 uls were collected, counted and the desired ones were pooled, precipitated and centrifuged as previously described. The resultant pellet was resuspended in 50 ul of distilled water and counted. 1.50 ug of [3H] double-stranded cDNA was obtained using E.coli DNA polymerase I large fragment. This ds-cDNA was extended using the AMV reverse transcriptase. For this reaction, the following mix was prepared at the final concentrations specified and to a final volume of 100 ul: 1.5 ug of [3H] ds-cDNA, 0.1 M Tris-HCl pH 8.3, 0.14 M KCl, 10 mM MgCl₂, 70 mM β-mercaptoethanol, 2 mM dNTPs, dH₂O. The reaction mixture was prewarmed at 42°C for 5 minutes and the reverse transcriptase was added at a concentration of 20 units per ug of ds-cDNA. The incubation was set for one hour.
at 42°C and the reaction was stopped with 10 mM EDTA. The extraction and purification steps were identical as above. The resultant pellet was resuspended in 60 μl of distilled water.

The size distribution of the ds-cDNAs was checked by running a small aliquot in an 8% polyacrylamide gel in Tris-borate buffer (10X TB: 0.5 M Tris, 0.66 M H$_3$BO$_3$, 10 mM EDTA, pH 8.0) and exposing the X-ray film (XAR-5 Kodak film, Picker Canada, Ottawa, Ontario) for a short period (4 to 6 hours) at room temperature using an intensifying screen (Kodak). From 1.5 μg of [$^3$H] ds-cDNA, 880 ng of extended [$^3$H] ds-cDNA were obtained of 200 to 2000 base pairs in length.

The resultant ds-cDNA preparation was then treated with nuclease S$_1$ (Boeringer-Mannheim Co., Dorval, Quebec). 30 units (1 unit per 30 ng ds-cDNA) of nuclease S$_1$ were used for 45 minutes at 37°C to destroy the hairpin loop located at the 5' end of each cDNA. A small aliquot of single-stranded cDNA was used as a positive control to determine when digestion was complete i.e. time when 0% resistance to S$_1$ was reached. The reaction was stopped with 10 mM EDTA then phenol/chloroform extracted and passed through a G-100 column. The desired fractions were pooled, precipitated and the ds-cDNA was resuspended in 50 μl of distilled water and counted. From 880 ng of [$^3$H] ds-cDNA, 67% was recovered after S$_1$ nuclease treatment, i.e. 590 ng of ds-cDNA. Both 5' and 3' ends were filled in using Klenow fragment of E.coli DNA polymerase I to obtain blunt ends. To this end, 280 ng of S$_1$ treated-[${}^3$H] ds-cDNA were added to 2 mM dNTPs, IX Klenow
buffer (100 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 5 mM DTT) and 5 units of Klenow enzyme in a final volume of 35 ul and incubated at room temperature for 30 minutes. Ligation of these complete ds-cDNAs to ³²P-end labeled BamH1 linkers (New England Biolabs, Beverly, Ma.) was performed using a ratio of linker/ds-cDNA of 2/1.

The end-labeling of BamH1 linkers was done as described by Maniatis et al, 1982. One A₂₆₀ unit of Biolab BamH1 linkers was dissolved in 100 ul of distilled water and stored at -20°C. To 2 ug of BamH1 linker, 30 uCl of [³²P]γ-ATP (Amersham Co., Oakville, Ontario, 10 uCl/ul, 3000 Ci/mmole), 70 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 5 mM DTT and 10 units of T₄ polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, Md.) were added in a final volume of 10 ul. Following an incubation of 15 minutes at 37°C, 70 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 5 mM DTT, 1 mM ATP and 10 units of T₄ kinase were added to the reaction mixture to a final volume of 20 ul which was further incubated for 30 minutes at 37°C. These kinased linkers were stored at -20°C. 600 ng of [³²P] kinased BamH1 linkers and 800 units of T₄ ligase (3 units per ng of ds-cDNA) (Boehringer-Mannheim Co., Dorval, Quebec) were added to the tube containing the blunt-ended ds-cDNAs and an incubation was set at 16°C overnight. The sample was then extracted once with phenol/chloroform (1:1 v/v), once with chloroform only and precipitated at -70°C for one hour with 0.1 volume of 3M NaOAc and 2.5 volumes of cold 95 % ethanol. It was then centrifuged at room temperature for 15 minutes and rinsed with 70 % cold ethanol, vacuum dried and resuspended in water. The ds-cDNA was then digested to completion with
BamHI (Amersham Co., Dorval, Quebec) and size fractionated over a sepharose 4B (Pharmacia Canada Ltd., Dorval, Quebec) mini-column equilibrated in TEN buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 100 mM NaCl). Fractions of approximately 100 ul (2 drops) were collected, counted and run in an 8% polyacrylamide gel to check if the first fractions were enriched in large fragments.

2.5.2 Vector Preparation

The plasmid vector pBR322 (6 ug) was digested to completion with BamHI using 10X medium salt buffer (500 mM NaCl, 100 mM Tris-HCl pH 7.5, 100 mM MgCl$_2$, 10 mM DTT) and 12 units of the enzyme. The sample was heated to 65°C for 15 minutes to inactivate the enzyme and 1.5 units of calf intestinal alkaline phosphate (CIAP) (Bethesda Research Laboratories, Gaithersburg, Md.) was added for 40 minutes at 37°C to dephosphorylate the ends of the vector to prevent self-annelling during the ligation reaction. Distilled water was then added (to 50 ul) and the sample was heated for 10 minutes at 65°C before extraction with chloroform/isoamyl alcohol (24:1 v/v). The phosphatase-treated pBR322 was ethanol precipitated and resuspended into TE buffer. 1 ug of BamHI cut phosphatase-treated pBR322 was added to each pool of fractions (about 200 ul) obtained during fractionation of BamHI cut/kinased ds-cDNAs. The resultant mixture was precipitated at -70°C for one hour with 0.1 volume of 3M NaOAC and 2.5 volumes of 95% cold ethanol. The pellet was rinsed with 70% cold ethanol and resuspended in TE buffer. The sample was incubated at 65°C for 10 minutes then set
on ice. Only 1/5 of the total reaction mixture of ds-cDNA-linker/vector was used for the ligation reaction which was performed at 15°C overnight in a final volume of 50 ul using ligase buffer (1X buffer: 50 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP) and 120 units of T₄ DNA ligase. The ds-cDNA was ligated to the vector at a weight ratio of vector/cDNA of 20:1. Approximately 10 ng of ds-cDNA was ligated to 200 ng of plasmid vector.

2.5.3 Transformation protocols for E.coli DH1 and RRI strains

E.coli strain DH1 (rec A⁻) (Low, 1968) given by Martin Tenniswood and E.coli strain RRI (rec A⁺) (Bolivar et al, 1977) given by Ken Dimock were used as recipient cells for the library.

Transformation of E.coli DH1 cells was carried out by using a high efficiency procedure adapted from Hanahan (1983) as described by Martin Tenniswood. A fresh plate of E.coli DH1 was prepared from a frozen stock of -70°C. The plate (5 g/liter Bacto-yeast extract, 20 g/liter Bacto-tryptone, 5 g/liter MgSO₄·7H₂O pH 7.6 with KOH, 14 g/liter Bacto agar) was incubated at 37°C overnight. A single fresh colony was picked and used to inoculate a 5 ml culture in Œb (Œa without agar, filtered prior to autoclaving). The culture was grown until OD₅₅₀ reached 0.3 (usually 4 hours). Half volume of the culture was used to inoculate 100 ml of Œb (prewarmed at 37°C) and cells were grown for a further 3 hours or until OD₅₅₀ reached 0.48. Cells were placed on ice 5
minutes and centrifuged in sterile 30 ml screw cap tubes in a Beckman J2-21M (JA-20 rotor) at 6000 rpm, for 5 minutes at 4°C. The cells were resuspended in 40 mls of TfBl (30 mM KODAC, 100 mM RbCl₂, 10 mM CaCl₂, 50 mM MnCl₂·4 H₂O, 15% glycerol (v/v), pH 5.8 with 0.2M acetic acid; sterilized by filtration). Cells were left on ice for 5 minutes then centrifuged at 6000 rpm at 4°C for 5 minutes. They were resuspended in 1/25 volume of TfbII (10 mM MOPS or PIPES, 75 mM CaCl₂, 10 mM RbCl₂, 15% (v/v) glycerol, pH 6.5 with HCl; sterilized by filtration) and left on ice for 15 minutes. Competent cells were frozen quickly in dry ice and stored at -70°C in aliquots of 200 µl into microfuge tubes using prechilled pipette and tubes.

Cells were thawed at room temperature for 5 minutes then were left on ice for 10 minutes. Half of the ligation mixture was used (5.5 ng of dscDNA/100 ng vector) for each transformation. Cells were transformed with the plasmid at a concentration of 0.5 µg/ml i.e. 200 µl of competent DH1 cells were added to the DNA which was then left on ice for 60 minutes before the heat shock at 42°C for 2 minutes. The cells were returned on ice for another 2 minutes and 1 ml of Ψb broth was added to the tube to allow growth for one hour at 37°C under constant gentle shaking. The plating was done on LB plates (5 g/l yeast extract, 10 g/l Bacto-trypoyne, 5 g/l NaCl, pH 7.5 with NaOH, 15 g/l Bacto agar) supplemented with 75 µg/ml ampicillin (Sigma Chemicals Cie., St.Louis, Mo.) and colonies were grown overnight at 37°C. With this method, 1 to 2 X 10⁸ transformants per µg supercoiled pBR322 were obtained.
Transformation of *E. coli* RR1 cells was performed using the CaCl₂ procedure described by Mandel and Higa (1970). 100 ml of LB broth was inoculated with 1 ml of an overnight *E. coli* RR1 culture. The cells were grown with vigorous shaking at 37°C until the OD₅₅₀ reached 0.2. Cells were then left on ice for 10 minutes. Following a centrifugation at 6000 rpm, 4°C for 5 minutes, the cells were resuspended in half of the original culture volume of an ice-cold, sterile solution of 50 mM CaCl₂ and 10 mM Tris-HCl pH 8.0. They were left sitting in an ice-water bath for 20 minutes. After a centrifugation at 8000 rpm, 4°C for 5 minutes, the supernatant was carefully discarded and the cells were resuspended in 1/15 of the original volume of the same ice-cold solution of CaCl₂/Tris-HCl pH 8.0. The competent cells were dispensed in prechilled tubes as 200 ul aliquots and left at 4°C overnight to increase the efficiency of transformation (Dagert and Ehrlich, 1979). Half of the ligation mixture was used (5.5 ng ds-cDNA/100 ng vector) for the transformation. Cells were transformed with the plasmid at a concentration of 1 ug/ml i.e. 100 ul of competent cells were used for each transformation reaction. Competent cells and DNA were left on ice 30 minutes then placed at 42°C for 2 minutes. 1 ml of LB broth was added to each tube and cells were incubated at 37°C for one hour before being spread onto LB ampicillin plates. Approximately 200 ul of transformation reaction was put on each plate. Colonies were grown at 37°C overnight. 5 x 10⁶ to 1 x 10⁷ transformants per ug intact pBR322 were obtained using this method.

Approximately 1500 transformants per nanogram of ds-cDNA were obtained in both cases (RR1 and DH1 cells).
2.6 Screening of the PI9 neuron-enriched cDNA library

Approximately 10,000 colonies (ampicillin resistant) were transferred in an ordered array to fresh LB agar/tetracycline plates (12.5 ug/ml) (Sigma Chemicals Cie., St. Louis, Mo.) at 50 colonies per plate and incubated at 37°C overnight. The Amp^r Tet^s colonies (about 9,800) were re-inoculated onto fresh ampicillin plates (75 ug/ml) using a 50 colony grid system and colonies were allowed to grow until they reached a diameter of 3-4 mm. The plates were then overlaid with sterile Biodyne circular membranes (1.2 micron; Pall Canada Ltd., Brockville, Ontario) for 15 seconds. The filters were placed on fresh LB ampicillin plates, incubated for 4 hours—at 37°C and then processed for hybridization as described by Grunstein and Hogness (1975). Three replicas of each of the original colony plates were prepared for future analysis. The bacterial colonies were lysed by placing the replica filters colony side up successively on top of Whatman 3MM paper saturated with 10% SDS for 3 minutes, 0.5 N NaOH/1.5 M NaCl (denaturing solution) for 5 minutes, 0.5 M Tris-HCl pH 8.0/1.5 M NaCl (neutralizing solution) for 5 minutes and 2X SSPE (0.3 M NaCl, 0.02 M NaH₂PO₄·H₂O, 0.002 M EDTA) for 5 minutes. Gentle scrubbing with a Kleenex tissue was performed if cellular debris were still visible on the filters. The filters, colony side up, were allowed to dry at room temperature for 30 to 60 minutes on top of a sheet of Whatman 3MM paper and were baked at 80°C for one hour in a vacuum oven. The processed colony filters were prehybridized at 42°C for 12 hours in a solution containing 50% formamide, 5X Denhardt's solution, 0.3% SDS, 5X SSPE and 250 ug/ml of Salmon sperm DNA. Hybridization was performed at 42°C in the
same solution containing 1-5 \times 10^5 \text{ cpm/ml} \text{ of cDNA probe synthesized from the various mRNA (P19 EC cells, P19 neurons, P19 fibroblasts.)}

Two different methods were used for the preparation of the radiolabeled probes. The first one involves the synthesis of \(^{32P}\) single-stranded cDNAs (Efstratiadis and Villa-Komaroff, 1979) and the second one consists in end-labeling the mRNA (Craig et al, 1979). Both procedures are detailed below.

A. \textbf{Synthesis of \(^{32P}\) Single-Stranded cDNAs}

4 \mu g of poly(A\(^+\)) RNA of any of the sources (P19 EC, P19 neurons and P19 fibroblasts) were added to a tube containing 100 \mu g/ml oligo(dT) primer, 100mM Tris-\(\text{HCl}\) pH 8.3, 0.14 M KCl, 10 mM MgCl\(_2\), 20 mM DTT, 1 mM dNTPs (dA, dT, dG), 0.1 mM dCTP, 50 uCi \(^{32P}\)-\(\alpha\)-dCTP (spec. act. 3000 Ci/mmole, 10 uCi/ul, Amersham Co.) and 25 units of AMV reverse transcriptase in a final volume of 100 ul. The incubation was made at 37°C for 2 hours. The template RNA was hydrolyzed by the addition of 2.5 ul of 3N NaOH at 65°C for one hour. The sample was then neutralized with 2.5 ul of 3N HCl and extracted once with phenol/chloroform (1:1 v/v) before being precipitated with 0.8 volume of 5 M ammonium acetate and 2.5 volumes of 95 % cold ethanol at -70°C for 15 minutes. Final purification was done by chromatography of the resuspended sample on a G-100 column. Fractions of about 500 uls were collected and counted, and those containing the radiolabeled probe were pooled, boiled for 5 minutes and transferred to the bags containing the filters in
prehybridization solution. The specific activity obtained with this method was between $1 \times 10^6$ - $1 \times 10^7$ cpm/ug mRNA.

B. End-labeling of mRNAs

2 ug of poly(A$^+$) of any of the sources (P19 EC or P19 neurons) were added to X ul of 50 mM Tris-HCl pH 9.5 to get a final volume of 27 ul. Paraffin (mineral oil) (about 50 ul) was added on top of the sample to prevent evaporation during alkaline hydrolysis. Following an incubation of 25 minutes at 90°C, the paraffin layer was removed using a very thin capillary. The efficiency of hydrolysis was verified by running an aliquot on a 10 % polyacrylamide gel. The RNA was hydrolyzed to an average size of about 100 bases. The hydrolyzed RNA sample was then brought to 50 ul in 0.04 M Tris-HCl pH 7.5, 10 mM MgCl$_2$, 2 mM DTT, 60 ucI$^{[32P]}$ $\gamma$-ATP (sp. act. 3000 Ci/mmole, 10 ucI/ul, Amersham Co.), 6.5 units/ug RNA of T$_4$ polynucleotide kinase (Amersham Co.) (final concentrations). Incubation was performed at 37°C for 35 minutes and the sample was set on ice. The RNA was purified by passage it through a G-100 mini-column (Maniatis et al, 1982). The $^{[32P]}$ end-labeled mRNA was counted and boiled for 5 minutes before being transferred to the bags containing the filters and prehybridization solution. The specific activity achieved with this method was about $3 \times 10^7$ - $10^8$ cpm/ug RNA.

In both cases, hybridization was performed at 42°C for 24 hours with constant shaking. Filters were then washed with three changes of 2X SSC/0.1-% SDS (0.3 M NaCl/0.03 M sodium
citrate) at room temperature, 30 minutes each and once with 0.2X SSC/0.01 % SDS at 65°C for 15 minutes. For higher stringency, three successive washes, 15 minutes each in 0.1X SSC/0.1 % SDS at 65°C were performed. For re-use, the filters were washed free of radioactive probe in 50 % formamide - 10 mM Tris-HCl pH 7.6 - 1 mM EDTA for one hour at 65°C. Specific hybridization was detected by exposure of filters to XAR-5 X-Ray film with Kodak intensifying screen at -70°C.

The second round of screening was done the same way but only colonies giving a positive signal with [32P] neuron ss-cDNA probe were picked and transferred all together on a fresh ampicillin plate. The colonies remaining positive for the [32P] neuron ss-cDNA after high stringency washes were kept for further analysis.

2.7 Storage of the P19 neuron-enriched cDNA library

Storage of the cDNA library was performed as described by Hanahan and Meselson (1980). Colonies were replica plated onto Biodyne circular membranes (1.2 u) (100 mm diameter) and grown overnight on fresh LB agar plates supplemented with glycerol (5 %). Each colony filter was covered with a sterile filter of the same size and these filters were sandwiched between several dry Whatman 3 MM papers plus one wet Whatman filter to maintain humidity. They were stored in individually sealed plastic bags (Fisher Scientific Ltd., Whitby, Ontario) at -70°C. When needed, the bags were removed from -70°C and brought to room temperature,
the filters were peeled apart, laid on fresh ampicillin plates (75 µg/ml) and incubated until small distinct colonies appeared (about 6 hours). They were then transferred onto fresh ampicillin plates supplemented with chloramphenicol (250 µg/ml) (Sigma Chemicals Co.) and further incubated for 12 hours at 37°C. The library was checked every eight months for possible loss of plasmids or inserts.

2.8 General RNA isolation

2.8.1 Extraction of total RNA from cells cultured in roller bottles

For large extraction of total RNA such as P19 EC undifferentiated cells, roller bottles were used. When cells reached confluency (3 x 10^8 cells per bottle), the medium was removed gently and cells were rinsed with 200 mls of ice-cold PBS. The bottle was immersed in an ice-water bath for 10-15 minutes to inhibit RNase. Then 30 mls of 3M LiCl/8M urea solution were added to the bottle which was swirled to allow spreading of the lysing solution all over the cells. The lysed cells were collected in 50 ml-polystyrene tubes (10 ml/tube) and DNA was sheared using a Polytron homogenizer set at 8 for 4 minutes. The RNA was allowed to precipitate overnight on ice. The preparations were transferred into 50 ml-polycarbonate tubes pre-rinsed with 10 % SDS and a centrifugation was set for 10 minutes, at 4°C at 6,000 rpm. The RNA pellet was rinsed twice with 3M LiCl (with centrifugation between each rinse) and resuspended finally in binding buffer supplemented with 0.1 % SDS and 10 mM EDTA. The RNA was
stored at -70°C. Approximately 4 to 6 mg of total RNA were extracted from P19 EC cells using this procedure.

2.8.2 Extraction of total RNA from cells cultured in dishes

Preparations of total RNA from P19 neurons, P19 fibroblasts and all other cell lines used, were obtained (as described in section 2.8.1) from fifteen to twenty non-confluent plates (2.5 x 10⁷ cells total). In each case, 1 to 2 mg of total RNA were extracted.

2.8.3 Total RNA extraction from various organs

Total RNA from 2 1/2 - 6 month-old adult mouse brain, spleen, liver, heart and kidney was isolated following a slight modification of Auffray and Rougeon's method (1980). The various organs dissected from mice were collected in a dish containing ice-cold PBS, cut in very small pieces using SDS-treated scissors and transferred to a 50 ml tube containing 10-15 mls of lysing solution (3 M LiCl/8M urea). The tissues were subjected to homogenization using a Polytron homogenizer set at 10 for 4 minutes on ice. The RNA was allowed to precipitate overnight at 4°C. The RNA suspension was then transferred to polycarbonate tubes, and centrifuged at 6,000 rpm, 4°C for 10 minutes. The pellet was rinsed three times with 3M LiCl to get rid of the urea, resuspended in 10 mM Tris-HCl pH 7.6/0.5 % SDS (solution prepared with DEPC water) and extracted with one volume of chloroform/isoamyl alcohol (24:1 v/v) until the interphase was clean. The RNA
was then precipitated with 0.1 volume of sodium acetate pH 5.5 (prepared in DEPC water) and 2.5 volumes of 95% cold ethanol at -70°C for two to three hours. The final RNA pellet was resuspended in binding buffer/0.1% SDS/10mM EDTA and stored at -70°C until used.

2.8.4 Isolation of mRNA from small preparation of total RNA

For small preparations of total RNA, poly(A⁺) and poly(A⁻) RNA fractions were isolated as outlined in section 2.5.1.

2.8.5 Isolation of poly(A⁺) from large preparation of total RNA: Batch method

When a very high amount of total RNA was used (6 mg) such as in the case of P19 EC cells, the batch method (modification of Maniatis et al, 1982) was chosen to isolate poly(A⁺) and poly(A⁻) RNA fractions. It consists of mixing the total RNA sample with oligo(dT) cellulose in 2X BB/0.1% SDS (1X BB is 0.4M LiCl/10mM Tris-HCl pH 7.6) and incubating the mixture at 37°C for one hour with constant gentle mixing. This allows for saturation of all active sites on the column by poly(A⁺) RNA. The column is then poured into a 3 ml syringe barrel prerinsed with 1% SDS and plugged with autoclaved siliconized glass wool. From then on, the steps are exactly the same as described in section 2.5.1.
2.8.6 Isolation of polysomal RNA

Polysomal RNA was prepared following the method described by Skup et al., 1982. Approximately $10^7$ - $10^8$ cells (10-15 confluent 100 mm plates) were trypsinized, collected in a polystyrene tube, diluted with half volume of ice-cold α-MEM medium and centrifuged at 2,000 rpm for 2 minutes. The cell pellets were pooled in one tube and rinsed twice with ice-cold PBS. 1 ml of hypotonic solution containing 50 mM Hepes (pH 7.6), 0.24 M KCl, 3 mM DTT, 0.25 M sucrose, 10 mM MgSO$_4$, 2% NP-40, 10 mM vanadyl-ribonucleoside complex (added to the solution prior to the cells) was added to the cells which were lysed by vortexing vigorously 2 minutes. The efficiency of lysis was monitored under the phase contrast microscope. Following a centrifugation for 15 minutes at 14,000 rpm, 4°C in a JA-20 rotor, J2-21 M Beckman centrifuge, the supernatant was transferred in a sterile tube in which 20 mM streptomycin sulfate was added (Sigma Co.) (use 0.2 M fresh stock). The polysomes were allowed to precipitate 6 hours or overnight at 0°C and were centrifuged at 6,500 rpm, 4°C for 15 minutes in a JA-20 rotor (Beckman J2-21 M centrifuge). The polysomal pellet was resuspended in 1 ml of 3M LiCl/8M urea and sheared by using a polytron set at 7 for 4 minutes. The RNA free of proteins was precipitated overnight at 4°C and purified as described in section 2.5.1.

2.8.7 Isolation of nuclear RNA

Nuclear RNA was isolated according to Wang et al., (1985). The cells (2 X $10^7$ cells total) were washed with
phosphate-buffered saline (PBS) and harvested with a rubber scraper in cold PBS. The cells were then washed twice by centrifugation at 2,250 rpm, 4°C, 5 minutes in JA-20 rotor (Beckman J2-21M) and lysed in 5.0 ml of ice-cold buffer A (0.3 M sucrose, 10 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 0.4 % Nonidet P-40, 0.5 mM dithiothreitol (DTT) by vortexing 10 seconds. The nuclei were quickly pelleted by centrifugation at 2,900 rpm, 5 minutes at 4°C in a Beckman JS-13 swinging bucket rotor. The pellet was resuspended in 5.0 ml buffer A, vortexed, and subjected to 3-5 strokes in a Dounce tissue homogenizer using the B pestle. The homogenate was then layered over 5.0 ml of buffer B (same as buffer A except the sucrose is 0.88 M) and the nuclei were purified by centrifugation at 2,900 rpm, 4°C, 10 minutes in a Beckman JS-13 swinging bucket rotor. The nuclear pellet was resuspended in 1.0 ml of buffer C (40 % glycerol, 50 mM Tris-HCl pH 8.3, 5 mM MgCl₂, 0.1 M EDTA) and pelleted once at 2,900 rpm for 5 minutes at 4°C. The pellet was resuspended in 1.0 ml of 3M LiCl/8M urea, subjected to shearing using a Polytron homogenizer set at 10 for 4 minutes. The nuclear RNA was precipitated overnight and centrifuged at 12,000 rpm in a microfuge tube for 5 minutes at 4°C. The pellet was rinsed twice with 3M LiCl, resuspended in binding buffer/0.1 % SDS/10 mM EDTA and stored at -70°C.

2.9 DNA isolation

2.9.1 Plasmid DNA extraction

Plasmid DNA was extracted from the various bacterial strains or selected clones by using a modification of the
alkaline lysis procedure of Birnboim and Doj (1979). Large (50-100-500 ml) and small (5-10 ml) preparations of plasmid DNA were prepared using the following method. From a single colony picked from a plate, 10 ml of LB broth supplemented with 75 μg/ml ampicillin were inoculated and grown overnight at 37°C. 250 ml of LB/ampicillin were inoculated with 2.5 ml of this overnight culture and left at 37°C overnight with gentle shaking. The cells were pelleted at 4,000 rpm, for 10 minutes at 4°C in a Beckman JA-10 rotor. They were resuspended by swirling gently in 10 ml of solution 1 (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA, 5 mg/ml lysozyme prepared fresh). An incubation was set for 15 minutes on ice, then 20 ml of solution 2 (0.2 N NaOH, 1% SDS) were added and the bottle was inverted for mixing. The cells were incubated on ice for a further 5 minutes before 15 ml of solution 3 (5 M potassium acetate pH 4.8) were added and mixed by gentle inversion. Following an incubation of 10 minutes on ice, the lysed cells were centrifuged at 8,000 rpm for 30 minutes at 4°C in a Beckman JA-10 rotor. The supernatant was collected into two 50 ml polystyrene tubes and extracted twice with chloroform/isoamyl alcohol (24:1 v/v). The sample was transferred into siliconized 30 ml Corex tubes. Then, 0.7 volumes of isopropanol was added to the supernatant, mixed by inversion several times and the mixture was left to precipitate for 30 minutes at room temperature. Centrifugation was done at 8,000 rpm for 45 minutes at room temperature in a Beckman JA-20 rotor. The pellet was rinsed with 70% isopropanol and resuspended in distilled water (100 ul).
The purification of plasmid DNA was done by treating the DNA with DNase-free RNase (100 mg/ml) at a final concentration of 10 ug/ml, for one hour at room temperature. Proteinase K (10 mg/ml) and 10 % SDS were added to the tube to a final concentration of 200 ug/ml and 1 % respectively. The incubation was set at 37°C for one hour. The sample was extracted twice with chloroform/isoamyl alcohol and precipitated with isopropanol the same way as above. The plasmid DNA was finally redissolved in 50-100 ul of double distilled water. Recovery was determined by taking an A₂₆₀ reading and purity by measuring the A₂₆₀/A₂₈₀ ratio. Average yield was 1 ug/ml of starting culture.

2.9.2 Genomic DNA extraction

Mouse genomic DNA was isolated by a modification of the procedure described by Blin and Stafford (1976). Cell monolayers (5 to 10 confluent plates, 10⁷-10⁸ cells) were washed twice with ice-cold PBS. 2 ml of solution A (10 mM Tris-HCl pH 8.0, 10 mM EDTA, 10 mM NaCl, 0.5 % SDS) were added per plate and using a rubber policeman, cells were scraped and pooled into a 50 ml polystyrene tube. 50 ug/ml proteinase K were added to the cell suspension which was incubated at 37°C for 3-4 hours. The sample was then treated with 50 ug/ml pancreatic RNAse and 2 ug/ml RNAse T₁ for one hour at 37°C and extracted three times with phenol/chloroform (1:1 v/v), then twice with chloroform/isoamyl alcohol (24:1 v/v). Precipitation was done at -70°C with 0.1 volume of 3M sodium acetate and 2.5 volumes of 95 % cold ethanol. The genomic DNA was pelleted at 8,000 rpm for 30 minutes at 4°C.
in a Beckman JA-20 rotor. It was rinsed with 70% cold ethanol, dried under vacuum and resuspended in 1 ml of TE buffer. The recovery and purity were determined as described in section 2.9.1. Average yield of DNA was about 100 µg/plate.

2.10 Southern blotting

Genomic DNA isolated from various cell lines was digested to completion with BamH1, HindIII, EcoR1, PstI and was subjected to electrophoresis in 1% (w/v) agarose gel in Tris-phosphate buffer (0.08 M Tris-base, 0.08 M phosphoric acid, 0.002 M EDTA). Lambda HindIII digested fragments were used as molecular weight markers to determine the length of the detectable bands on filter. The gels stained with ethidium bromide (0.5 µg/ml) were soaked in denaturing solution (500 mM NaOH/150 mM NaCl) for one hour at room temperature then in neutralizing solution (500 mM Tris-HCl, pH 8.0, 150 mM NaCl) for one and a half hours at room temperature with shaking. The gels were then transferred onto Biodyne nylon membranes (1.2 micron) by the method of Southern (1975) for 12-16 hours. The filters were baked for one hour at 80°C in a vacuum oven then prehybridized for 12 hours at 65°C in a solution containing 5X SSPE (1X SSPE is 0.15 M NaCl, 0.01 M NaH2PO4·H2O, 0.001 M EDTA), 5X Denhardt's solution, 0.1% SDS, 250 µg/ml carrier DNA or at 42°C in the same solution supplemented with 50% formamide. The hybridization was done in a final volume of 15 to 20 mls at 65°C or 42°C with nick-translated selected plasmid for 24 hours. Usually 5 X 10^6 cpm/ml were used per bag. Filters were
washed successively with 2X SSC/0.1 % SDS (three changes, 30 minutes each) at room temperature then with 0.1X SSC/0.1 % SDS (three changes, 15 minutes each) at 65°C. They were exposed to XAR-5 X-Ray films (Kodak) at -70°C with Kodak intensifying screens.

2.11 Northern blot analysis

RNA samples (total, poly(A⁺), poly(A⁻), polysomal, nuclear) were denatured in 50 % formamide, 6.5 % formaldehyde, 10X MOPS pH 7.0 at 65°C for 15 minutes. This solution was added to the RNA as a ratio of 3:1 denaturing solution/RNA sample. The RNA was quickly cooled on ice then fractionated by electrophoresis in 1 % (w/v) formaldehyde (0.6 %) agarose gel in MOPS buffer (10X buffer is 200 mM MOPS, 50 mM NaOAc, 3H₂O, 10 mM EDTA pH 7.0 with acetic acid stored in the dark). The gels were stained with ethidium bromide (0.5 ug/ml) and directly transferred onto Biodyne nylon membranes (1.2 micron) as described by Thomas (1980) and Pall Manufacturer. After 12-16 hours, the blots were baked at 80°C for one hour in a vacuum oven, prehybridized for 12 hours and hybridized 24 hours at 42°C in the presence of formamide as described in section 2.10. Nick-translation plasmid DNA (5 X 10⁶ cpm/ml/bag) was boiled 10 minutes in the presence of oligo(A) (1 mg/ml-stock: 20 mg/ml) and placed at 42°C for 5 minutes to allow the annealing of oligo(T) stretch present in the inserts to the oligo(A) and thereby avoid the non-specific binding of the probe to all poly(A)-tailed RNA blotted on the filter. The probe was then directly added to the bags containing the prehybridization
solution. The blots were washed at high stringency as described in section 2.10. Dextran sulfate (10% w/v) was used in the hybridization solution only when a very low specific activity was obtained ($10^4 - 10^5$ cpm/ml) (such as in the case of M13 single-stranded probes) to increase the efficiency of hybridization (Wahl et al, 1979; Meinkoth and Wahl, 1984).

2.12 Nick-translation of plasmid DNA

Nick-translation of whole plasmid DNA was performed using the Amersham Nick-translation kit as follows. In a tube, 1 μg of plasmid DNA was mixed with 10 μl of nucleotide solution (100 μM dATP, 100 μM dGTP, 100 μM dTTP in 250 mM Tris-HCl pH 7.8, 25 mM MgCl$_2$, 50 mM 2-mercaptoethanol), 6 μl [$^{32}$P]dCTP (spec act. 3000 Ci/mmole, 1 mCi/μl, Amersham Co.), 28 μl water and 5 μl enzyme solution (2.5 units DNA polymerase I and 50 μg DNase I in Tris-HCl pH 7.5, MgCl$_2$, glycerol and BSA). The reaction mixture was incubated for 40 minutes in a 14°C water bath. The percentage of [$^{32}$P]dCTP incorporated was determined by TCA precipitation: 2 μl of the reaction mix was diluted in 200 μl of 0.2 M EDTA. 20 μl of that preparation was added to 50 μl of salmon sperm DNA (carrier DNA) at 2-4 mg/ml and 10 μl of ice-cold 10% TCA solution. The sample was left on ice for 15-20 minutes and the resultant precipitates were collected on a glass filter (Whatman GF/C) and washed twice with 10 ml of 10% TCA under vacuum. The filters were counted along with an aliquot of the unprecipitated diluted sample. The nick-translated DNA was then passed through a G-100 mini-column buffered with a solution containing 50 mM Tris-HCl pH 7.5, 10 mM EDTA,
150 mM NaCl, 0.1 % SDS. The purified probe was boiled 10 minutes and added to the bags containing the prehybridization solution at 42°C. A specific activity of about $1 \times 10^8$ cpm/ug DNA was obtained with this procedure.

2.13 DNA sequence determination

Sequence of the selected clones was determined by the Sanger dideoxynucleotide procedure (Sanger et al, 1977; 1980).

2.13.1 Isolation of M13mp18 RF particles

One blue plaque from M13mp18 (given by Bob Korneluk)/JM109 (given by Torben Beck-Hansen) transformation experiments was picked and used to inoculate 10 mls of LB medium. Fresh exponential JM109 bacterial cells (100 ul) were added to the culture which was incubated at 37°C for about 6 hours. From this culture, 2 mls were transferred into 200 mls of LB plus 2 mls of exponential JM109. This culture was grown for 8 hours at 37°C. The M13mp18 replicative forms (RF circular particles) were isolated using the procedure described in section 2.9.1 except that the precipitation step was done with 0.1 volume of 3 M sodium acetate and 2.5 volumes of 95 % cold ethanol at -70°C for 30 minutes instead of isopropanol at room temperature. The concentration of RF particles was determined by running an aliquot on a 1 % agarose gel and comparing it to a known concentration of lambda DNA.
2.13.2 Subcloning of the repeat element (selected cDNA insert) into M13mp18 vector

M13mp18 RF particles (2 µg) were digested to completion with BamHI and dephosphorylated using calf intestinal alkaline phosphatase (0.4 unit) at 37°C for 40 minutes. The sample was heated to 65°C for 10 minutes then extracted once with chloroform/isoamyl alcohol (24:1) to inactivate the CIAP. Following precipitation with sodium acetate/ethanol, the sample was resuspended in 200 µl of TE.

The cDNA clone containing the insert was cut with BamHI and fractionated on an 8% polyacrylamide gel in Tris-borate buffer (0.089 M Tris-base, 0.089 M boric acid, 0.002 M EDTA). The insert located by staining the gel with 0.5 µg/ml ethidium bromide, was cut from the gel and extracted using the crush and soak method (Maxam and Gilbert, 1980). The band was crushed to a paste-texture using a siliconized glass rod in an Eppendorf tube. 400 µl of a solution containing 500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS, 10 µg/ml transfer RNA was added to the paste and an incubation of 24 hours was set at 37°C with vigorous shaking. The sample was then passed through a mini-column (Pipetman P1000 disposable pipet tip plugged with siliconized glass wool or Kimwipes) by centrifugation at 3,000 rpm, 2 minutes. The column was rinsed with 200 µl of the same solution. Both eluates were pooled and ethanol precipitated (no salt added) at -70°C. The sample was re-precipitated twice and finally resuspended into 50 µl of TE. The recovery was determined by running an aliquot in an 8% polyacrylamide gel. The BamHI cut-insert was ligated to BamHI cut-M13mp18 vector at a
molar ratio of 2:1 (insert/vector) in a final volume of 35 ul. The reaction was incubated at 12°C for 6 hours.

Transformation of JM109 with M13mp18 hybrid RF particles was done as follows: DNA sample (10-20 ng) was mixed with 300 ul of JM109 competent cells. After an incubation of 40 minutes on ice and the heat shock at 42°C for 2 minutes, 200 ul of exponential JM109 cells were added to the tube along with 10 ul 100 mM IPTG and 50 ul of 2 X X-gal and 3 ml of top agar (0.6 %). The whole mixture was poured onto YT plates and grown overnight at 37°C. 4000 recombinants (white plaques) per ug of BamHI cut-RF were obtained as compared to 1.6 X 10^5 blue plaques/ug uncut RF (control).

2.13.3 Isolation of single-stranded DNA from M13mp18 recombinants

A series of 5 mls of LB broth were inoculated with 50 ul of an overnight culture of JM109. The cells were grown for one and a half hour at 37°C with shaking. Individual white plaques were picked from the transforming plates using a sterile pasteur pipette and were transferred into each 5 ml exponential JM109. The cultures were further incubated at 37°C for 7 hours with shaking. 1.5 ml of each culture was collected and centrifuged for 10 minutes in a microfuge. 1.2 ml of supernatant was transferred to a clean tube and 300 ul of 20 % PEG (polyethylene glycol 8000)/2.5 M NaCl (final concentration: 5 % PEG/0.625 M NaCl) was added and mixed by inverting the tube 3-4 times. After an incubation of 30 minutes at room temperature, the M13mp18 single-stranded DNA
was pelleted by centrifugation in a microfuge for 10 minutes. The excess of PEG was wiped from the inside of the tube with a kleenex and the pellet was resuspended in 100 ul TE pH 8.0.

The DNA sample was further purified by extracting once with TE-saturated phenol, once with chloroform and precipitation with 0.1 volume of 3M sodium acetate and 2.5 volumes of 95% cold ethanol at -70°C for 15 minutes. The DNA was centrifuged for 15 minutes at 4°C in a microfuge, rinsed with 70% cold ethanol, dried under vacuum and resuspended in 15 ul TE pH 8.0. An aliquot was run on a 1.3% agarose gel in Tris-phosphate buffer.

2.13.4 Sequence determination of selected M13mp18 hybrid clones

Screening for positive recombinants was facilitated by the alpha-complementation test. White plaques were picked and the T test was performed on each plaque to assess the orientation of the insert within the phage ss-DNA. To this end, 1 ug of ss-DNA was mixed with 5 ng of 15 nt BRL primer and BRL 1X Polymerase buffer (10X buffer is: 70 mM Tris-HCl pH 7.5, 70 mM MgCl₂, 500 mM NaCl) in a final volume of 10 ul. The sample was boiled for 3 minutes then placed in a tube filled with 90°C water at room temperature for 45 minutes to allow the annealing of the primer to the ss-DNA. The following reaction mixture was prepared: 3 ul [³²P]-dCTP (sp. act. 3000 Ci/m mole), 20 ul T₀ mix (20 ul dGTP 0.5 mM, 20 ul dATP 0.5 mM, 1 ul dTTP 0.5 mM, 20 ul 10X Polymerase buffer), 20 ul 1 mM ddT and 4 units of Klenow fragment (large
fragment of *E. coli* DNA Polymerase I). 5 ul of that reaction mixture were added to each tube containing the annealed primer-as DNA. Following an incubation of 15 minutes at 30°C, 1 ul of 0.5 mM dCTP was added. The reaction was incubated for a further 15 minutes at 30°C then stopped with 5 ul formamide dye [0.1 % (w/v) xylene cyanol, 0.1 % (w/v) bromophenol blue, 10 mM Na₂EDTA, 95 % (v/v) deionized formamide]. The samples were fractionated by electrophoresis in 8 % urea-polyacrylamide gel (0.4 mm thick) set at 1200 volts (constant power 60 watts) for 2 hours in Tris-borate buffer. Gels were covered in Saran Wrap and were exposed at -70°C to XAR-5 X-Ray film (35 X 43 cm) with Dupont Cronex Lightning Plus intensifying screens.

The complete sequence of the selected cDNA clone from the P19 neuron cDNA library was determined using two M13mp18 hybrid clones identified as presenting the positive and negative orientation of the insert. These were referred to as clone 3(+) and clone 9(-). 1 ug of ss-DNA from each clone was mixed with 5 ng of BRL 15 nucleotide primer, 1X BRL Polymerase buffer in a final volume of 10 ul. The sample was boiled 3 minutes then left 45 minutes on the bench in a tube filled with 90°C water. Then, 1.5 ul [³²P] α-dCTP (sp.act. 3000 Ci/mmole), 1 unit of Klenow fragment and 1 ul distilled water were added to the tube, mixed well and an equal volume was dispensed into each of the following tubes: reaction A (54:1 ddA/dATP), reaction C (20:1 ddC/dCTP), reaction G (54:1 ddG/dGTP), reaction T (72:1 ddT/dTTP). An incubation was set at 30°C for 15 minutes. To each of the reactions, 1 ul 0.5 mM dCTP was added and the tubes were incubated for a further 15 minutes at 30°C. The dideoxynucleotide reaction
was stopped by adding 5 ul of formamide dye (same recipe as above) to each tube. Half of each sample was loaded on an 8 \% urea-polyacrylamide gel (18 X 85 cm), (0.4 mm thick) in Trisborate buffer and run at constant power 60 watts, 3000 volts for 2 hours. Then, a second loading was made and samples were run for a further 4 1/2 hours at same voltage. Gels were exposed for 4 to 16 hours to Kodak RP-5 films at -20\(^{\circ}\)C without intensifying screens.

2.14 Uniformly labeled M13mp18 single-stranded probes

Separate strand probes of the selected insert from the P19 neuron-enriched cDNA library were obtained by labeling M13mp18 clone 3(+) orientation and clone 9(-) orientation as follows. 1 ug of ss-DNA of each clone was mixed with 5 ng of BRL 15 nt primer and 1X BRL Polymerase buffer in a final volume of 10 ul. The sample was boiled 3 minutes and left 45 minutes on the laboratory bench in a tube filled with 90\(^{\circ}\)C water. Then, 4 ul \(^{32}\)P-\(dCTP\) (sp.act. 3000 Ci/mmole), 4 ul \(^{32}\)P-\(dATP\) (sp.act. 3000 Ci/mmole), 1.5 ul dGTP 1 mM, 1.5 ul dTTP 1 mM, 6 units Klenow fragment were added to the reaction. An incubation was set at 30\(^{\circ}\)C for 25 minutes. The samples were chromatographed on a G-50 mini-column, ethanol precipitated at -70\(^{\circ}\)C with 0.1 volume 3 M sodium acetate and 2.5 volumes of 95 \% cold ethanol, resuspended in 10.5 ul water and digested to completion with Smal (Amersham) at 37\(^{\circ}\)C for 3-4 hours. 5 ul of sequencing loading dye (formamide dye) were added to each tube and following the boiling step at 90\(^{\circ}\)C, 3 minutes, the samples were loaded on an 8 \% urea-polyacrylamide gel and run for 16 hours at 100
volts in Tris-borate buffer. Radiolabeled pAT153 cut with HpaII was used as a molecular weight marker. The gel was exposed at 4°C, 1-3 hours to XAR-5 Kodak film in a cardboard cassette. The radiolabeled strands were located on the X-Ray film, cut from the gel and extracted using the crush and soak method described in section 2.13.2. After the precipitations at -70°C, the samples were redissolved in 100 ul water. Oligo(A) (1 mg/ml) (stock: 20 mg/ml) was added and tubes were left at 42°C for 5 minutes. Then, they were transferred to respective bags with prehybridization solution. Average specific activity obtained with this method: 3 X 10^7 cpm/ug ss-DNA.

2.15 Single-stranded probes labeled using M13 hybridization primer

In this case, the priming reaction takes place a few nucleotides downstream from the BamH1 site in which the insert has been subcloned. Therefore, the single-stranded clone 3 and clone 9 are labeled within their M13 sequence rather than within their insert-hybrid sequence. In this case, there is no need to separate the cold from the labeled strands since only the mp18 portion will be labeled. The labeling was done as follows: 1 ug of ss-DNA from each clone was mixed with 125 ng of M13 hybridization primer (Boeringer-Mannheim) and 1X BRL Polymerase buffer in a final volume of 15 ul. The sample was boiled at 90°C for 3 minutes and left on the bench in a tube filled with 90°C water. Then, 2.5 ul [32P]α-dCTP (sp.act. 3000 Ci/m mole), 2.5 ul [32P]α-dATP (sp.act. 3000 Ci/m mole), 0.6 ul dATP 0.5 mM, 0.6 ul dCTP 0.5 mM, 1 ul dGTP 1 mM, 1 ul dTTP 1 mM and 6 units Klenow
fragment were added to each tube which were incubated at 30°C for 20 minutes. Samples were put on ice a few seconds then passaged through a G-50 mini-column to remove the non-incorporated [32P] label. An alkaline gel (Maniatis et al., 1982) was prepared to determine the size range of the labeled fragments. The samples were incubated at 42°C with oligo(A) (1 mg/ml) for 5 minutes, then were transferred in the respective bags containing prehybridization solution. Specific activity was about 10^7 cpm/ug ss-DNA.
CHAPTER III

EXPERIMENTAL RESULTS

3.1 Establishment and differential screening of the cDNA library

P19 cell cultures were RA-treated and enriched for neurons by the procedure outlined in section 2.1. The RNA isolated from these cultures was used as the template for the synthesis of double-stranded cDNA in sizes spanning 200 to 2800 base pairs (Estradiatis and Villa-Komarovff, 1979). The cDNA was ligated to BamHl linkers and inserted into BamHl restricted pBR322 plasmid DNA. The recombinant constructs were then introduced into competent E. coli DH1 and RR1 strains (Manahan, 1983) and a cDNA library of more than 10,000 clones was established through selection on ampicillin-agar plates. Of these, approximately 9800 were sensitive to tetracycline indicating that they contained a cDNA insert within the BamHl site in the tetracycline gene.

2500 recombinant clones were thus selected at random and re-seeded individually in an ordered array on fresh ampicillin-agar plates and incubated at 37°C for 18 hours. Each of these "master" plates (supporting 50 colonies) was then replicated three times on hybridization membrane discs. Replicate filters were incubated at 37°C on ampicillin-agar for 6 hours in order to enhance bacterial growth and processed for hybridization by the method of Grunstein and Hogness (1975). Hybridization to each set of triplicate filters was
carried out with $^{32}$P-labeled single-stranded cDNA synthesized on mRNA isolated from 1) undifferentiated, parental P19 cell cultures, 2) fibroblast or 3) neuron-enriched cultures of P19 cells. The first round of differential hybridization screening yielded 75 colonies demonstrating a distinctly elevated hybridization signal to probe 3) relative to probes 1) and 2) in at least two of three sets of triplicate filter series prepared. Clones of transformed E.coli were re-seeded and put through a second round of screening (again in triplicate) and 11 of the original 75 colonies still scored positive for probe 3) after an additional wash at very high stringency (0.1X SSC, 0.1% SDS at 65°C). Examples of some of the clones showing preferential hybridization to neuron messages are shown in Figure 3.1.

Table 3.1 summarizes the main features of the P19 cDNA library following the second round of screening. 67% of the colonies did not hybridize with any of the probes used. These probably contained sequences corresponding to low abundant mRNAs or inserts that were too short and were washed off during the washes. Within the remaining 33% which hybridized to at least one probe, many (9.9%) contained sequences which were shared by all cell types i.e. which were present in parental EC cells as well as in their differentiated derivatives. 6.4% of the colonies hybridized preferentially to EC messages. Only a very small proportion of the library (0.5%) was represented by clones bearing neuronal-specific sequences.
FIGURE 3.1

Autoradiographs from the first screening of the P19 neuron-enriched cDNA library. Triplicate filters (50 colonies per filter) were respectively hybridized at 42°C for 24 hours with [32P] single-stranded cDNA synthesized from P19 EC cell mRNA, P19 neuron mRNA and P19 fibroblast mRNA. The specific activity was 5 × 10^6 cpm/ug mRNA in each case. After high stringency wash with 0.1X SSC/0.1% SDS at 65°C for 45 minutes, filters were exposed to X-ray film for 2 days at -70°C with intensifying screens. Colonies showing preferential hybridization to the neuron probe are indicated by these arrows (→). Colonies reactive with all probes are shown by (↔). Colonies hybridizing with both P19 EC-P19 neurons or P19 neurons-P19 fibroblasts or P19 EC-P19 fibroblasts are indicated by (↔→).
Probe

$P_{19}$-EC cell

$P_{19}$-NEURON

$P_{19}$-Fibroblast
TABLE 3.1

Characteristics of the cDNA library constructed from P19 neuron mRNA. The double-stranded cDNA was inserted into the tetracycline-resistance gene of pBR322 at the BamHI site after the addition of kinased BamHI linkers and transfected into E.coli RR1 and DH1 strains. Differential hybridization was used to detect clones containing sequences specific for the neuronal lineage along with those sharing homology with the parental P19 EC cells and/or P19 derived-fibroblasts. Percentages indicated are from the initial screening and represent a gross estimation of the abundance of the sequences within the library.
**TABLE 3.1**

Characteristics of the cDNA library* after the first round of screening

<table>
<thead>
<tr>
<th>FEATURES</th>
<th>NUMBER (N)</th>
<th>PERCENTAGE (%)</th>
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</thead>
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<tr>
<td>Total recombinants</td>
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<td>-</td>
</tr>
<tr>
<td>Amp&lt;sup&gt;R&lt;/sup&gt; Tet&lt;sup&gt;S&lt;/sup&gt; recombinants</td>
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<td>98.0</td>
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<tr>
<td>Screened recombinants</td>
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<td>25.5</td>
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<tr>
<td>Positive recombinants for P19 - neurons only</td>
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<td></td>
</tr>
<tr>
<td>P19 - fibroblasts only</td>
<td>130</td>
<td>5.2</td>
</tr>
<tr>
<td>P19 - undifferentiated EC</td>
<td>159</td>
<td>6.4</td>
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<tr>
<td>P19 - neurons, fibroblasts</td>
<td>51</td>
<td>2.0**</td>
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<tr>
<td>P19 - neurons, EC</td>
<td>32</td>
<td>2.5**</td>
</tr>
<tr>
<td>P19 - fibroblasts, EC</td>
<td>110</td>
<td>4.4</td>
</tr>
<tr>
<td>P19 - neurons, fibroblasts, EC</td>
<td>238</td>
<td>9.5**</td>
</tr>
<tr>
<td>None of the probes</td>
<td>1,675</td>
<td>67.0</td>
</tr>
</tbody>
</table>

* P19 neuron-enriched cultures:
  - 90% neuronal cells
  - 10% fibroblast-like cells

** Percentages indicated after the second round of screening (utilizing the 75 clones showing preferential hybridization to neurons only) were: 0.5% for P19-neurons, 2.3% for P19-neurons-fibroblasts, 4.2% for P19-neurons-EC, 9.9% for P19-neurons-fibroblasts-EC.
3.2 Partial characterization of the 11 potential neuronal-specific cDNA clones

3.2.1 Size of insert

Plasmid DNA was prepared from each of the selected clones, digested with BamHI and electrophoresed on 1.3% agarose gels or 10% polyacrylamide gels to determine the length of the inserts. The size of inserts ranged from 120 to 1000 base pairs (data not shown).

3.2.2 Northern Blot Analysis

To further confirm the neuronal specificity of the 11 recombinant plasmids and determine the size of the mRNA homologous to the cDNA sequence found in each of them, Northern blot hybridization was performed. Total RNA was isolated from a variety of cell sources, electrophoresed on denaturing agarose gels and transferred onto nylon membranes. Figure 3.2 (panels a-f) show copies of the same filter probed with each of the eleven nick-translated plasmids isolated from the selected cDNA clones.

To ensure that all tracks contained equal amounts of RNA, the blot was hybridized with a cloned mouse \( \alpha \)-tubulin gene which detects its homologous transcript in a variety of murine cell types (plasmid MAT 1.1 \( \alpha \)-tubulin; kindly provided by Dr. Paul Dobner). All tracks showed a band of 2.1 kb and of similar intensities (Figure 3.2, panel g). Photographs of the ethidium bromide stained gel prior to and following
Northern blot analysis of the eleven cDNA clones selected for their preferential hybridization with $^{32}$P single-stranded cDNA probe from P19 derived neurons. Total RNA from a variety of cell sources was fractionated on a 1% formaldehyde agarose gel, transferred to nylon filters and hybridized at 42°C in the presence of 50% formamide with each of the nick-translated recombinant plasmids (specific activity of $5 \times 10^7$-$10^8$ cpm/ug). Exposure was for 2 days at -70°C with XAR-5 films with intensifying screens. For panels a (AD$_2$32), b (AD$_4$50) and c (AD$_2$46), 20 ug of total RNA of the following samples were used: P19 undifferentiated EC cells (lane 1), P19 neurons (lane 2), adult mouse brain (lane 3), mouse spleen (lane 4), mouse liver (lane 5), mouse kidney (lane 6), rat L6 myoblast cells (lane 7), mouse heart (lane 8), P19 RAC65 D muscle cells (lane 9) and F9 clone 9 endoderm cells (lane 10). The position of 28S and 18S ribosomal RNA is shown.
FIGURE 3.2 (continued)

For panels d (BD₉₅), e (BD₉₃₀), and f (BD₁₁₂₁, BD₁₈, BD₁₄₉, BD₁₃₂₉, AR₄, AR₅₀), 20 µg of total RNA from P19 EC cells (lane 1), P19 neurons (lane 2), adult mouse brain and heart (lanes 3,4) was used.
FIGURE 3.2 (continued)

Panel g shows an autoradiograph of a copy of the filters used in panels a, b, c which was hybridized to α-tubulin cDNA sequence. This internal standard was used to ensure that the same amount of RNA was present in each lane.

Panels h and i are the ethidium bromide stained photographs of the gel before and after its transfer onto a nylon filter. The same amount of RNA was used in panels a, b, c in each track and as expected, small and middle size RNA was more efficiently transferred than large (>28S) RNA.
| TABLE 3.2 |
| Characteristics of the eleven cDNA clones isolated after two rounds of screening by differential hybridization with $^{32}$P single-stranded cDNA probes. |
### TABLE 3.2

Characteristics of the potential neuronal-specific cDNA clones isolated from the P19-neuron cDNA clone bank

<table>
<thead>
<tr>
<th>CLONE</th>
<th>SIZE OF INSERT (bp)*</th>
<th>SIZE OF RNA SPECIES (nt)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD₂32</td>
<td>560</td>
<td>900</td>
</tr>
<tr>
<td>AD₂46</td>
<td>600</td>
<td>2,100</td>
</tr>
<tr>
<td>AD₄50</td>
<td>1,050</td>
<td>900, 2,100</td>
</tr>
<tr>
<td>BD₉5</td>
<td>1,000</td>
<td>2,100</td>
</tr>
<tr>
<td>BD₉30</td>
<td>700</td>
<td>2,100</td>
</tr>
<tr>
<td>AR₄4</td>
<td>275</td>
<td>200 - 600</td>
</tr>
<tr>
<td>AR₈50</td>
<td>190</td>
<td>200 - 600</td>
</tr>
<tr>
<td>BD₆18</td>
<td>120</td>
<td>200 - 600</td>
</tr>
<tr>
<td>BD₁₁21</td>
<td>225</td>
<td>200 - 600</td>
</tr>
<tr>
<td>BD₁₄9*</td>
<td>175</td>
<td>200 - 600</td>
</tr>
<tr>
<td>BD₁₃29</td>
<td>275</td>
<td>200 - 600</td>
</tr>
</tbody>
</table>

* Insert size was determined by migration of the BamHI fragment on a 10% polyacrylamide or 1.3% agarose gel and comparison was made to pAT153/HpaII digest fragments.

** Summary of data from Northern blot analysis [Figure 3.2 (panels a - f)].
transfer onto the nylon membrane are also shown as proof that lanes contain comparable amounts of RNA (Figure 3.2, panels 1). As anticipated, small and middle size RNAs are transferred more efficiently than the larger messages i.e. >28S (5.4 kb).

Table 3.2 summarizes the characteristics of the 44 potential neuronal-specific clones selected from the P19 neuron cDNA library. None of the clones were neuron-specific. Each of them presented a particular hybridization profile. None of them cross-reacted with rat L6 myoblast RNA. This suggested that their cDNA insert was mouse specific.

The pattern of hybridization revealed by clone AD$_2^{32}$ (panel a) indicates no specificity for neurons although the intensity of a band corresponding to 900 nucleotides is more intense in the brain than in EC cells. It was also very high in heart, kidney and F9 endoderm cells.

Clone AD$_2^{50}$ (panel b) hybridized to an RNA species of about 900 nt. The abundance of this RNA varied, being abundant in heart and less abundant in P19 EC cells, F9 endoderm cells and kidney. Although this clone was not neuronal specific, the presence of an extra band comigrating with 18S rRNA (about 2,100 nt) and found only in the heart and F9 endoderm renders the study of that particular clone more interesting since it may represent a case of differential splicing (alternative RNA processing).
Clone AD-46 (panel c) also showed no tissue-specificity revealing a band of about 2,100 nt in all samples analysed except for the tat L6 cells. The intensity of the band fluctuated, being very high in the heart, brain, F9 endoderm cells. In some instances, a second band was detected but the level of hybridization was very low.

The pattern of hybridization for clone BD-5 (panel d) was also disappointing. A band of approximately 2,100 nt was seen in all samples assayed and it was intense in heart. In addition, two extra bands of about 1,350 and 850 nt were detected in brain and heart while only the larger one was seen in P19 EC cells and embryonic neurons. The intensity of these bands also varied between the embryonic (EC cells, neurons) and adult samples (brain, heart). Thus, clone BD-5 seemed to recognize a developmentally regulated mRNA.

Clone BD-30 (panel e) hybridized to a transcript that comigrated with 18S rRNA but was present in EC cells as well as neurons, brain and heart.

The last six clones, BD-9, BD-18, BD-21, BD-29, AR-4, AR-50 (panel f) gave the same pattern of hybridization. The hybridization signal consisted of a smear (spanning a 200 to 600 nt region) rather than discrete bands and was more intense in P19 EC cells than in P19 neurons. It was not detected in brain and heart RNA. These cDNA clones seemed to contain the same sequence which appeared to be developmentally regulated. Chapter IV will be dedicated to the complete characterization of one of these sequences which may represent genetic information required in the earliest events of
differentiation which is repressed in the later stages of differentiation.

While the characterization of the eleven cDNA clones was underway, another screening of the library was performed using mRNA from P19 EC cells and P19 neurons. This mRNA was first broken up to an average size of 250 nucleotides then end-labeled. Using this screening procedure, the 11 clones discussed above reacted with probes from both P19 EC cells and their neuronal derivatives suggesting that this $^{32}$P-mRNA procedure was better. A completely different cDNA abundance profile within the library was obtained. Compared to the distribution profile that was established with $^{32}$P-single stranded cDNA probes, this time looking at 1500 clones, 28% (420) of the clones hybridized to at least one probe and most of them (18%) (270) contained sequences which were shared by both cell populations (P19 EC cells and P19 neurons). Approximately 9% (135) of the colonies hybridized to EC abundant sequences. Less than 1% (8 clones) of the library was represented by clones bearing neuronal-specific sequences. No further analysis was performed to confirm the specificity of these new "neuronal-specific" colonies.

Both screening procedures yielded different patterns of hybridization (data not shown) i.e. different abundance profile for the cDNAs. However, when referring to the 11 putative neuronal cDNA clones isolated during the screening with $[^{32}\text{P}]$ ss-cDNAs, the second approach using $^{32}$P end-labeled mRNA, although further trials will have to be made, seemed to have provided a better way of identifying possible neuronal cDNA clones and eliminating false positives. The
overall procedure seemed to be more sensitive than the one involving \(^{32}\)P-single stranded cDNA as probes. Less material is used in the preparation of probes and a 10-50 fold increase in the specific activity is obtained as compared to the one for the single-stranded cDNAs.
CHAPTER III

DISCUSSION

During animal development a pattern of differential gene expression is established, resulting in mRNA populations that are both qualitatively and quantitatively distinguishable from one cell type to another (Hastie and Bishop, 1976). The first step in determining how such differential distribution of mRNA is achieved is to prepare pure DNA probes for genes expressed preferentially in one cell type. With the availability of pure DNAs that are complementary to a broad set of tissue-specific mRNAs, it should be possible to define the regulatory step(s) in mRNA biosynthesis.

To identify abundant mRNAs that become expressed in early neuronal development, a cDNA library of 9,800 recombinants was generated from P19 neuron-enriched cultures and screened by differential hybridization to $^{32}$P single-stranded cDNA synthesized from undifferentiated P19 cells, P19 derived-fibroblasts and P19 neuron-enriched cultures (90% neurons, 10% fibroblast-like cells). This procedure identified 11 potential neuron-specific cDNA clones. In order to eliminate false positives, the screening was repeated three times. Unfortunately, further characterization of the 11 candidate sequences by experiments involving Northern blotting indicated that none contained sequences specific to the neuronal lineage. A second round of screening, this time using $^{32}$P end-labeled mRNA extracted from both the parental P19 and
the neuron-enriched cultures, gave a completely different pattern of hybridization and did not include the 11 "neuron" clones previously identified. Thus, this $^{32}$P-mRNA procedure should be a much more efficient mean of identifying differentially abundant RNA species.

Whereas the construction of a cDNA library is a relatively straightforward process, that of identifying and selecting the molecular clones of interest is less so. In order to minimize discrepancies, the screening procedure is usually repeated several times under constant parameters. In our study, the screenings were repeated three times or more for each probe series used (with new filters in some cases) and such variables as method of probe preparation (always prepared fresh with comparable final specific activity from the same mRNA stocks), hybridization conditions (including probe concentration and specific activity) and autoradiographic exposure were kept constant.

We suspect that the most important step is the preparation of the replica filters. Variation in the amount of bacteria transferred from the master plates to the replica filters may be the cause of our screening problems. Three replica filters were generated from every master plate and each, randomly chosen, was then hybridized to a particular probe. The lifts, thus being made in succession deposit more bacteria on the first filter and gradually less on those following. This would not have been a serious consideration if the hybridizations had been performed sequentially using the same filter for each probe. The amount of DNA lost during the steps for probe removal (or stripping) is minimal.
(Meinkoth and Wahl, 1984) and as a consequence the interpretation of the hybridization patterns is facilitated. With this approach, hybridization signals are usually reproducible between repeated rounds of screening and are not influenced by the number of plasmid-bearing bacteria originally transferred.

Notwithstanding technical difficulties, several other factors inherent to the biological system at hand may have hampered our efforts to identify neuronal messages. Reports on neuronal differentiation using EC cells have demonstrated the de novo expression of proteins characteristic of the differentiated state (Eddé et al, 1983; Jones-Villeneuve et al, 1983). Yet, although these data are suggestive of corresponding de novo transcribed mRNA, it is possible that the new neuronal proteins arise by post-transcriptional regulation of mRNAs whose levels do not vary during differentiation. If this were indeed the case, then the clones we have identified as sharing sequences with P19 EC, P19 EC-P19 neurons and P19 EC-P19 fibroblast transcripts should be the focus of experiments aimed at understanding this aspect of gene regulation during early neuronal development.

Work by Garrels and Schubert (1979) and Burnstein and Greene (1978) provided evidence that post-transcriptional/translational regulation is involved during neuronal differentiation. Although they demonstrated that the RA-induced neuronal differentiation of PC12 pheochromocytoma cells requires RNA synthesis, the use of high resolution two-dimensional polyacrylamide gel electrophoresis revealed no
quantitative change in the expression profile of more than 800 proteins. It was thus suggested that RA, like nerve growth factor, may induce differentiation by modulating protein synthesis rather than inducing qualitative changes in the pattern of gene transcription (Levi et al., 1985). Retinoic acid-induced neuronal differentiation of P19 EC cells may proceed the same way.

A possibility remains that new neuronal proteins may be encoded by low abundance mRNAs. If that were the situation, the representation of these mRNAs within the cDNA library would be correspondingly low. In this case, cDNA clones of such representative transcripts may have only been selectable by screening more than 2,500 colonies. Recently, a cDNA library was generated from differentiated F9 cells using "subtractive" hybridization in order to remove all house-keeping sequences and enrich the library for sequences specific to the differentiated state (Daniel Skup, Institut du Cancer de Montréal). However, following the characterization of several clones selected on the basis of their abundance within the library, it soon became evident that none were the result of transcription initiated de novo. Each sequence was shown to be present in the parental EC cells and showed substantial changes in levels of expression following induction (D. Skup, personal communication). Thus, de novo transcribed mRNAs may only be present in low abundance. One example of such low abundant mRNA de novo transcribed is that encoding the 58K neurofilament protein (F. Grosveld, personal communication).
Two different procedures were used to screen our cDNA library: the use of labeled single-stranded cDNAs and end-labeled mRNAs. Both yielded different abundance profile for the cDNAs. However, in reference to the 11 putative neuronal cDNA clones isolated during the first screening which showed no specificity for neurons, the second approach seemed to have provided a better way of eliminating false positive cDNA clones and identifying clones of interest. This statement is based on the assumption that a preferential representativeness of the 3' end sequence of most or all poly(A+)
RNAs used in the preparation of the single-stranded cDNAs was achieved; the priming reaction with oligo(dT) taking place at the poly(A) tail (3' end of the mRNA). Thus, two mRNAs sharing the same coding sequences but having different 3' ends would be detected as different with the single-stranded cDNA probes whereas with the end-labeled mRNA probes, they would be classified as similar. This indicates that cDNA library screening is technically tricky and should probably be done using labeled RNA rather than cDNA.

Our screening process did not yield neuronal specific coding sequences but has allowed the isolation of a group of clones bearing a genetic element which has some unusual properties and which may play an important role in gene expression. These clones will be the object of Chapter 4.
CHAPTER IV

EXPERIMENTAL RESULTS

Six of the eleven recombinant plasmids isolated from the P19 neuron cDNA library were retained for analysis because they reacted strongly to small transcripts of approximately 200-600 nucleotides in P19 EC cells, and that the hybridization signal was significantly reduced in P19 neurons and disappeared completely in mouse adult brain tissue. The level detected in neurons was estimated as approximately nine fold lower than that encountered in parental, undifferentiated P19 EC cells.

4.1 The six cDNA clones represent mouse repetitive sequences

Southern hybridization experiments were undertaken in order to assess the representation of the sequences found in each of the six clones within the mouse and human genomes. Genomic DNA was isolated from P19 EC cells, human EJ cells, 3RAl cells as well as NIH 3T3 cells and restricted with BamHI, EcoRI, HindIII, PstI and TaqI, fractionated by electrophoresis through 1% agarose gels, transferred onto nylon membranes and hybridized to $^{32}$P-nick translated plasmid prepared from each clone. The hybridization pattern obtained in each case was typical of that of a repetitive sequence (Figure 4.1). The sequence was not detected in the human EJ cell DNA thus suggested that it was mouse specific. In ad-
FIGURE 4.1

Southern blot analysis of one of the six cDNA clones selected from the P19 neuron-enriched cDNA library. Genomic DNA (20 ug) isolated from mouse P19 EC cells, 3RARl cells, 3T3 cells and human EJ cells was digested to completion with BamHI, EcoRI, PstI or HindIII, fractionated on a 1% agarose gel, transferred onto nylon filters and hybridized at 65°C respectively with each individual nick-translated cDNA clone (specific activity 10^7 - 10^8 cpm/ug) for 24 hours. Samples are P19 cut with BamHI (lane 1), EcoRI (lane 2), HindIII (lane 6), PstI (lane 7), TaqI (lane 8); human EJ cut with BamHI (lane 3), HindIII (lane 9), PstI (lane 10); 3RARl cut with BamHI (lane 4), HindIII (lane 11), PstI (lane 12); NIH 3T3 cut with BamHI (lane 5). The pattern of hybridization obtained after an overnight exposure at -70°C with intensifying screens was typical of a murine short repetitive element.
dition, the hybridization profiles revealed no discrete bands in any of the lanes as would be expected in the case of long interspersed repeated sequences such as MIF (Bennett and Hastie, 1984). Thus, this suggested that the six cDNA inserts may rather be related to short interspersed repeated elements.

No cross-hybridization experiments were done to determine how many of the clones, if any, represented the same sequence. However, the profiles of hybridization obtained for the Northern and Southern experiments (Fig. 3.2 and 4.1) which were the same for the six clones, suggested that the six cDNA inserts were either the same or closely related. Only one cDNA, BD11 21, was chosen among the six candidates and used for further analysis.

4.2 The complete base sequence of BD11 21

To further characterize the structure of this repetitive element and further investigate the relationship between this element and the murine SINES families B1 and B2, Sanger dideoxynucleotide sequencing was performed. The insert (about 225 bp) was removed from the plasmid by digestion with BamHI restriction enzyme, isolated from a 10% polyacrylamide gel and subcloned into M13mp18 vector. The T test which consists of sequencing using only one of the four sequencing reactions (in this case the T nucleotide reaction), was used to establish the orientation of the insert within the M13mp18 hybrid clones. The full sequence of two of the recombinant M13
clones referred to as clone 3 and clone 9 representing both orientations of the insert was then obtained (Figure 4.2, panels a, b).

A comparison of the sequence obtained (Figure 4.3) with the B2 consensus sequence indicated only nine mismatches scattered throughout the 207 bp sequence. Fifteen (15) nucleotides at the "5" end of the element are not part of the consensus.

The sequence of the element shows the presence of an RNA polymerase III split promoter and termination signal as well as a polyadenylation signal followed by a 13 nucleotide stretch of oligo(A). The entire repeat element consists of 192 nucleotides with an additional 15 nt, which may be part of an exon or an intervening sequence. This sequence is referred to as the positive (+) strand of B2. The full 207 bases were sequenced in the opposite orientation and no inconsistencies were found (See Figure 4.2 panels a,b).

4.3 Drop in the level of small B2 transcripts during P19 cell differentiation

To further investigate the kinetics involved in the decrease of the steady state level of the small transcripts in differentiated P19 derivatives, a time course of transcript abundance was established using plasmid BD11 to probe RNA extracted at various stages from PA-treated P19 cells (Figure 4.4) The level of transcripts decreased rapidly
FIGURE 4.2

(Panel a) Sequencing gel showing the complete sequence of clone M13mp18-3 as determined by Sanger dideoxynucleotide procedure. The BD$_{11}$21 sequence can be read off from the bottom to the top of each 8% urea "king kong" gel (0.4 mm thick). These gels were run for 6-7 hours at 3200 volts. The BamHI linkers flanking the repeat are shown as well as the oligo(A) stretch of 13 nucleotides; one of the features of the repeat. Each reaction is denoted at the top of each lane by a letter (A, C, G, T).
FIGURE 4.2 (continued)

(Panel b) Sequencing gel showing the complete sequence of clone M13mp18-9 representing the negative orientation of BD\textsubscript{14}21 repeat.
FIGURE 4.3

Complete base sequence of the positive strand of the repetitive element found in BD121 cDNA clone as determined by Sanger dideoxynucleotide method. The clone M13mp18-3 was used. The numbering is with reference to the nucleotide that immediately follows the BamHI linker used to construct the cDNA library. Nucleotides which differ from the B2 consensus are indicated by arrows. The RNA polymerase III split promoter is boxed. The termination signal and the polyadenylation signal are underlined. The 15 nt stretch which may be part of an exon or an intervening sequence is indicated by dashes. The entire sequence is 192 nucleotides not including the 15 nucleotides specific to this B2 copy. This sequence representing the RNA polymerase III transcribed strand of the B2 repeat is referred to as the positive strand because it contains the RNA polymerase III promoter.
Time course of disappearance of B2 RNAs in P19 RA-treated cells over a 30 day period. Total RNA was isolated from P19 cell cultures on daily intervals. 20 ug of total RNA was fractionated on a 1% formaldehyde gel, transferred onto nylon filters and hybridized at 42°C in the presence of 50% formamide with nick-translated BD11 plasmid and oligo(A) for 24 hours. Samples were as follows: P19 EC RNA (lane 1), P19 RA-treated cell RNA after 1, 2, 3, 4, 5, 7, 30 days exposure to RA (lanes 2-8). Exposure was for 2 days at -20°C with intensifying screens. The positions of 28S and 18S ribosomal RNA are shown.
during the first day of exposure to the drug. These transcripts were barely detectable 7 days and 30 days following RA treatment.

Total RNA was thus prepared from aggregated P19 cells harvested at intervals during the first day of induction with RA (Figure 4.5). A significant decrease in transcripts homologous to B2 occurred during the first hour of drug exposure. By cutting the corresponding areas out of the filters and determining the amount of hybridized $^{32}P$-labeled probe by liquid scintillation counting, the steady state level of the smaller transcripts was observed to drop from 100% in parental P19 EC cells, to 82% in cells treated for 2 hours with RA and 37% after 20 hours of treatment. The transcripts were not detectable in adult mouse brain tissue as shown in Figure 4.6. The data are also presented in another form in Figure 4.7, in order to highlight the kinetics of disappearance of both transcripts either individually or in combination during the initial 24 hour period with 5 X $10^{-7}$ M RA. The first few hours of exposure to RA lead to an increase in the level of the 200 nt RNA species while the counts for the 600 nt transcripts decreased. After approximately 4 hours of treatment however, both levels were comparable and continued to decrease.

In an attempt to ensure that the rapid decrease in small messages was due to the presence of RA in the medium rather than to extraneous events brought about by subculturing the cells as monolayers or aggregates, a Northern blot was prepared using total RNA from untreated as well as RA-treated monolayers of P19 cells harvested at different times
FIGURE 4.5

Level of B2 related-RNAs within a 24 hour RA exposure (5 x 10^{-7} M). Total RNA from P19 RA-treated aggregates was isolated after 0, 1, 2, 4, 6, 8, 10, 14, 18 and 20 hours exposure to the drug. 20 ug of each RNA was fractionated on a 1% formaldehyde gel, blotted on nylon filter, hybridized for 24 hours at 42°C in the presence of 50% formamide with BD_{1121} nick-translated plasmid (spec. act. of 5 x 10^{7} cpms/ug). The position of 28S and 18S rRNAs as well as the two diffuse bands of approximately 200 and 600 nucleotides which share homology with the repeat is indicated.

Panel a shows the ethidium bromide picture of the gel used for blotting. Panel b shows the filter following probing with pBD_{1121} and exposure to X-ray film.
Developmental regulation of 32 small RNAs during neuronal development. 20 ug of total RNA was treated as previously described. Samples were as follows: P19 EC cells (lane 1), P19 RA-treated aggregates at 2 hours (lane 2) and 20 hours (lane 3), mouse adult brain cells (lane 4).
Kinetics of disappearance of the B2 small transcripts within a 20 hour induction of differentiation into neuroectodermal cell derivatives. Bands from the previous exposed blots were cut using a razor blade and counted to determine the hybridized radioactivity (\(^{32}\)P-RBD \(_{21}\) DNA) (■) 200 nt RNA species; (○) 600 nt RNA species; (▲) both transcripts combined. Results are representative of three experiments.
within a 10 hour period. Figure 4.8 presents evidence that the addition of RA to the culture medium is responsible for reducing the steady state level of the small transcripts homologous to B2. Control samples at 0 and 8 hours gave hybridization signals of comparable intensity but comparison of the 8 hour control to the corresponding 8 hour RA-treated cells revealed a significant reduction (approximately 27%) in small transcript levels.

Taken together, these results indicated that these small B2-related transcripts are developmentally regulated. Their level decreased dramatically upon RA-induced differentiation of EC cells into neuroectodermal derivatives.

4.4 Expression of small B2 RNAs in various tissues

To further investigate the expression of small B2 transcripts and determine if the reduction in the level of small B2 RNAs was only restricted to RA-treated cell cultures, a Northern blot was prepared with total RNA isolated from embryonic samples of neurons, endoderm, fibroblasts, myoblasts and adult tissue samples of brain, spleen, heart and liver. Three different species were analyzed: mouse, rat and human (Figure 4.9, panels a, b).

As compared to undifferentiated EC cells, a lower level of small B2 RNAs was observed in all murine differentiated embryonic samples analysed. The post-natal samples (with the exception of human neuroblastoma cells used as negative controls) showed very faint signals.
FIGURE 4.8

Northern blot showing the effect of RA on the expression of B2 small transcripts. 20 ug of total RNA from untreated P19 EC cell monolayers and their corresponding RA-treated derivatives were fractionated on a 1% formaldehyde agarose gel, blotted onto a nylon filter and hybridized with BD121 nick-translated plasmid. The following samples were used: P19 EC untreated cells at 0 and 8 hours (lane 1, 3), P19 treated cells after 2 and 8 hours exposure to the drug (lane 2, 4). Panel a represents the ethidium bromide picture of the gel blotted and showed in panel b.

When counted, the hybridized radioactivity was for t = 0,8 hours untreated: 100% ; t = 2 hours in RA: 82% and t = 8 hours in RA: 73%.
Northern blot analysis showing the presence of the small B2 RNAs in various tissues. 20 μg of total RNA from various cell lines and species were treated as previously described. BD11 nick-translated plasmid was used as a probe. Panel a includes the following samples: P19 EC cells (lane 1), P19 RA-treated aggregates (24 hours) (lane 2), adult brain (lane 3), 3RAP1 cells (lane 4), mouse NIH 3T3 (lane 5), P19 (ras⁺)-1 (lane 6), P19 (ras⁺)-1 fibroblasts (lane 7), P19 (ras⁺)-2 fibroblasts (lane 8), heart (lane 9), spleen (lane 10), liver (lane 11), human neuroblastoma cells (lane 12), F9 clone 9 endodermal cells (lane 13), rat L6 myoblast cell line (lane 14), P19 RAC65 (lane 15) and P19 RA-treated RAC65 (lane 16). Panel b: P1901Al EC cells (lane 1), P19 DMSO-treated aggregates (2 hours) (lane 2), P19 D3 (lane 3), P19 DMSO-treated D3 (lane 4), P19 DMSO-treated RAC65 D⁺ cells (lane 5).

The decrease in small B2 RNAs is not tissue- or lineage-specific but is rather associated with differentiation events induced either by RA (panel a) or DMSO (panel b).
EC lines similar to P19 showed the same extent of hybridization (panel a: lanes 1, 6 15; panel b:1). The slight hybridization to both 200 and 600 nucleotide transcripts observed in rat L6 myoblast cells was probably attributed to cross-hybridization since repetitive sequences very similar to the murine B2 have been detected in the rat genome.

Immortalized fibroblast cell lines such as P19 (ras$^+$)-1 fibroblast, 3RAR1, NIH 3T3, along with two mutant cell lines derived from P19 (RAC65 and D3) were also analysed for the expression of small B2 RNAs.

The immortalized cell lines showed a reduction in the level of B2 RNAs as compared to EC cells (panel a: lanes 4, 5, 7, 8). However, the decrease observed in each of these cell lines (except for P19(ras)$^+$-2 fibroblasts) is not as significant as any of the other differentiated embryonic samples analysed. Both mutant P19 cell lines (which are unable to differentiate upon induction with RA or DMSO) showed the same pattern of hybridization as P19 EC cells before and after exposure to the drugs. However, and although this picture (Figure 4.9, panel b: lanes 1,2) does not reveal the decrease in the smaller B2 RNAs in DMSO-treated P19 01A1 cells, P19 01A1 treated for 2 hours with DMSO (1 %) showed a $10 \%$ reduction in B2 RNA level as compared to untreated cells. This decrease is less than the one shown for RA-treated cells (Figure 4.7 after 2 hours of treatment: $18 \%$ reduction). In addition, RAC65 D$^+$ which produces more muscle cells than the usual P19 01A1 cell lines showed a significant decrease in the level of expression of small B2 homologous RNAs as compared to EC cells.
Results in panel a (Figure 4.9) indicate that the disappearance of the small transcripts is seen in all cell lineages and correlates with the commitment of cells to differentiate following RA induction. Panel b (lanes 1,5) show that differentiation induced by DMSO also gives rise to a measurable decrease in the level of the small B2 RNAs when compared to parental cells. Hence, differentiation per se appears to be associated with the decrease of the small B2 RNAs.

4.5 Distribution of the small B2 RNAs within P19 EC cells and their neuronal derivatives

In order to gain further insight into the possible role(s) played by the small B2 RNAs in the mouse cell, their distribution was studied within undifferentiated P19 EC cells and their neuronal derivatives. The cells were fractionated into total RNA, poly(A⁺), poly(A⁻), polysomal and nuclear RNA and each individual strand of the B2 element was hybridized to Northern blots prepared with these various samples. In this experiment, B2(+) and B2(−) refer respectively to the strand to which the probe is reactive to. The blot shown in panel a (Figure 4.10) was probed with the complementary strand of B2(−) therefore the RNA is recognized by B2(−). The blot presented in panel b was probed with the complementary strand of B2(+) thus the RNA is recognized by B2(+).
FIGURE 4.10

Distribution of the small B2 RNAs in the mouse Pl9 undifferentiated EC cells and their neuronal derivatives. Both Pl9 EC cells and Pl9 derived-neurons were fractionated into nuclear, polysomal, poly(A\(^+\)), poly(A\(^-\)) and total RNA, run through a 1% formaldehyde agarose gel, blotted onto nylon membrane and hybridized with single-stranded probes M13mp18-3 (B2+) or M13mp18-9(B2-) made by primer extension (using M13 hybridization primer and two radiolabeled nucleotides) through the M13 phage portion leaving the B2 insert unlabeled and single-stranded (see Materials and Methods, Section 2.15). Specific activities were 2 x 10\(^6\) cpm/ug. Samples used were as follows: Pl9 EC total RNA (lane 1), Pl9 EC poly(A\(^+\)) (lane 2), Pl9 EC poly(A\(^-\)) (lane 3), Pl9 EC polysomal RNA (lane 4), Pl9 EC nuclear RNA (lane 5), Pl9 neuron total RNA (lane 6), neuron poly(A\(^+\)) (lane 7), neuron poly(A\(^-\)) (lane 8), neuron polysomal RNA (lane 9), neuron nuclear RNA (lane 10). 20 ug of total RNA, poly(A\(^-\)), polysomal, nuclear RNA and 4 ug of poly(A\(^+\)) RNA were used. The position of 28S and 18S ribosomal RNAs is indicated.

Panel a shows a blot hybridized with complementary strand of clone 9 (B2-) while panel b was hybridized with the complementary strand of clone 3 (B2+).

Panel c shows the same blot but hybridized with α-tubulin (message common to both EC and neurons).
Blots shown in Figure 4.10, panels a and b, revealed an interesting feature of the B2 elements: small B2-homologous RNAs (low molecular weight (LMW)-RNAs) are identified exclusively as B2(+) whereas the large B2-containing transcripts (high molecular weight (HMW)-RNAs) are primarily B2(-). Some HMW-RNAs are B2(+), but their level is much lower than HMW-B2(−) RNAs. This suggested that the positive strand of B2 featuring the RNA polymerase III promoter sequences gets transcribed primarily into small RNAs.

These blots indicated also that both small B2 RNAs and large B2-containing transcripts are polyadenylated (panels a, b; lanes 2, 7).

A good proportion of the small B2(+) RNAs (mainly the 600 nt-B2 small RNAs) was located in the nuclear fraction where the large B2(-) transcripts were exclusively found. A small portion of the small B2 (+) RNAs (primarily the 200 nt-B2 RNAs) was detected in polysomes whereas traces amount of large B2(-) transcripts were found in this fraction. The comparison of lanes 1, 4 and 5 or 6, 9 and 10 in panel b suggested that a large population of small B2 RNAs (200 nt B2 RNAs) are located outside the nucleus and not associated with polysomes.

Panel c which represents the filter used in panels a, b but hybridized with tubulin, indicated that the RNA fractionation was only partially successful. Poly(A+) and poly (A−) fractions (panel c, lanes 2, 3, 7, 8) showed degradation which explains the different apparent transcript sizes.
observed in panel a. In addition, poly(A⁺) fractions (c3, e8) were not devoid of α-tubulin mRNA. The nuclear fractions (c5, e10) had lots of mature α-tubulin mRNA and did not reveal an unprocessed pre-mRNA precursor, suggesting that the preparations were not clean.

This distribution profile of B2 elements in mouse P19 EC cells and their neuronal derivatives indicated that in the nucleus, small B2(+) RNAs (600 nt RNA species) and large B2(-) containing hn RNAs were both detected in high levels, especially in P19 EC cells. In the cytoplasm, the majority of small B2(+) RNAs (200 nt RNA species) seemed to be located in the non-polysomal RNA fraction although a subset of the 200 nt RNAs was detected in the polysomal fraction of both EC cells and neurons.
CHAPTER IV

DISCUSSION

We have described the identification of a transcribed repetitive element isolated from a cDNA library made from mRNA prepared from P19 cells induced with RA to differentiate into neurons. Whereas elevated expression of these transcripts is manifest in parental, undifferentiated EC cells, transcriptional levels of the message drop dramatically following exposure to retinoic acid \(5 \times 10^{-7} M\). Furthermore, this murine repetitive sequence (which shares extensive homology with the B2 element) is not expressed in adult brain tissue. These observations on B2 expression during neuronal differentiation supplement previous studies documenting such reduction in the level of small B2 RNAs during F9 endodermal differentiation (Murphy et al., 1983) and in some normal tissues such as heart and liver (Kramarov et al., 1982; Grigoryan et al., 1985). However, this is the first demonstration that the reduced expression of the small B2 RNAs occurs very early and precedes or is concomitant with the commitment of P19 EC cells to differentiate into neuroectodermal cell derivatives (Campione-Piccardo et al., 1985). Thus suggesting that B2 repeat sequences may be involved either in the earliest events of differentiation, in the process of determination, or in maintaining or modulating the proliferation state of the cells prior to receiving the stimuli responsible for the initiation of differentiation.
The kinetics of disappearance established for both 200 and 600 nt B2-homologous RNAs (Fig. 4.7) suggest that the smaller species may represent the products of processing of the larger 600 nt transcripts. To determine if both 200 and 600 nt RNAs are distinct species or if the 600 nt RNA is the precursor of the 200 nt RNA, the experiments described for B1 should be done (Adeniyi-Jones and Zasloff, 1985). Such situation prevails in the B1 family (murine AluT equivalent) in which the 210-nt primary transcript is processed to yield a smaller RNA (Adeniyi-Jones and Zasloff, 1985). A similar phenomenon may take place in the B2 family.

The length of the poly(A) tail during B2 polyadenylation may also give rise to size variations in B2 transcripts. Size heterogeneity may also result from the extension of transcription beyond the usual 3'-terminus due to the loss of one T from the TCTTT sequence. Sequence TCTT does not terminate transcription and RNA polymerase III moves until it meets another oligo(dT) block. Kramerov et al (1985) isolated many of these extended-transcripts from a cDNA library made from the small B2 RNAs suggesting that this may be a major way, along with the length of poly(A) tail, of producing a population of B2 RNAs with different sizes.

Many previous reports suggested that B2 RNA synthesis may be related to cell division or cell proliferation (Vasseur et al, 1985a; Edwards et al, 1985; Singh et al, 1985). Our results showing the reduction of B2 RNAs in immortalized cell lines such as P19(ras\(^+\))-1 fibroblast or NIH 3T3 indicate that B2 may not be related to cell proliferation but rather be associated with the transformed state itself. The same
observation was made by Grigoryan et al (1985) when comparing the level of small poly(A)$^+$ B2 RNA in normal and tumor cells of the same origin (LLC and lung, liver and hepatoma, MOPC-21 plasmacytoma and spleen).

The results obtained for RA- and DMSO-treated cells showing a reduction in the level of the small B2 RNAs suggest that both drugs initiate intracellular changes which result in lower levels of the small B2 RNAs. This further suggests that the changes observed in B2 level are not the direct effect of the drugs but are directly related to the intracellular changes mediated by the induction of differentiation.

These quantitative changes in the abundance of B2 transcripts occurring during in vitro differentiation of EC cells parallel those detected during in vivo development of mouse embryos (Vasseur et al, 1985a). Transcription of B2-containing RNAs takes place very early and persists in pluripotential embryonic cells until day 7 of embryogenesis (corresponding at that stage to EC cells), suggesting the involvement of B2 in the early events of differentiation or determination or any of the possibilities mentioned earlier.

Previous efforts which focused on demonstrating the transcriptional activity of B2 sequences during cellular proliferation have provided evidence suggesting that synthesis of small B2 transcripts is elicited by RNA polymerase III stimulating factors (Svensson and Akusjarvi, 1985). Since nothing is currently known about regulatory mechanisms underlying the repression of such transcripts, the modulation
of B2 sequence transcription may thus be achieved through events which influence the interaction of RNA polymerase III with active or potentially active chromatin by such stimulating (or repressor) factors.
CHAPTER V

CONCLUSIONS

The construction of a cDNA library from P19 neuronal derivatives allowed us to isolate a repetitive sequence (the murine B2 element) which is expressed and quantitatively regulated during differentiation of EC cells. Our observation of the disappearance of small B2 transcripts during neuronal differentiation is provocative and suggestive of a role for B2 repeat sequences in the differential regulation of gene expression. Several important questions however have yet to be answered. What are the molecular events involved in regulating the transcription of B2 sequences? Can the retinoic acid induced repression of B2 RNA synthesis be attributed to a failure to initiate transcription or to an increase in transcript turnover? What purpose do B2 sequences serve in the murine genome and how do they influence or participate (if at all) in the regulation of gene expression?

One way to distinguish between the two possibilities (i.e. failure to initiate transcription or increase in turnover rate) is to perform run-off transcription assays and compare the levels of newly synthesized B2 RNAs in EC cells and neuron populations. If regulation of B2 is at the level of transcription, then this would suggest that the site of RA action and/or the early differentiation signals would affect rate-limiting event in polymerase III transcription of B2.
It remains unclear as to how RA brings about the reduction of B2 RNA levels. Does RA act directly or does it initiate, stimulate or participate in a cascade of events leading to the repression of B2 transcripts? Reports indicate that the uptake of RA by the cell enhances the concentration of cAMP-protein kinase (Anderson et al, 1983) and suggests that the ensuing stimulation or activation of a protein phosphorylation cascade may ultimately regulate B2 expression by phosphorylating the RNA polymerase III-transcriptional factors (TFIII A, B, C).

The mechanism of action of RA (i.e. what follows the uptake of RA by the cell via the membrane, its interaction with a cytoplasmic cellular RA-binding protein (cRABP) and the translocation of this complex into the nucleus) remains obscure (Jetten and Jetten, 1979; McCue et al, 1983; Sherman et al, 1983). It is known however that RA can simultaneously activate and suppress gene expression in cultured cells as well as in the whole animal and it does so very rapidly, within one to two hours after its uptake by the cell (Omori and Chytil, 1982). However, the way by which RA might interact with the genetic script to initiate these effects has yet to be demonstrated. An interesting proposal suggests that cRABP-RA complex could interact directly with the genome and alter chromatin structure such as to favor or hinder its transcription (Chytil and Ong, 1979; Sherman et al, 1983; Reeves (1984).

On the basis of this hypothesis and of our data, the following models are suggested to explain the mechanism of action of RA on B2 transcription, to define its involvement
in the reduction of the small B2 RNAs and envisage how the small B2 RNAs could act as repressors of gene expression in early events of differentiation.

Model 1: RA as a regulator of RNA polymerase III activity.

This model proposes that RA regulates RNA polymerase III activity directly by binding to it or indirectly by blocking the transcription of some or all of the genes encoding the transcriptional factors TFIIIA, B, C required for the formation of the protein-complex to which RNA polymerase III should bind to activate B2 transcription (Lassar et al, 1983; Enver, 1985).

If a certain affinity exists between RA/cRABP complex and polymerase III or any of the RNA polymerase III transcriptional factors, one would expect a decrease in RNA polymerase III activity in P19 neurons as compared to P19 EC cells. Therefore, a determination of the activity of RNA polymerase III in both cellular populations via the analysis of the transcription profile of both 5S RNA and tRNA (other RNA polymerase III transcribed RNAs) could validate the model.

By affecting RNA polymerase III activity, RA would interfere with B2 transcription and would prevent the formation of RNA-RNA duplexes thereby allowing the processing and translation of the "differentiated" messages to occur (See Model: B2(+) RNAs as repressors of gene expression).
Model 2: RA with a specific affinity for the B2 elements.

This model suggests that RA associated with specific protein (eCRABP) B2 elements and consequently prevents RNA polymerase III from gaining access to promoter sequences and initiate B2 transcription. This model also provides us with an explanation for the various patterns of cellular differentiation generated by treatment of P19 EC cells with various concentrations of RA. Neuroectodermal cell derivatives develop when P19 EC cells are exposed to 5 x 10^{-7} M RA whereas 10^{-9} - 10^{-8} M RA is sufficient to give rise to mesodermal cell types such as skeletal and cardiac muscle. We propose that neuronal genes host more B2 elements than the muscle genes. Thus, to block B2 transcription, neuronal genes would require higher concentrations of RA.

To determine if RA has any affinity for B2 sequences, one could prepare columns in which the complex eCRABP/RA would be covalently bound and pass B2-labeled sequences over that column. Alternative methods to use would be the biotinylated-cellulose column described by D. Ward (Yale University, personal communication) or the affinity column described by Levens and Howley, 1985. However, the ultimate experiment would be to transfect proper recipient cells, via the DNA-mediated gene transfer, with a B2-depleted gene and study its expression after RA treatment. If B2 sequences are required for RA to initiate changes which trigger expression of that particular gene then, this B2-depleted gene should not be expressed.
In order to determine if neuronal genes contain more copies of the B2 repeat sequence than muscle genes, one could use neuronal specific cDNA to retrieve corresponding genomic neuronal sequences and ultimately, entire neuronal genes. Thereafter, by comparing the hybridization profile of restricted genomic DNA clones specifying either neuronal or myogenic sequences using a B2 sequence probe, a reasonable estimation of the abundance of B2 sequences within these genes would be obtained. One may also undertake the more labour intensive task of DNA sequence comparison in order to map the distribution of B2 repeat elements within cellular genes.

Model 3: RA may induce new factors competing for B2 promoter sequences.

RA could promote the transcription of factors specific to B2-neuronal and B2-myogenic factors which would compete with RNA polymerase III-transcriptional factors for B2 sequences. At $10^{-8} \text{M}$, only certain factors would be produced and at $10^{-7} \text{M}$ yet other factors would be elicited to match the corresponding B2 sequences. Although this model offers another explanation to why different concentrations of RA give rise to various cell types, it also implies that each set of lineage-specific genes is characterized by a specified B2 element. Previous reports have indicated that all B2 copies differ in only 5 to 10% of their bases and that these differences usually spread through the sequence and do not give rise to any significant variation between the B2 repeats. Thus, B2 copies are all considered identical.
Among these models suggested, only one (Model 2) is more likely to give a realistic view of the situation that could prevail in the RA-treated P19 cell cultures. The argument against Model 1 is that the inhibition of RNA polymerase III by RA/cRABP complex would prevent the production of 5S RNA and tRNA which are required throughout the cell cycle. This model would thus result in cell death. Model 3 implies that at least some B2 repeats have lineage- or tissue-specificity therefore have variations in the nucleotide sequence and structure. However, all B2 repeats so far isolated from various tissues or cell lineages were shown to be identical.

Before testing Model 2, an interesting experiment to perform would be to determine which of the RNA polymerase III transcriptional factors are involved in the transcription of the B2 elements. The presence of the RNA polymerase III split promoter within the B2 sequence and failure to inhibit B2 RNA transcription by concentrations of α-amanitin which would otherwise inhibit RNA polymerase II suggested that B2 elements are transcribed by RNA polymerase III. However, no one has ever demonstrated the binding of RNA polymerase III transcriptional factors (TFIII A, B, C) to B2 sequences. This could be demonstrated with relative ease by following one of several procedures now available to isolate DNA-binding proteins. The methods described by Sancar and Rupp, 1983, Miskimins et al, 1985, Levens and Howley, 1985 or the one by D. Ward (personal communication) could all be used to define which of the TF III's bind to B2 sequences. As a final experiment, an in vitro transcription assay using RNA polymerase III and the proper TF III, would show the transcription of the B2 elements by RNA polymerase III.
The previous models are suggestive of a role for RA as inhibitor of the small B2 RNA expression. Our data on the distribution of small B2 RNAs within various subcellular fractions of P19 EC cells and their neuronal derivatives suggested that B2 RNAs may be involved in post-transcriptional (RNA processing) regulation. The striking observation was that small 600 nt B2(+) RNAs and large B2(-) RNAs (B2(-) containing hnRNAs) were both detected in the nucleus in high levels, especially in P19 EC cells. The ensuing model presents the small B2 RNAs (600 nt RNA species) as repressors of gene expression. We speculate that these small transcripts interfere with RNA processing/splicing/translation by forming RNA-RNA duplexes with large B2-containing hn-RNAs via their complementary sequences. This hypothesis is not without precedents. The control of translation via RNA-RNA duplex formation has been extensively documented in prokaryotes (Mizuno et al., 1984; Coleman et al., 1985; Izant and Weintraub, 1985) and has recently been described in eukaryotes (Izant and Weintraub, 1984, 1985; Rosenberg et al., 1985). Many occurrences (both in vitro and in vivo) of antisense inhibition as a means controlling translation have been described (For review, see Izant and Weintraub, 1985). Inhibition can be cytoplasmic (translational) as well as nuclear (post-transcriptional/RNA processing/splicing). Double-stranded RNA hybrid molecules have thus been detected, showing that this may well be a useful mechanism for the control of gene expression.

An important step in the maturation of mRNAs is the assembly of hnRNA into RNP complexes termed hnRNP particles. The removal of intron transcripts and splicing of
mRNA sequences takes place in hnRNP particles (Pederson, 1983). If one or more duplexes of B2(+)/B2(−) are formed in any transcript (nascent RNA chain), this event may interfere with the secondary structure of the transcript and the subsequent packaging of the ribonucleoproteins around the RNA chain, thereby preventing proper polyadenylation (3' termini) and splicing of the RNA. We suspect that such a phenomenon takes place in the P19 cells where numerous B2(+) RNAs are detected. In P19 neurons where the levels of B2(+) RNAs are much lower, hnRNPs would be assembled properly, and allow the processing and the ensuing translation of the "differentiated" messages to occur.

According to our hypothesis, repression of the small B2 RNAs is required for the initiation of differentiation. RA could block the formation of B2 small RNAs via any one of the previously elaborated models and thereby prevent the formation of duplexes between small and large B2 transcripts and induce differentiation. One way to determine the extent of B2 involvement in post-transcriptional regulation would be to isolate the RNA-RNA duplexes from P19 EC cells and P19 neurons. If the model is correct, more duplexes should be isolated from the parental EC cells. Alternatively, one could remove all B2 sequences from a neuronal gene and study the expression of that B2-depleted gene after its reintroduction by DNA mediated gene transfer into proper recipient cells. These experiments would give possible biological functions to the B2 repeats whose role(s) remain obscure.

Although our observations suggest that short B2 repeat sequences may play a role in post-transcriptional regulation,
we have no reason to dismiss the possibility that the B2 elements may serve multiple functions within the mouse genome. Further experimentation is undoubtedly needed to clarify and understand the physiological purpose of these abundant, short repeat sequences.
REFERENCES


Svensson, C. and G. Akusjarvi (1985) Adenovirus VA-RNA1 mediates a translational stimulation which is not restricted to the viral mRNAs. EMBO J. 4: 957-964.


