THE UNIVERSITY OF CALGARY

The modulation of the neuronal expression of the ATBF1-A gene.

by

Duane Hewitt

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

CALGARY, ALBERTA

SEPTEMBER, 1996

Abstract

Tissue specific gene expression is a complex problem in molecular biology. The mechanisms underlying the control of the level of transcription alone have been the object of study for many years. Transcription factors control the expression of specific genes which perform characteristic functions in the tissues as well as a complex cascade of transcription factors. ATBF1 is a transcription factor that appears to be involved in neuronal development. It is induced early on in the neuronal differentiation pathway in cell culture. The factors which act on the upstream promoter region and establish the neuronal specific expression pattern of the ATBF1-A isoform were of interest in this study. Biochemical methods were used to characterize the factors capable of binding the ATBF1-A promoter region. These assays included the electrophoretic mobility shift assay (gel shift) and DNase I footprinting. It was found that the transcription factor AP-2 bound the ATBF1-A promoter and AP-2 is implicated in the regulation of the ATBF1-A promoter.

Acknowledgements

I would like to thank Dr. Tamaoki and Dr. Miura for their assistance in the timely completion of this project and for their hospitality during my term in Dr. Tamaoki's laboratory. Many thanks to the members of my Supervisory Committee for their helpful suggestions. I would also like to thank Dr. Schultz for the arrangements made so that I could complete the requirements for my thesis. Finally, I would like to thank my wife, Lana Wylie, for her support throughout my degree program.

Table of Contents

List of Figures

Introduction

The control of gene expression is a problem of breathtaking scope and complexity. Many different levels of control have been discovered to date and it is likely that several more will be uncovered in the years to come. Some examples of these levels that have been characterized are pre-transcriptional, transcription initiation, transcriptional elongation, post-transcriptional, translational and post-translational. Of these examples, the level that is arguably the most well characterized is the initiation of transcription (Andrin and Spencer, 1994 and Harrison, 1990). This level has been studied extensively but the examination seems to raise more questions rather than providing satisfying answers. A good model system to study the control of gene expression is the developmental switch such as the beta globin gene family (Orkin, 1990, Andrin and Spencer, 1994 and Harrison, 1990). In such a system a specific event causes a dramatic change in the expression levels of one or several genes at the appropriate time in the appropriate tissues.

The mechanisms underlying tissue specific gene expression are currently being elucidated by a wide variety of techniques (Orkin, 1990, Andrin and Spencer, 1994 and Harrison, 1990). The interplay of transcription factors and gene expression as well as post-translational modifications all play a role in this process. The control elements in gene expression include

but are not solely comprised of sequences in upstream promoters and enhancer elements (Orkin, 1990). The specific occupancy of these sites by DNA binding proteins modulates the efficiency of transcription. Tissue and temporal regulation of gene expression may be modulated in different ways. One mechanism is through the presence of transcription factors in a tissue that bind to specific elements that activate genes in the proper context.

The beta-globin genes were the first such system cloned from mammals and have been of interest ever since their initial characterization (Orkin, 1990). These genes demonstrate specific, reproducible changes in expression patterns during development (Harrison, 1990). Despite the extensive characterization of these genes there remain many open questions regarding the actual mechanisms by which tissue specific gene expression is modulated. The binding of transcription factors to specific DNA sequences in promoter and enhancer regions of these genes has been well established (Orkin, 1990). Specific developmental changes in the occupancy of promoter and enhancer binding sites have been demonstrated *in vitro* as well as *in vivo* using the globin genes (Fraser *et al.*, 1993). However, relating these changes to direct mechanisms for the correlated changes in gene expression remains incomplete. Therefore, further study of these genes along with other such systems is required to elucidate the

basic mechanisms underlying the control of transcription initiation.

Developmental switches are often controlled by a corresponding "master" gene which potentiates the transcription of several genes which are expressed in specific tissues at specific times (Muscat, 1995 and Orkin, 1990). In erythroid genes this role is fulfilled by the transcription factor GATA-1 (Orkin, 1990). GATA-1 has been demonstrated to be involved in the appropriate expression of an array of erythroid specific genes. Furthermore, a GATA-1 enhancer site was shown to mediate distal enhancer activity through the beta globin TATA box found in chickens (Engel, 1991). The difficulty in fully characterizing this system lies in the fact that the control of transcription results from multiple regulatory modules acting synergistically (Engel, 1993). The Locus Control Region of the beta globin gene cluster responds to varying concentrations of a limited number of transcription factors (Barton *et al.*, 1993). GATA-1 has been shown to interact during chromatin assembly with a stage specific factor to potentiate expression of the appropriate globin family member (Emerson, 1991).

Multiple factors may compete for binding to similar or overlapping sites in a promoter (Yu *et al.*, 1990, Rahuel *et al.*, 1992 and Fisher *et al.*, 1993). Distinct factors may also cooperate in binding to a promoter (Yu *et al.*, 1990, Fischer

et al., 1993 and Andersson *et al.*, 1994). This increase in complexity allows for a finer level of control. Competition among transcription factors for similar or overlapping binding sites makes possible large changes in gene expression with relatively subtle changes in the concentration of transcription factors (Bacon *et al.*, 1995). GATA-1 has been shown to specifically displace a repressor from its binding site in the glycophorin B promoter in an erythroid specific manner (Rahuel, *et al.*, 1992). Therefore, the study of the beta-globin gene cluster has uncovered many clues to the mechanisms regulating gene expression(Andrin and Spencer, 1994). However, other systems of tissue specific gene expression need to be studied to determine the features in common with the beta-globin genes and the unique features of each system.

Another example of a tissue specific transcription factor is the protein HNF-1 (Mendel and Crabtree, 1991) which activates the alpha-fetoprotein (AFP) gene in hepatoma cells but not in HeLa cells (Sawadaishi, S. *et al.*, 1988). A binding site for HNF-1 is also found in the albumin promoter (Sawadaishi, S. *et al.*, 1988). This illustrates that a single transcription factor may participate in the tissue specific expression of multiple genes.

The AFP gene is another example of a transcriptionally modulated developmental switch in which a marked decline of

mRNA production occurs rapidly after birth (Sawadaishi, S. *et al.*, 1988). The AFP gene is often induced in hepatoma cell lines(Sawadaishi, S. *et al.*, 1988). This makes it an interesting study because the transcription factors involved in its regulation may also play a role in liver cancers. From the characterization of factors that bind AFP regulatory sequences a transcription factor that binds an AT-rich sequence was cloned (Morinaga, *et al*, 1991).

The AFP enhancer possesses a site that binds HNF-1 with ten-fold less affinity than the site found in the promoter region (Morinaga, *et al*, 1991). An additional factor has been discovered that binds this sequence and has been named ATBF1 (Morinaga, *et al*, 1991). ATBF1 is unique in a number of ways. It is extremely large for a transcription factor, measuring 306 kDa and was the largest DNA binding factor cloned up to that point. It is also unusual in that it possesses seventeen zinc finger motifs and four homeodomain motifs (Morinaga, *et al*, 1991). It was the first protein discovered to combine these two DNA binding motifs. ATBF1 has been shown to be a repressor of AT-rich elements in the human AFP gene (Yasuda, *et al.*, 1991). It has not been determined whether ATBF1 is the primary repressor of AFP *in vivo*. ATBF1 mRNA is detected in HeLa cells but no footprint is detected with HeLa nuclear extracts (Sawadaishi, *et al.*, 1988) which may indicate that ATBF1 is regulated post-transcriptionally.

The homeodomain genes encode transcription factors that are implicated in development (Wright *et al.*, 1989 and Manak and Scott, 1994). The homeodomain is an evolutionarily conserved 60 amino acid motif that binds DNA (Manak and Scott, 1994). These genes were initially identified as playing a role in the determination of segment identity and in differentiation along the anterior/posterior axis in *Drosophila* (Wright *et al.*, 1989). Several homeodomain proteins have been shown to be important during neurogenesis (Doe *et al.*, 1993).

The homeodomains in ATBF1 show from 27-43% homology with other homedomain transcription factors (Morinaga *et al.*, 1991). The homology of ATBF1 is higher with the LIM family of homeodomains than with the POU family (Morinaga *et al.*, 1991). The highest levels of conservation are found in the third helix which is the DNA site recognition helix (Morinaga *et al.*, 1991). Upstream of the first homeodomain in ATBF1 is a stretch of 131 amino acids that are characteristic of the POU specific A box (Morinaga *et al.*, 1991).

Recently a homolog of ATBF1 has been cloned in *Drosophila* (Fortini, *et al*, 1991). This homolog is similar to ATBF1 except that it has sixteen zinc fingers and three homeodomains (Fortini, *et al*, 1991). This homolog has been named zfh-2 and has been implicated in neurogenesis (Lai *et al*, 1991 and Lundell and Hirsh, 1992). Strong identity (63%-78% homology)

has been detected between the first three homeodomains of ATBF1 and the corresponding domains in zfh-2. The strongest identity (78%) occurs in the first homeodomain motif with several unusual residues being conserved. This has led to this unusual domain being termed a "pseudo-homeodomain" (Fortini *et al*, 1991). Some of the zinc fingers also show amino acid sequence homology (51-69%). The homology between zfh-2 and ATBF1 outside the DNA binding domains has significantly lower identity (30-40%) than within these regions(Fortini, *et al*, 1991). The zfh-2 gene product has been implicated in the regulation of the neuron specific DOPA decarboxylase gene (Lundell and Hirsh, 1992). The fact that this large protein is evolutionarily conserved as one unit between mice, men and fruit flies seems to indicate that it serves important functions as a single unit.

ATBF1 mRNA has been detected in the brains of 1-5 day old neonatal mice as well as in 17 day old embryos (Ido *et al*, 1994). ATBF1 transcripts have also been detected within three hours of retinoic acid induced neuronal differentiation of a mouse embryonal cell line (P19) (Miura, *et al.*, 1995). This transcription factor is not detected in DMSO induced differentiation of P19 into skeletal and cardiac muscle cells (Ido, *et al.*, 1994) ATBF1 was undetectable prior to induction of P19 cells with retinoic acid (Miura, *et al.*, 1995). These

results suggest that ATBF1 may play a role in the development of certain regions of the brain.

The P19 cell line is currently being studied as a model system for neuronal differentiation (Bain *et al.*, 1994). P19 are a line of pluripotent mouse embryonal carcinoma cells. The differentiation of these cells can be induced by retinoic acid (RA) and dimethyl sulfoxide (DMSO) (McBurney, 1993). These two drugs have been shown to act through distinct pathways (Edwards *et al.*, 1983 and Jones-Villeneuve *et al.*, 1983). P19 cells possess a normal karyotype, are multipotential, divide rapidly, maintain the ability to differentiate over multiple passages and are stably transfected at high frequency (McBurney, 1993). These cells display the neuronal cell surface markers when assayed with antibodies (McBurney *et al.*, 1988). These characteristics combined with the ability to be inducibly differentiated into neurons make P19 an ideal system in which to study neuronal development.

There are multiple pathways along which P19 cells may be induced to differentiate. The neuronal pathway appears to be mediated by the retinoic acid receptors (RAR) which are members of the ligand dependent transcription factor superfamily (McBurney, 1993). Neuronal cell surface markers do not appear until at least three days after induction and these markers become abundant 5-6 days after induction (McBurney *et al.*, 1988). A mutant cell line has been isolated that does not

differentiate in response to retinoic acid (Pratt *et al.*, 1990). This cell line expresses some but not all retinoic acid responsive genes upon induction (Pratt *et al.*, 1993). When P19 cells are induced with DMSO along a muscle differentiation pathway ATBF1 message is not detected.

It takes as little as two to four hours of exposure to retinoic acid in order for the cells to commit to a neuronal differentiation pathway (Berg and McBurney, 1990). Significantly this corresponds to the time of induction of ATBF1 mRNA which can be detected at high levels as early as three hours of retinoic acid induction (Miura, *et al.*, 1995). This evidence indicates that ATBF1 may be involved directly in the commitment of P19 cells to the neuronal differentiation pathway or may be an early marker for the commitment event.

Recently it has been discovered that there are two promoters from which ATBF1 mRNA is produced (Miura, *et al*, 1995). The two distinct isoforms are referred to as ATBF1A and ATBF1B respectively. The ATBF1 mRNA has been shown to be induced upon the neuronal differentiation of P19 cells (Miura, *et al.*, 1994). A 400 base pair fragment has been shown to be sufficient for a portion of the neuronal specificity of this promoter (Figure 1). From an examination of the DNA sequences of this fragment there are several matches to the consensus binding sites of transcription factors previously implicated in neuronal specific gene expression. The characterization of

this promoter will lead to a better understanding of the mechanisms underlying neuronal development and the temporal and spatial control of gene expression.

Some candidates that may be involved in the neuronal specificity of ATBF1A are AP-2, zif268/egr-1, the neuronal specific silencer element (NRSE), and POU domain proteins such as Oct-3, Brn-2 and Emb. All of these factors have previously been shown to be involved in neuronal differentiation. There are several putative binding sites for AP-2 in the promoter region that is sufficient for neuronal specificity (Figure 1). There are also sites for Sp1 in this region. An interesting site that appears from the sequence is one half of the direct repeat required to bind the retinoic acid receptor (RAR) which is adjacent to one of the AP-2 binding sites of the ATBF1A promoter. It is conceivable that the retinoic acid receptor may interact with AP-2 in some sort of heteromeric complex to activate ATBF1 expression in retinoic acid induced P19 cells.

AP-2 has a molecular mass of 52 kDa and has been shown to bind DNA as a dimer (Williams and Tjian, 1991). This transcription factor has been cloned and expressed in bacteria and binds the consensus sequence 5'CCCCAGGC 3'(Williams *et al.*, 1988). It contains a proline rich activation domain along with an alpha-helical DNA binding domain (Williams *et al.*, 1988). AP-2 is an immediate early gene which means that

Figure 1- DNA sequence of the ATBF1A promoter fragment that has been shown to confer neuronal specificity in CAT assays. Sequences of interest are indicated by underlining for restriction enzyme sites which are labelled in italics and by bold and underlining for putative transcription factor binding sites. The matches of the AP-2 sites to the consensus sequence are as follows:

Site	Sequence	Matches
A	CCCCTCGG	5/8
B	CCCCAGGC	8/8
C	CCCAAGGC	7/8
D	CCCAAGCG	6/8
E	GCGGGTGCG	8/9

AP-2 Consensus CCCCAGGC

Egr-1 Consensus GCG(G/T)GGGCG

AGGCCTCAGGCTCCTTTTAGTGGCCGAGGGCGCGTCCCTTTCTTCTCGCCCGATTCTAG

Stu I

GCCGGCCCCTGACCTTTGATGAGCGAGGGGTCAGCCCCTCGGGGAGGCCCTGGGCTTTGT

NaeI RAR(1/2) AP-2[A],

TCCCGCCGGGGCCGGTTCCCGGGCGGCACAGCCCGAGCGGCGAGGTTCCTGCCCCAG*GC*

Sp1 AP-2[B]

*GGGTGCG*CGGGACCCCCGGTCTGCCCCAAGGCCCGCGCCCCAAGCGGAGCGAGCG

zif-268[E] AP-2[C]{*StyI*} AP-2[D]

CAGCTGAGCGCCCGGGTCccgcgcgtccctgtgcgtccccgggtccctgcgggcgggcgg

PvuII

gcgcggtcgccgccgagcaaccggcctgcgcccggcacgactgtagatgtcaggctttgc

ccggggagccgagcggcagcgggctgtgagttttcaaattaaccttccgctttgttgctg

tgtaatgtggatcc

BamH1

exposure of serum starved cells with growth factors (Mitchel *et al.*, 1991). AP-2 is a transactivator that has demonstrated expression in the developing nervous system of the mouse and binding activity in several neuronal specific promoters (Maden and Holden, 1992 and Andersson *et al.*, 1994).

The expression pattern of AP-2 *in vivo* also suggests a role in neuronal development (Mitchell, *et al.*, 1991). The main expression of AP-2 was found in the neural crest lineage of the mouse and RNA levels peaked in the 11.5 day embryo (Mitchell, *et al.*, 1991). AP-2 has also been shown to be induced by retinoic acid during P19 neuronal differentiation (Philip *et al.*, 1994). This roughly correlates to the expression of ATBF1 during development and P19 neuronal differentiation (Ido *et al.*, 1994 and Miura *et al*, 1994).

Due to the presence of multiple putative AP-2 sites in the ATBF1a promoter (Figure 1) and the demonstrated role in neuronal development, AP-2 is the prime suspect for the modulation of neuronal specificity of the ATBF1 promoter. However, there is limited data on the precise role of AP-2 in neuronal development. Presumably it participates in a cascade of regulatory factors which lead to the expression of neuronal specific genes. Some possible downstream targets of AP-2 are Brn-2 and acetylcholinesterase (Maden and Holden, 1992).

In P19 cells it has been determined that retinoic acid induced neuronal differentiation is accompanied by increased

expression of AP-2 (Phillipp *et al.*, 1994). AP-2 was not detected during DMSO induced differentiation of P19 cells even when retinoic acid was present nor during retinoic acid induced differentiation of F9 cells which do not differentiate into neurons (Phillipp *et al.*, 1994). This indicates that the induction of AP-2 is not solely in response to retinoic acid but is specific for the neuronal differentiation pathway. The induction of AP-2 peaks at day 11 of retinoic acid induction in P19 cells (Williams, *et al.*, 1988). The major source of these AP-2 transcripts is correlated with the number of nondividing neurons (Phillipp *et al.*, 1994). This protein is highly conserved between frogs, mice and humans which suggests an important role in vertebrate development (Winning *et al.*, 1991).

The expression of AP-2 has also been shown to be enhanced during retinoic acid induced differentiation of NT2 cells (Luscher *et al.*, 1989). AP-2 was shown to peak at 48-72 hrs and decline thereafter. There are several genes that are activated by retinoic acid induced differentiation that contain AP-2 binding sites (SV40 enhancer, MHC class I genes, tissue plasminogen activator gene, and Hox 1.6 gene). No elevation of AP-2 was observed at 3, 6 nor 12 hours of NT2 induction with retinoic acid but it was clearly elevated at 24 hours (Lushcer *et al.*, 1989). This study demonstrated that retinoic acid induces AP-2 at the transcriptional level and it

is believed to be a secondary rather than a primary response in this cell line.

Recently, multiple isoforms of AP-2 have been detected. They are produced through alternative splicing and alternative transcription start sites (Meier *et al.*, 1995). A total of four isoforms of AP-2 have been detected to date in mice (Meier *et al.*, 1995). The third isoform is the major mRNA species in the nervous system of developing embryos during days 8.5-12.5. This isoform is also the major species of AP-2 activated (150-fold induction) during P19 neuronal differentiation (Meier *et al.*, 1995).

The existence of multiple species of this transcriptional activator allows for the possibility of regulation of downstream genes through binding of heterodimers as well as homodimers. All the AP-2 isoforms are coexpressed in similar tissues but the third isoform is the predominant species in the central and peripheral nervous systems (Meier *et al.*, 1995). The different functional roles played by these isoforms remain to be determined. The first three isoforms have also been detected in humans (Meier *et al.*, 1995). AP-2 is abundant in some fibroblast cell lines such as 3T3 and HeLa cells (Phillipp *et al.*, 1994).

Another possible candidate for control of ATBF1 expression levels is zif268/egr-1 which is also an immediate early gene (Cao, *et al.*, 1990). It has a molecular mass of 80

kDa and is a zinc finger DNA binding protein (Christy and Nathans, 1989). It has a consensus sequence of GCGG/TGGGCG for its high affinity binding site (Christy and Nathans, 1989 and Lemaire *et al.*, 1990). This transcription factor is expressed in mouse fibroblasts and is induced during neuronal differentiation of P19 cells (Thiel *et al.*, 1994). Even though its binding site is similar to that of Sp1 it does not recognize Sp1 sites (Lemaire *et al.*, 1990). This transcription factor has been shown to regulate the neuronal specific synapsin I gene during P19 retinoic acid induced differentiation (Thiel, *et al.*, 1994). During P19 retinoic acid induced differentiation zif-268 is induced on day five and the synapsin I gene is activated on day eight (Thiel, *et al.*, 1994). It was also demonstrated that an adjacent NRSE site interfered with zif-268 transactivation in the synapsin I promoter (Li *et al.*, 1993). This transactivator may be involved in the regulation of the ATBF1A promoter due to the presence of a sequence that resembles its binding site (Figure 1). This is also supported by its documented role in neuronal development and therefore it may play a role in the neuronal specificity of the ATBF1A transcript.

The NRSE, as mentioned previously, has been documented to be involved in the repression of several neuronal genes in nonneuronal cells (Li *et al.*, 1993, Thiel *et al.*, 1994, and Mori *et al.*, 1992). This element appears to be the major

determinant of the neuronal specific expression of the SCG10 gene (Wuenschell, *et al*, 1990). This was demonstrated using both CAT assays and transgenic mice (Wuenschell, *et al*, 1990). There does not appear to be a good match for the NRSE consensus in the ATBF1 promoter fragment but this does not totally preclude binding by it or related silencing factors in nonneuronal cells or at some other point in the ATBF1a promoter and contributing to the overall nonneuronal suppression of this gene.

The role of retinoic acid in the neuronal differentiation of P19 cells has been well documented (Maden and Holden, 1992). It has been shown that a RAR/RXR complex occupies a site within an hour of P19 RA induction (Dey *et al.*, 1994). The occupancy of this RAR binding site precedes the assembly of other elements on this promoter (Dey *et al.*, 1994). The retinoic acid receptor (RAR) is a ligand dependent DNA binding transcription factor (Green and Chambon, 1988). It binds to DNA as a dimer to a site that is found as a direct repeat (Leid *et al.*, 1992). Other members of this ligand dependent transcription factor superfamily have been shown to inhibit differentiation by binding to other transcription factors (Blobel, *et al.*, 1995). There is also a recent report that RAR can form a complex with c-jun that prvents it from binding AP-1 responsive genes (Schule *et al.*, 1991). It has been shown that RAR forms herteromeric complexes with RXR and binds more

efficiently to its site *in vitro* as a heterodimer (Dey *et al.*, 1994). RXR has been shown to form heterodimers with other receptor transcription factors such as the thyroid hormone receptor (Mader *et al.*, 1993). The binding of RAR and RXR homo and heterodimers has been examined and has been shown to have some flexibility (Mader *et al.*, 1993). Therefore it will be interesting to see if the half consensus site adjacent to an AP-2 (Figure 1) site plays a role in activating ATBF1A expression in retinoic acid induced P19 cells.

Another set of factors that have previously been shown to be involved in neuronal differentiation but do not have any obvious sites in the 400 bp fragment are some members of the POU domain family (Fujii and Hamada, 1993 and Okamoto *et al.*, 1993). Specifically implicated are the factors Oct-3 which inhibits neuronal differentiation as well as Brn-2 and Emb which are expressed at high levels in the central nervous system of the developing mouse (Shimazaki *et al.*, 1993). Oct-3 expression is extinguished in differentiating cells and therefore Oct-3 may act to suppress tissue specific gene expression (Shimazaki *et al.*, 1993). Brn-2 is predominantly expressed in the nervous system, binds octamer sites and is selectively induced upon P19 neuronal differentiation (Fujii and Hamada, 1993). Recently Brn-2 has been implicated in the development of the hypothalamus (Nakai *et al.*, 1995). Some flexibility of Brn-2 site recognition has been previously

reported (Li, *et al*, 1993). However, even taking this flexibility into consideration it still does not seem likely that Brn-2 is participating in binding the 400bp fragment that yields neuronal specificity.

Another POU family member has been described that is predominantly expressed in the central nervous system (Okamoto, *et al*, 1993). It has been named *Emb* and it also recognizes the octamer sequence. Since there are no obvious octamer binding sites in the ATBF1A promoter sequences, the octamer binding proteins are not likely to be involved in modulating the neuronal specificity of this promoter. However, it is possible that they may participate in achieving the full expression level detected in CAT assays with the full 5.5kb upstream promoter region. Alternatively the members of the POU family may be operating upstream of the ATBF1a gene in the modulation of its neuronal specific gene expression.

Proposed Experiments and Strategy

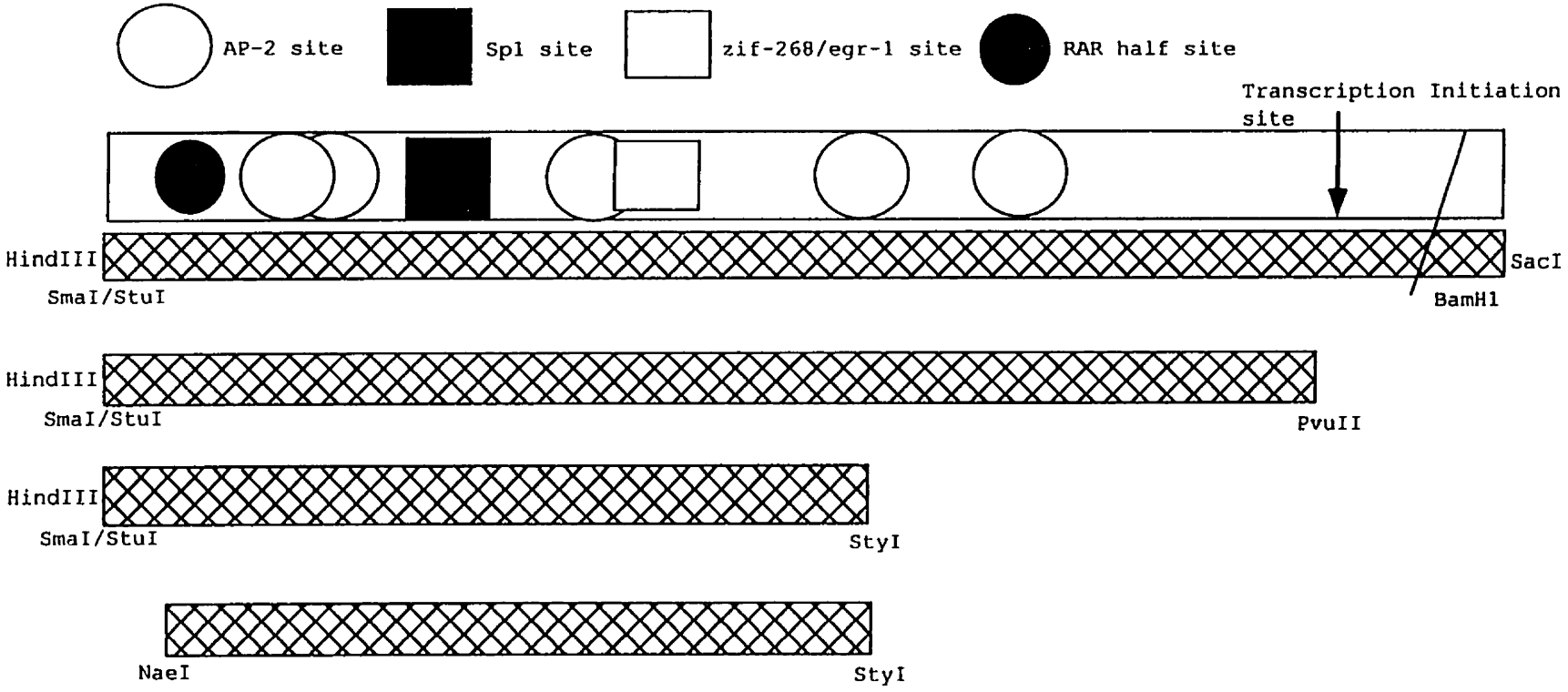
Previous experiments have implicated a 400bp fragment of ATBF1 sequences(Figures 1 and 2) as being sufficient to endow a portion of neuronal specificity upon a CAT construct (Miura *et al.*, 1995). The 400 bp sequence contains approximately 230 bp of upstream promoter sequences and 170 bp of the 5' untranslated region(UTR). (Lower case sequences in Figure 1

indicate 5' UTR). Initially this fragment was cloned into Bluescript II KS+ in order to be conveniently prepared in large quantities for electrophoretic mobility shift assays (EMSA) and DNaseI footprinting.

From an examination of the sequence of the promoter region in this fragment it can be discerned that several matches to transcription factor binding sites are present (Figure 2). There are multiple sites which resemble the consensus sequence for AP-2 which vary from a perfect match for the AP-2 consensus (CCCCAGGC) to sites with from one to three mismatches. In the region of interest there are also sites for Sp1 and a site that resembles an egr-1/zif-268 binding site. As indicated in Figure 2 there is some overlap of binding sites on this promoter fragment. Two adjacent sequences which resemble an AP-2 binding site are the most upstream sites present on this fragment. The perfect consensus AP-2 site overlaps the putative zif-268/egr-1 site.

The gel shift assays were performed with nuclear extracts from induced and uninduced P19 cells and with different portions of the promoter region(Filled rectangles on Figure 2). The *StyI* restriction site coincides with one of the AP-2 sites(Figure 1). This offers the opportunity to assay the capacity of the whole fragment as well as parts of it to bind P19 nuclear extracts. Further experiments such as competitive

Figure 2 Schematic representation of the ATBF-1 promoter fragment that is sufficicient to confer neuronal scpecificity in P19 cells. The unfilled rectangle represents the full fragment (Not drawn to scale). The hatched figures represent the fragments used for gel shift assays and the shapes represent putative transcription factor binding sites. The *StuI* site of the promoter region was cloned into the *SmaI* site of Bluescript II KS. At the other end of the insert the *BamH1* site of the 5' UTR was cloned into the *BamH1* site of the vector. The *SacI* site is indicated because it was used to digest the clone in order to yield fragments which could be uniquely end labelled. The sites of transcription factor binding are represented by circles and squares.


AP-2 site
Sp1 site
zif-268/egr-1 site
RAR half site
Transcription Initiation site
HindIII
SmaI/StuI
SacI
BamH1
HindIII
SmaI/StuI
PvuII
HindIII
SmaI/StuI
StyI
NaeI
StyI

gel shifts and comparisons with commercially available protein were performed in order to identify the transcription factor that binds to the ATBF1 promoter fragments in neuronally differentiating P19 cells. Differences in mobility patterns were noted and used in order to identify candidate sites of interaction with transcription factors.

In order to refine the identification of transcription factors and the sites occupied DNase I footprinting was used. The footprinting was performed using the full length promoter fragment and nuclear extracts from induced and uninduced P19 cells. Footprinting experiments were also performed on this promoter using commercial AP-2 protein.

Definitions of Abbreviations and Terms

AP-2- transcription factor (Williams *et al.*, 1988)

ATBF-1- human homeodomain/zinc finger transcription fator (Morinaga *et al.*, 1991, Ido *et al.*, 1994, Miura *et al.*, 1995, Sawadaishi, *et al.*, 1988 and Yasuda *et al.*, 1994)

Brn-2- octamer family transcription factor implicated in neuronal development (Nakai *et al.*, 1995)

F9- mouse embryonal cell line (Phillip *et al.*, 1994)

Egr-1- transcription factor, homolog to zif-268

Emb- octamer family transcription factor

GATA-1- transcription factor involved in erythroid differentiation (Orkin, 1990)

HeLa- human cervical carcinoma cell line

LIM- structural domain subfamily of homeodomain transcription factors

NRSE- neural restrictive silencer element (Li *et al.*, 1993, Mori *et al.*, 1990, Mori *et al.*, 1992 and Wuenschell *et al.*, 1990))

NT2- human neuroblastoma cell line

Oct-3- member of the octamer family of transcription factors known to inhibit neuronal differentiation

POU- structural domain subfamily of homeodomain transcription factors

P19- a mouse embryonal carcinoma cell line (McBurney, 1993)

SCG10- early marker gene for neuronal cells (Mori *et al.*, 1990 and Mori *et al.*, 1992)

Sp1- general transcription factor (Fisher *et al.*, 1993)

RA- retinoic acid, a morphogen

RAR- retinoic acid receptor (Mader *et al.*, 1993)

RXR- retinoid X receptor (Mader *et al.*, 1993)

zfh-2- Drosophila homeodomain/zinc finger transcription factor (Fortini *et al.*, 1991)

zif-268- transcription factor, homolog to egr-1

Materials and Methods

Media

Luria Broth- 10g of Tryptone, 5g of Yeast Extract, and 10 g of NaCl were dissolved in water and 0.2mL of 5N NaOH was added and the media was autoclaved.

SOB- Dissolve 20g tryptone, 5g Yeast Extract, and 0.5g NaCl in 950mL of water. Add 10mL of 250mM KCl, 0.2mL 5N NaOH volume up to 1L and then autoclave. Before use add 5mL of sterile 2M MgCl.

SOC- SOC is the same as SOB except that it contains in addition 20mM glucose.

Terrific Broth- 12g of Tryptone, 24 g of Yeast Extract and 4 mL of Glycerol were mixed in 900 mL of water and autoclaved. 100 mL of TB salts were added.

TB Salts- 0.17M KH_2PO_4, 0.72M K_2HPO_4

LB Plates were prepared through the addition of 15 g/L of Agar. Antibiotic was added after the plates had cooled to 55 degrees Celsius.

Ampicillin was prepared as a stock at 50 mg/mL and used at a final concentration of 50 ug/mL.

Alpha-MEM supplemented with 10% Fetal Calf serum was used to grow P19 cells.

Strains and Plasmids

P19 cells are a mouse embryonic stem cell line that has been described by McBurney (McBurney, 1993).

Bluescript II KS+ as described by Stratagene was used for subcloning.

PA5.5 was the source plasmid for the 400 bp fragment. This plasmid contains 5.5 kb of the ATBF1A upstream promoter region and was obtained from Y. Miura.

DH5 alpha was the *E. coli* strain used for maintenance and amplification of plasmids.

Solutions and Buffers

Tris containing solutions were diluted from a 1M pH 8.0 stock.

EDTA containing solutions were diluted from a 0.5M pH 8.0 stock.

TE was prepared as a 10mM Tris and 1mM EDTA solution.

10% SDS was prepared in water solution as a 10% w/v solution.

PAGE Elution Buffer 10mM Tris pH 8.0, 1mM EDTA pH 8.0, 1M NaCl

TBE was prepared with 54 g of Tris Base, 27.5 g of boric acid and 20 mL of 0.5M EDTA in a total volume of 1 L for a 5X stock. Gels were run at 0.5X strength.

Gel Shift/Competitor Oligonucleotide Sequences

The Zif-268 and AP-2 DNA oligonucleotides were synthesized at the University of Calgary core facility.

Zif-268

5' TGC CTT CGC CCC CGC CTG GCG G 3'

3' ACG GAA GCG GGG GCG GAC CGC C 5'

AP-2

5' GAT CGA ACT GAC CGC CCG CGG CCC GT 3'

3' CTA GCT TGA CTG GCG GGC GCC GGG CA 5'

The following competitor oligonucleotides were part of the Stratagene Gelshift Kit.

NF-1

5' ATT TTG GCT TGA AGC CAA TAT G 3'

3" TAA AAC CGA ACT TCG GTT ATA C 5'

Sp1

5' GAT CGA TCG GGG CGG GGC GAT C 3'

3' CTA GCT AGC CCC GCC CCG CTA G 5'

AP-3

5' CTA GTG GGA CTT TCC ACA GAT C 3'

3' GAT CAC CCT GAA AGG TGT CTA G 5'

Ligations

Takara Kit for ligations was used when available for cloning. Ligation reactions were performed with 2uL total of vector and insert. To this is added 16 uL of Buffer A (Reaction Buffer) and 2 uL of Buffer B (Takara enzyme). The reaction was allowed to proceed for at least 30 minutes at 16 degrees Celsius. One-half of this reaction was then used to transform competent bacteria. The other half was frozen for future use.

T4 DNA Ligase from Pharmacia was used for sticky end ligations at 14 degrees celsius and for blunt-end ligations at room temperature.

Restriction Enzyme Digests

Restriction enzymes from Boehringer-Mannheim, New England Biolabs, and Pharmacia were used. The reaction conditions were set up using the supplied buffers and the appropriate temperature water bath. On those occasions that double digests were performed they were typically done in Pharmacia's One-Phor-All Plus buffer unless the enzymes did not operate well in that buffer.

Preparation of Plasmid DNA from Bacteria

Wizard Mini-preps were performed as specified in the protocol provided with the kit from Promega.

Maniatis protocol (Sambrook *et al.*, 1989) was typically used to screen clones after a ligation and transformation.

Large Scale Plasmid Preparation was performed as per the Alkaline Lysis protocol (Sambrook *et al.*, 1989). For footprinting reactions the plasmid DNA was purified twice on a cesium gradient, for other applications such as cloning plasmid DNA was only run once on a cesium gradient.

Agarose Gel Electrophoresis of Nucleic Acids

0.8%, 1.5% and 2% gels were used depending on fragment size.

Preparation of DNA fragments

DNACell apparatus from Takara was used on occasion to prepare large fragments (>500bp) from agarose gel slices for cloning.

Electroelution from Agarose gels with dialysis bags was performed to isolate large fragments (>500bp) for cloning.

Preparation of fragments for Gel shifts and DNase I footprinting was performed as follows to isolate fragments less than 500bp including the promoter fragments used for gel shift and footprinting experiments.

Bluescript II DNA with the 400bp ATBF1A promoter fragment was digested as 10ug in 100uL with HindIII-SacI. The labelled DNA was then Phenol:Chloroform extracted and ethanol precipitated. The digested plasmid was then labelled with ^{32}P-dCTP by the Klenow fragment as described below. After labelling the ATBF1 promoter fragment is separated from the vector on a 5% polyacrylamide gel. Once the gel has been run sufficiently (Xylene cyanol three quarters of the way to the bottom) the gel and one glass plate are wrapped in plastic wrap and exposed to film for one minute. The film is developed and then used to cut out the appropriate section of the gel which is placed in 500uL of high salt elution buffer for three hours at 37 degrees Celsius or overnight at room temperature (Ausubel, *et al.*, 1994). The eluted DNA is passed through a

syringe filter and ethanol precipitated (no additional salt required). The pellet is resuspended in 100uL TE and 1uL is counted in a scintillation counter in order to determine the cpm/uL.

Proteins

Human AP-2 proteins was purchased from Fisher/Promega as footprint optimized preparation. Recombinant AP-2 was catalogue number E307 and was in a concentration of 1 footprinting unit per uL. This unit is defined as being sufficient to footprint 35 fmoles of a SV40 promoter.

HeLa nuclear extract was used from the Stratagene Gel Shift Kit.

Bovine Serum Albumin was used from New England Biolabs 10mg/mL reagent which is supplied as a supplement for restriction digests.

Antibodies to Egr-1 and AP-2 for gel supershift assays were purchased from Santa Cruz Biochemicals.

Preparation of Nuclear Extracts

Nuclear extracts from P19 cells were prepared from uninduced and induced P19 cells as per the procedure found in

section 12.1 in Current Protocols in Molecular Biology (Ausubel *et al.*, 1994). The cells were isolated, washed and resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT) and then homogenized. The nuclei are then pelleted and resuspended in a low-salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT). High-salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT) is added slowly with constant mixing which releases soluble proteins from the nuclei without lysis. The nuclei are then removed and the nuclear extract is then dialyzed and frozen in aliquots. A cocktail of protease inhibitors is used in each buffer which is composed of final concentrations as follows: 1 mM AEBSF, 20 ug/mL aprotinin, 5 ug/mL leupeptin, 25 ug/mL antipain, 1ug/mL pepstatin A and 1mM benzamidine.

Induction of P19 Cells with Retinoic Acid

On the first day the cells are removed from the petri dish with treatment with trypsin (0.25%), EDTA for 2-3 minutes at room temperature. The cells are transferred to bacterial grade dishes with $1.0x10^6$ cells to each 10 cm dish in alpha-MEM containing retinoic acid at a concentration of 3.0×10^{-7} M. The next day 5mL of medium containing retinoic acid is added to each plate. On the third day the medium is exchanged

by allowing the cells to settle in a 50 mL Falcon tube by standing (not by centrifugation) and then exchanging the supernatant. On the fourth day 5mL of medium containing retinoic acid is added. On the fifth day the medium is again renewed and the cells are plated back on to cell culture grade dishes. The cells are allowed to adhere and differentiate for several days (changing the media every second day) and then harvested on day ten.

Transformation of Competent Cells

Cells prepared by Hanahan (Hanahan, 1983) protocol and kept frozen at -80 degrees celsius. The cells were thawed and then aliquoted in prechilled tubes at 100 uL per tube. Either 10uL of a ligation reaction or 10ng of plasmid DNA was added to each tube. The mixture was incubated on ice for 20 minutes and then heat shocked for one minute at 42 degrees Celsius. The cells were incubated on ice for one minute and then 1mL of SOC medium was added and the cells were incubated at 37 degrees Celsius for 30 minutes. The cells were spun down and the SOC was replaced by 150 uL of fresh SOC medium. Aliquots of 5uL, 25 uL and 100 uL were plated on LB/Amp plates and the colonies were screened.

DNA Sequencing

The DNA Core Facility at the University of Calgary provided support for DNA sequencing when necessary using an Applied Biosystems automated sequencing apparatus.

Maxam-Gilbert Sequencing ladders

Were prepared as per the protocol in Maniatis (Sambrook *et al.*, 1989) or as in the express protocol (Belikov and Wieslander, 1995).

DNA Sequence Analysis

The DNA Strider program for the Macintosh was used to construct restriction maps and search for transcription factor binding sites in the ATBF1A promoter.

Site-directed Mutagenesis using PCR

The strategy was based upon the methodology described in section 8.5 of Current Protocols in Molecular Biology (Ausubel, *et al.* 1994).

Labelling of DNA Probes

Klenow Labelling of the fragments used for gel shifts and footprinting was performed using reagents from the Random Primer labelling kit from Boehringer-Mannheim. The footprinting probe was a HindIII-SacI fragment. Since SacI has

a 3' overhang and HindIII has a 5' overhang the Klenow treated fragment is uniquely labelled at the HindIII end of the fragment.

The DNA size markers were labelled as follows: A pBluescript HpaII digest (50ng) was mixed with dNTP mix (excluding dCTP), 5x buffer, 1mM DTT, 3uL alpha-labelled dCTP, and 1 unit of Klenow enzyme. The mix is incubated at 30 degrees Celsius for 30 minutes and then frozen at -20 degrees Celsius. Dilutions are run adjacent to footprinting reactions as 1/250, 1/25 and 1/10.

Calf Intestinal Phosphatase was purchased from Pharmacia. The digests were performed at alkaline pH and at 42 degrees Celsius.

Gel Shift

Nuclear extracts (0.8ug) were incubated with 5,000 cpm of labelled probe and 1.5 ug poly dIdC as a nonspecific competitor in 25mM HEPES pH7.5, 10 mM KCl, 1mM EDTA, 5mM $MgCl_2$, 1mM dithiothreitol, 1ug BSA (Kozmik *et al.*, 1990) and 10% glycerol at room temperature for 20 minutes. The reactions were then loaded onto a 5% polyacrylamide gel that was run in 0.5xTBE. Gels were dried and autoradiographed overnight at -70 degrees celsius with an intensifying screen. Competitor oligonucleotides were included in a molar excess as indicated

in the appropriate figures usually 10-fold, 100-fold and 250-fold. In some cases the incubation buffer was used from the Stratagene Gelshift Kit as detailed in the instruction manual (Stratagene protocol, 1990).

DNase I Footprinting

Deoxyribonuclease I was purchased from Pharmacia and Promega. The Pharmacia enzyme (Catalog# 27-0512) provided optimal activity under the assay conditions used. The activity was 2941 u/mg and was resuspended and stored in 5mM sodium acetate(pH 4-5), 1mM $CaCl_2$, 50% glycerol at -20 degrees Celsius.

The footprinting reaction conditions were optimized and the buffer conditions suggested in the AP-2 technical data sheet were used. The reaction took place in a volume of 200 uL and the following buffer 25 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 6.25 mM $MgCl_2$, 10% glycerol, and 0.5 mM DTT. The reactions with nuclear extract include 1.5ug poly dIdC as a non-specific competitor. Immediately before digestion 5 uL of Pharmacia DNase I is diluted into 100 uL of 10 mM Tris-HCl (pH 8.0) and 3 uL of this dilution is used in each reaction. The labelled probe is added from 10,000 to 50,000 counts for each reaction. The reactions are incubated with or without added protein for 20 minutes and then DNase I is added. Each reaction is incubated for 2 minutes with DNase I and then 25 uL of DNase

I stop solution is added and followed by the addition of an 125 uL of phenol chloroform. The mixture is centrifuged for four minutes at 6000 rpm and the aqueous phase transferred to a new tube. The digested probe is precipitated with 300 uL 95% ethanol and incubation at -80 celsius for 15 minutes.

Electrophoretic conditions: Denaturing polyacrylamide gels were made with the following 31.5 g of urea, 9 mL of 5xTBE, 7.5 or 12 mL (5% or 7% respectively) of Long Ranger concentrated gel solution from J.T. Baker. Polymerization was catalyzed with 37.5 uL of TEMED and 375 uL of 10% Ammonium Persulfate. The gel was run until the xylene cyanol dye was halfway down the gel (7%) or five-sixths of the way to the bottom (5%).

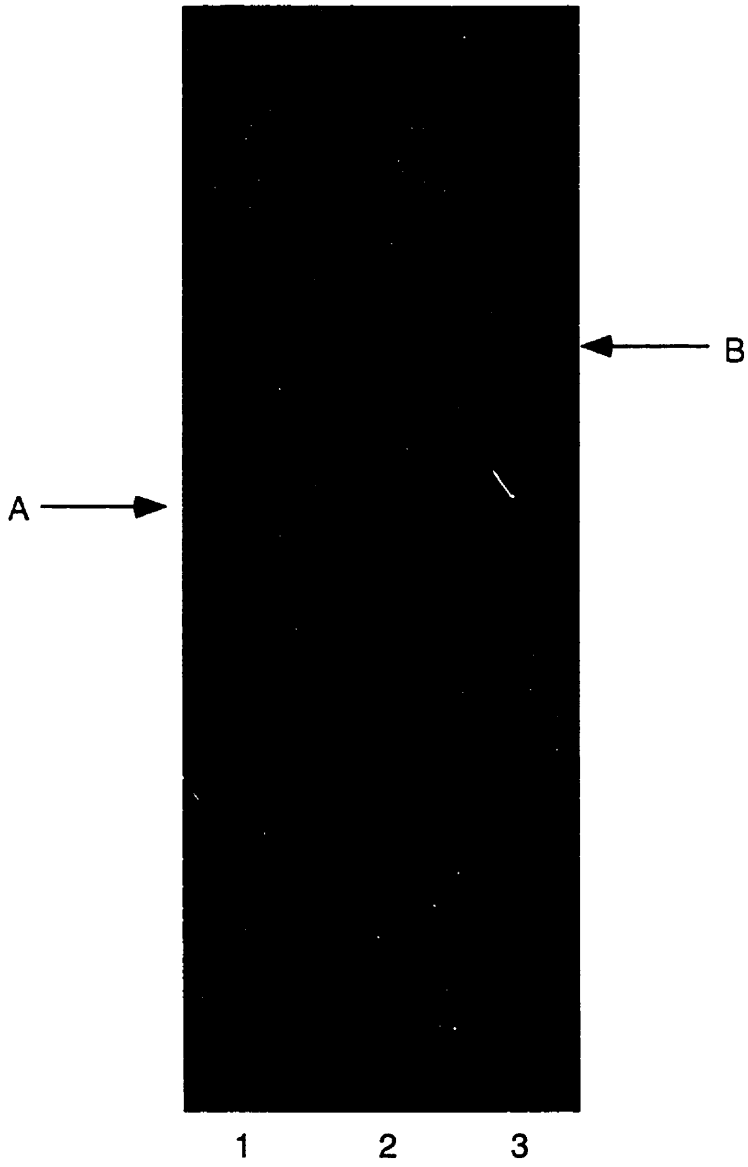
Results

Gel mobility shift assays

It has previously been shown that the 400 bp BamH1-StuI fragment (Figures 1 and 2) of the ATBF1 promoter is sufficient for 50% of the neuronal specificity in CAT assays (Y. Miura, personal communication). This fragment was subcloned into the SmaI and BamH1 sites of Bluescript II KS+ from PA5.5 (from Y. Miura) in order to enable the preparation and isolation of large quantities of this fragment for use in gel shift assays and DNase I footprinting reactions. Conditions were established for gel shift reactions with respect to the amount of nuclear extract and probe required to obtain shifting of the probe (data not shown).

Reproducible differences were observed between the shifted bands from cells induced with retinoic acid [RA(+)] and uninduced cells [RA(-)] (Figure 3). The RA(+) induced shift was reproducible as a single shifted band. (Indicated by the letter A in Figure 3 and 4) The RA(-) induced shifts were less consistent in reproducibility (Figures 3, 4, 5 and 7). The bands formed by the uninduced P19 nuclear extracts were heterogeneous in size and most of these bands were of a higher molecular weight than the RA(+) complexes (Indicated by letter B in Figures 3 and 4 and also observed in Figure 7). However, in some cases a band did appear that was similar in mobility

Figure 3 Gel shift of NaeI-StyI fragment of the ATBF1a promoter region. The lanes are negative control without protein (1), P19 nuclear extract from cells induced with retinoic acid (2), and P19 nuclear extract from uninduced cells (3). The specific shift obtained with induced cell extracts is clearly visible in lane 2 at position A. Heterogenous shifting is observed with RA(-) extracts as is indicated by the letter B.

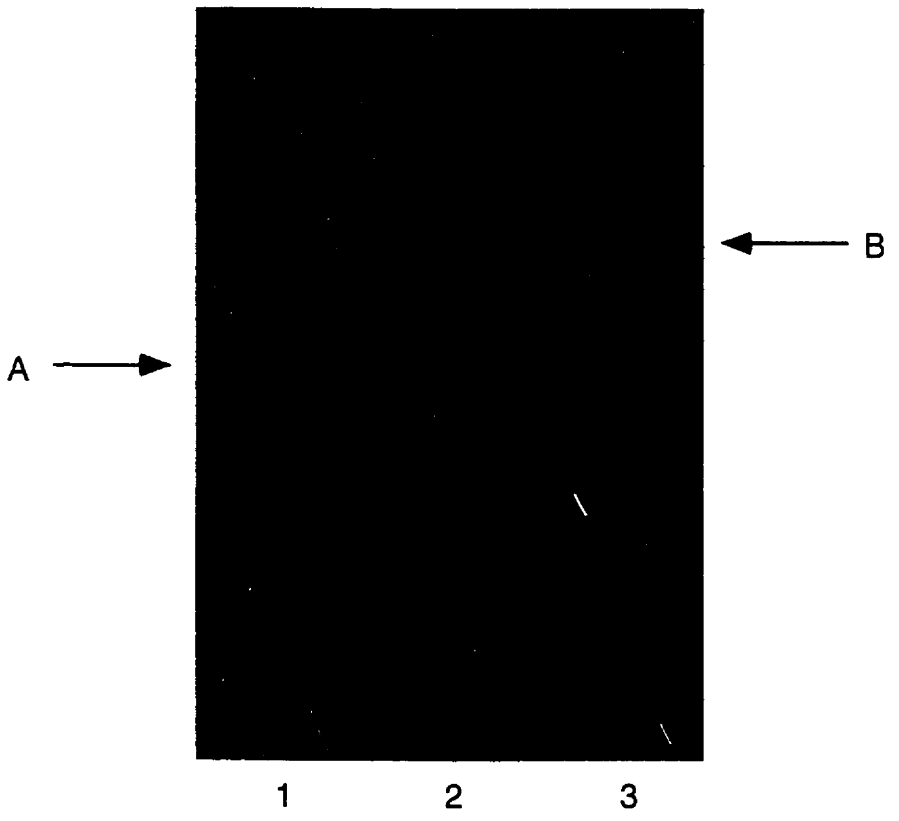

A
B
1
2
3

to the single band observed in RA(+) nuclear extracts (Figures 4, 7 and 8). This band is observed when using a longer fragment (HindIII-StyI) than in Figure 3 and may be due to binding at a site other than the ones contained within the smaller fragment (NaeI-StyI). This complex appears to have a lesser mobility shift than the shift derived from RA(+) extracts (Figures 4, 7 and 8).

The RA(-) gel shift complexes appeared to be sensitive to various factors such as the number of freeze-thaw cycles undergone by the sample. The RA(-) shifts faded and eventually disappeared with more than three freeze-thaw cycles of an aliquot (Figure 5, lane 2). Other factors that altered the RA(-) shift were buffer conditions, amount of competitor present, which nuclear extact preparation was used and the number of binding sites on the fragment that was used for the mobility shift assays (data not shown). The RA(+) complexes did not vary with changes in these conditions. These results indicate that the RA(-) shift is labile. This may be due to degradation or instability of the factors that bind the promoter in RA(-) extracts.

In fact, the RA(+) nuclear extracts yielded a single band with both the NaeI-StyI fragment, a HindIII-StyI fragment and the full length HindIII-BamH1 fragment (data not shown). It appears that there is a single specific protein binding site that is present even in the smallest fragment. This fragment

Figure 4 Gel shift of the HindIII-StyI fragment of the ATBF1a promoter. The lanes are negative control without protein (1), P19 nuclear extract from cells induced with retinoic acid (2), and P19 nuclear extract from uninduced cells (3). The specific shift obtained with induced cell extracts is present in lane 2 at position A. The heterogeneous shifting that is consistently observed with RA(-) extracts is indicated by the letter B. An additional band that is observed in the second preparation of RA(-) extracts migrates closely to the A band of RA(+) extracts but appears to have a slightly higher mobility.


A
B
1
2
3

includes three putative AP-2 sites, the Sp1 site and the juxtaposition of the AP-2 and zif-268/egr-1 sites (Figure 2).

Mobility shifts with oligonucleotide competitors

Once it had been established that a difference existed between the mobility shifts of RA(+) and RA(-) nuclear extracts it was necessary to identify the protein responsible for the RA(+) shift. Further experiments were performed to identify its binding site and relate occupation of that site to the neuronal specific expression pattern of the ATBF1A promoter.

An experiment that was performed in order to identify the protein causing the RA(+) shift was the oligonucleotide competition assay (Figures 5, 6 and 7). Separate competitor oligonucleotides were used containing consensus binding sites for AP-2 (Figures 5, 6 and 7), Sp1 (data not shown) and egr-1 (Figure 7). Additional competitions were performed with the negative control oligonucleotides NF-1 (data not shown) and AP-3 (Figure 6). Sites for two these factors were not present in the ATBF1A promoter sequences and thus they can help determine whether an observed competition is specific. It was found that the AP-2 oligonucleotides could successfully compete with the ATBF1A promoter fragment for the RA(+) shift. (Figures 5, 6 and 7). The RA(+) shift was specifically competed with the AP-2 oligonucleotides and did not respond to

Figure 5 Gel shift of NaeI-StyI fragment. From left to right the lanes are no protein control (1), RA(-) P19 nuclear extract (2), RA(+) nuclear extract (3), RA(+) with 10-fold molar excess of AP-2 competitor (4), RA(+) with 100-fold molar excess of AP-2 competitor (5) and RA(+) with 250-fold molar excess of AP-2 competitor (6).

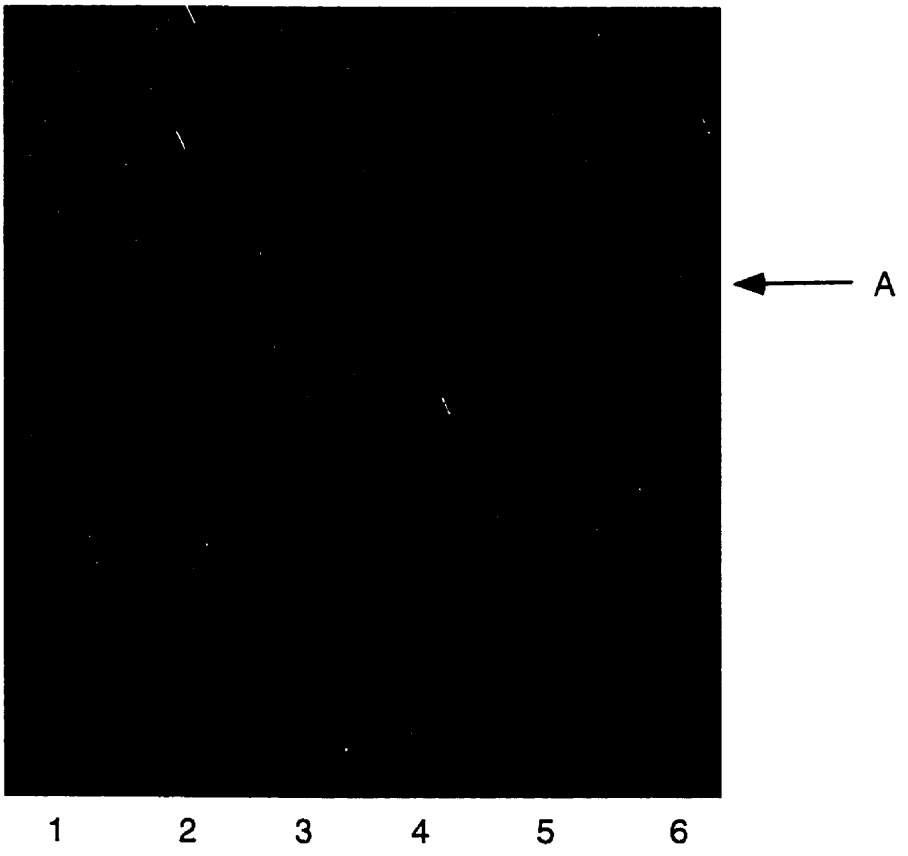
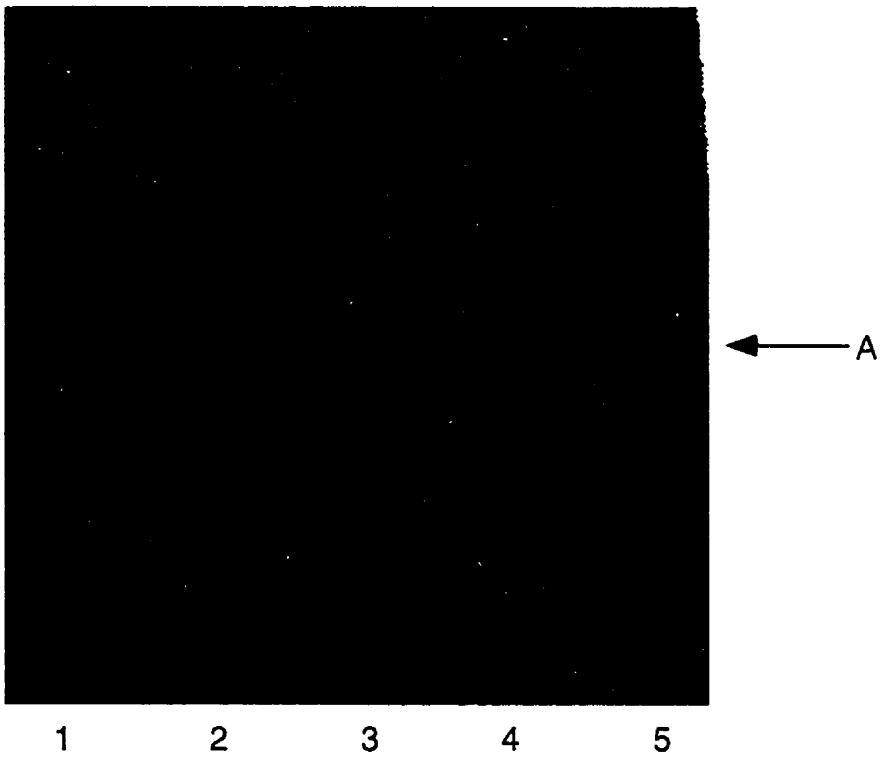

A
1
2
3
4
5
6

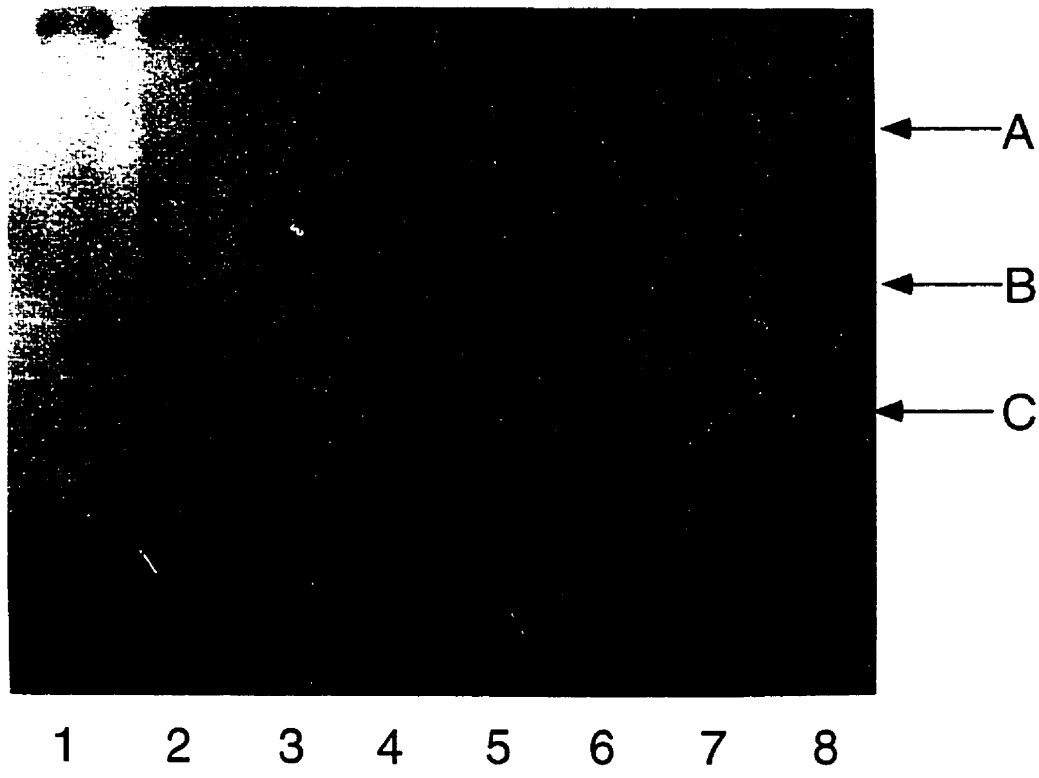
Figure 6 Gel shift of NaeI-Sty fragment. From left to right the lanes are no protein control (1), RA(+) nuclear extract (2), RA(+) with 100-fold molar excess AP-2 competitor (3), RA(+) with 100-fold Egr-1 competitor (4) and RA(+) with 100-fold AP-3 competitor (5).


A
1
2
3
4
5

any of the other competitors (Figure 7 and data not shown). As the amount of AP-2 competitor oligonucleotide was increased from 10-fold molar excess to 100-fold the amount of the shifted band decreased (Figures 5, 6 and 7). This evidence supports the hypothesis that AP-2 is the factor binding the ATBF1A promoter fragment from RA(+) nuclear extracts. Therefore the case for AP-2 playing a role in the regulation of ATBF1A expression is strengthened.

It was also observed that one of the bands in the RA(-) shift was competed by AP-2 oligonucleotide (Figure 7, Lane 7, Arrow B). A possible explanation for this result is that there is some AP-2 protein present in uninduced P19 cells. This is consistent with the fact that AP-2 transcripts are detectable prior to retinoic acid induction (Phillipp *et al.*, 1994). This protein may be inactive or present in low quantities *in vivo* but may be activated or extracted in sufficient quantities to be detected by the gel mobility shift assay. In this case the larger molecular weight complexes from uninduced nuclear extracts may contain AP-2 as well as containing other factors which may prevent AP-2 from activating neuronal specific promoters like ATBF1A. It is interesting to note that the complex formed by RA(-) extracts on the longer fragment (HindIII-StyI) which migrates close to the RA(+) shift was not competed by AP-2 oligonucleotide. This suggests that this complex is caused by the binding of some factor other than

Figure 7 Gel shift of HindIII-StyI fragmet. The lanes are no protein (1), RA(+) nuclear extract (2), RA(+) nuclear extract and 100-fold molar excess AP-2 competitor (3), RA(+) and 100-fold molar excess of egr-1 competitor (4), RA(+) nuclear extract and 100-fold molar excess of NF-1 competitor (5), RA(-) nuclear extract (6), RA(-) nuclear extract and 100-fold molar excess of AP-2 competitor (7), and RA(-) extract and 100-fold excess of egr-1 competitor(8). The arrow denoted by A indicates the putative AP-2 band and the specific competition that occurs in lane 3. Arrows B and C indicate the heterogeneous bands that appear in the RA(-) shifts. The band denoted by B also appears to be competed by AP-2 oligonucleotide in lane 7.

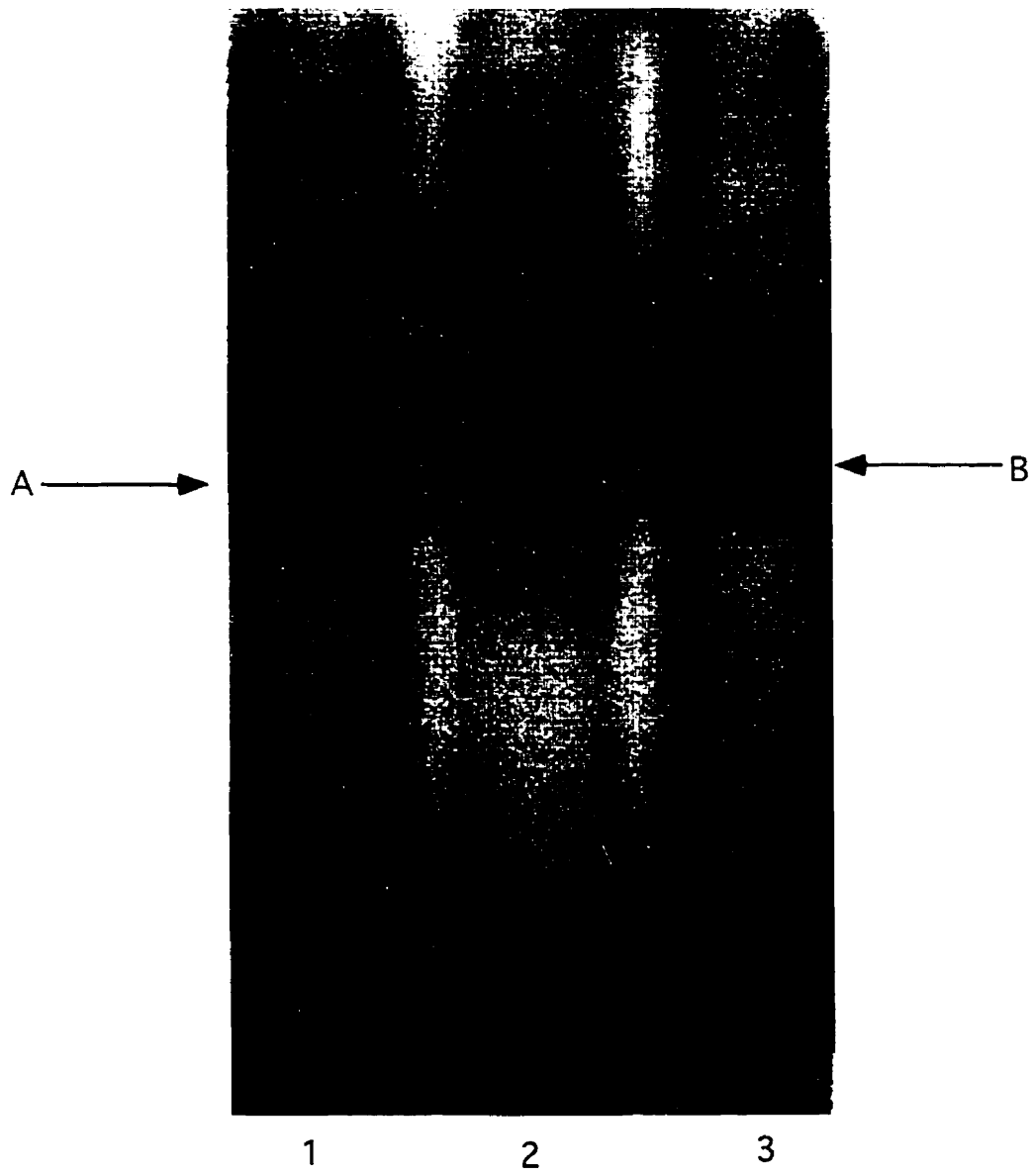

A
B
C
1
2
3
4
5
6
7
8

AP-2 from the uninduced extracts. This indicates that the conformation of the promoter is different between uninduced and induced P19 cells.

Mobility Shifts with Commercial AP-2 protein

Another experiment that was performed and further supported the hypothesis that the binding factor is AP-2 was a comparison of the RA(+) shifted band with the same promoter fragment shifted with commercially available AP-2 protein (Figure 8). The commercial AP-2 yielded a shift that was similar but not completely identical to the RA(+) produced shift (Figure 8). A significant possibility for the differences in mobility may be that the major isoform induced during P19 neuronal differentiation (47 kd) is of a lesser molecular weight than the recombinant protein (52 kDa) (Meier *et al.*, 1995). This corresponds with the data which shows a slightly greater mobility shift for the recombinant protein complex versus the RA(+) derived factor complex (Figure 8, Lanes 2 and 3). The small difference observed may also be due to a variation in post translational modifications between P19 RA(+) derived AP-2 and the commercial product which is derived from expression in E. coli. In some experiments multiple bands were observed with the commercial AP-2 preparation. (Figure 8, lane 2) This may due to the purified protein being present in a much higher molar concentration and thus occupying more than

Figure 8 Gel shifts of the NaeI-StyI promoter fragment. The lanes are are RA(-) nuclear extracts (1), AP-2 commercial protein (2), and RA(+) nuclear extract.

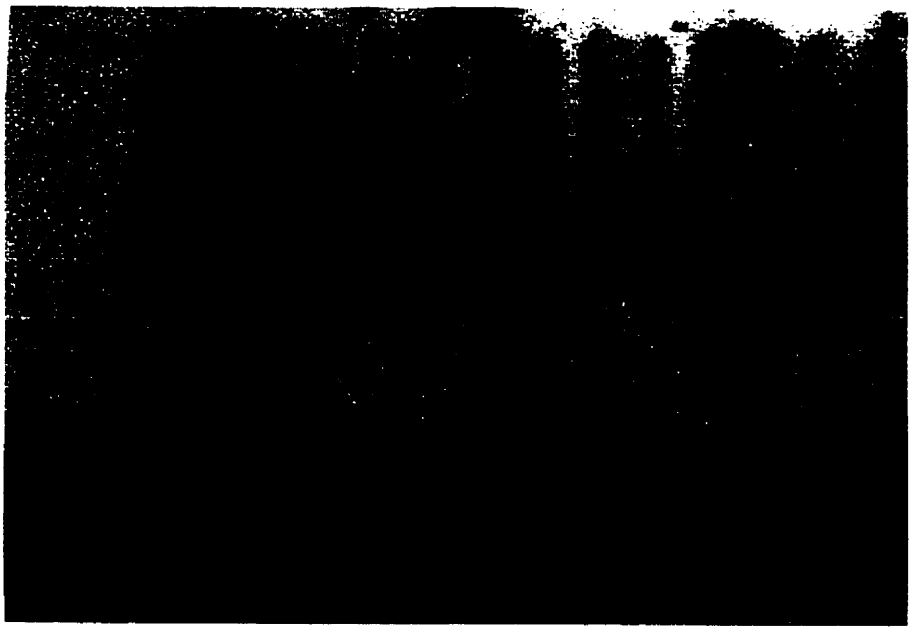

A
B
1
2
3

the single site occupied by the protein from RA(+) nuclear extracts. This may also be due to differing specificities of the different isoforms of AP-2.

Mobility shifts with Antibodies

A third set of experiments that were attempted in order to confirm the hypothesis that AP-2 was the factor causing the shift in RA(+) nuclear extracts. These experiments involved the use of antibodies in an attempt to "supershift" the band in order to identify the bound protein directly as AP-2 or egr-1. Therefore commercial antibodies were obtained that had been specifically prepared for the supershift assay. However a supershift was not observed in these experiments. The expected higher molecular weight complexes were not observed in the antibody containing lanes (Figure 9, Lanes 5 through 8). This differs from the expected result of super-shifted bands with the AP-2 antibody. However it was not possible to obtain a supershifted band even with the commercial AP-2 protein shift (data not shown) and therefore these experiments are inconclusive. In a previous expermiment (Phillipp *et al.*, 1994) a gel shift with an AP-2 specific oligonucleotide produced a band that did not supershift upon addition of antibody. It may be that the antibody only recognizes certain isoforms of AP-2. Further experiments are required to clarify this point.

Figure 9 Supershift experiments with HindIII-StyI promoter fragment. No protein control (1), RA(+) nuclear extract (2), RA(-) nuclear extract (3), Commercial AP-2 protein (4), RA(+) with AP-2 antibody (5), RA(+) with egr-1 antibody (6), RA(-) with AP-2 antibody (7), and RA(-) with egr-1 antibody (8).



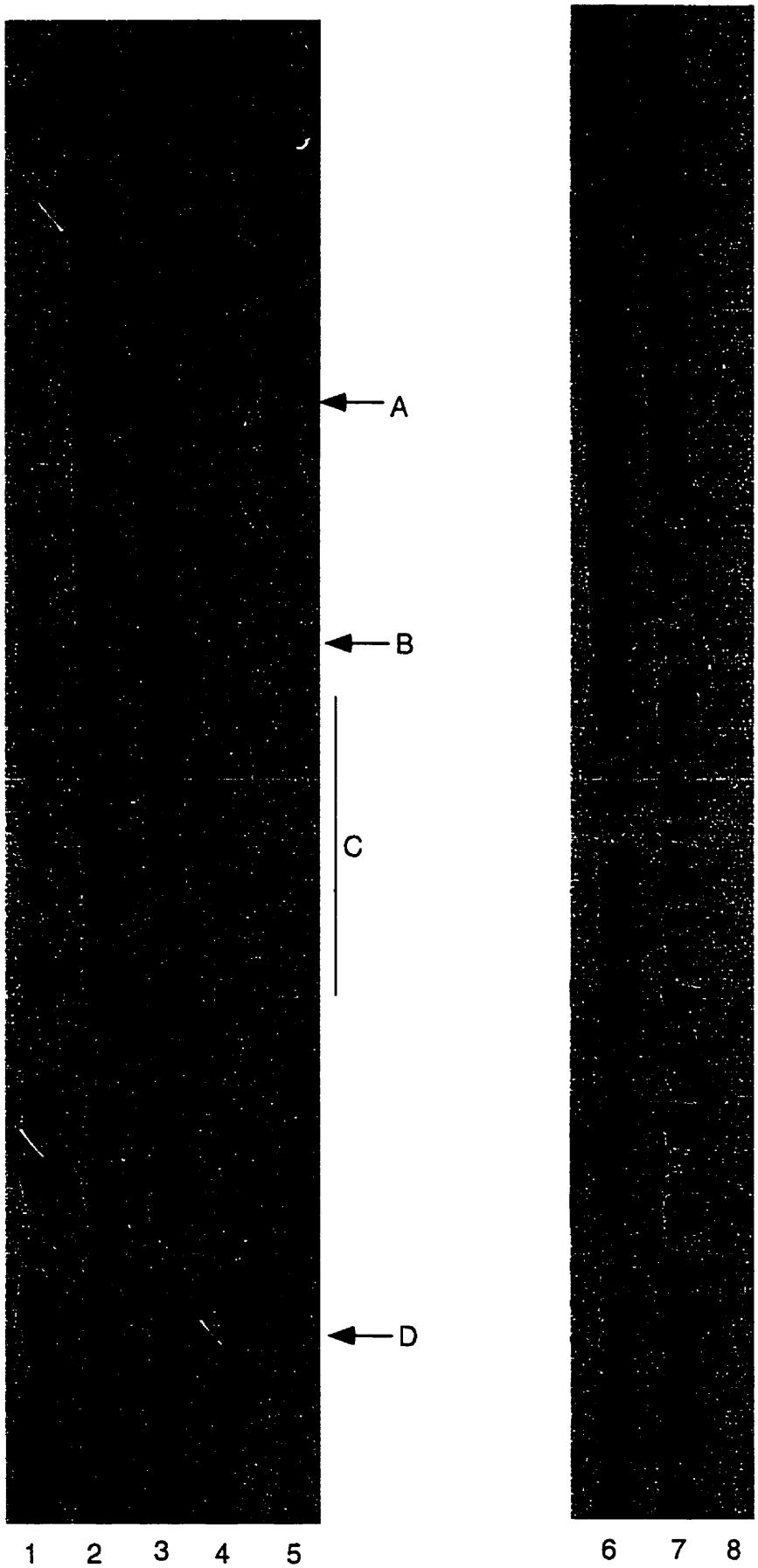
1 2 3 4 5 6 7 8

DNase I footprinting

In order to further identify and characterize the binding of the RA(+) derived factor to the ATBF1A promoter DNase I footprinting experiments were performed. In these experiments the protection patterns from RA(+) extracts, RA(-) extracts and commercial AP-2 protein were compared. The initial experiments were performed using RA(+) and RA(-) nuclear extracts (Figure 10). The P19 retinoic acidinduced nuclear extract displays a protection pattern distinct from the nuclear extract from uninduced cells. Several areas of preferential protection by the RA(+) nuclear extracts were detected (Figure 10 lanes 4 and 5 as indicated by the arrows and filled bars). When aligned to a DNA size standard, the perfect consensus AP-2 site (Figure 1, AP-2[B]) matches the position of the arrow adjacent to the letter A in Figure 10 (size marker not shown). The downstream protections may be attributed to the proximal AP-2 sites (Figure 1, Letters C and D). At the highest concentrations of protein the RA(-) extracts completely protect the ATBF1A promoter in what appears to be a nonspecific manner while the retinoic acid induced extracts show areas of increased protection (Figure 10 letters A-D).

However upon inclusion of commercial AP-2 protein in a DNase I footprint reaction a strong, distinct and reproducible footprint was obtained (Figure 11 as indicated by the letter

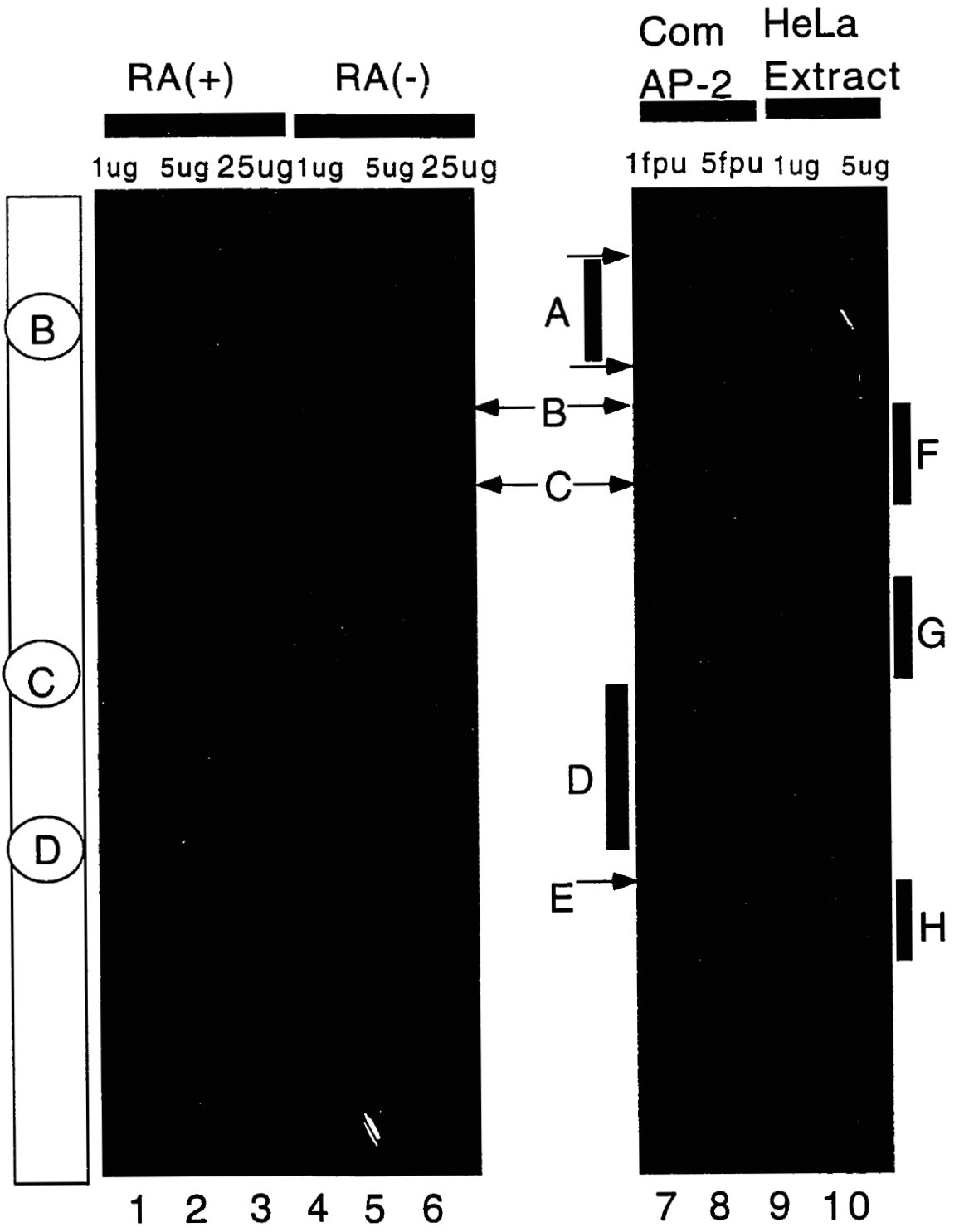
Figure 10 DNaseI footprinting of HindIII-SacI fragment of the ATBF1A promoter. The lanes are as follows naked DNA digested with DNase I (1), 5ug of RA(+) P19 nuclear extract (2), 25 ug of RA(+) P19 nuclear extract (3), 100 ug of RA(+) P19 nuclear extract (4), 100 ug of a different RA(+) P19 nuclear extract than lanes 2-4 (5), 5 ug of RA(-) P19 nuclear extract (6), 25 ug of RA(-) P19 nuclear extract (7), and 100 ug of RA(-) P19 nuclear extract. The lower strand was labelled in these assays.


A
B
C
D
1
2
3
4
5
6
7
8

A). By aligning molecular weight markers (data not shown) that were electrophoresed under identical conditions with the commercial AP-2 footprint the binding site corresponds to the perfect consensus AP-2 site which is present on all fragments which yield the RA(+) specific gel shift of the ATBF1A promoter (Schematic in Figure 11). Therefore the perfect consensus site [B] efficiently binds AP-2 protein. The same area displayed some protection with a control HeLa extract which is known to contain AP-2. In fact the HeLa protection pattern (Figure 11, Lanes 9 and 10) more closely resembled the P19 RA(+) protection pattern than that of the recombinant AP-2 protein. This may be due to modifications of the AP-2 protein or to interactions between the upstream Sp1 site and the AP-2 site which give a different pattern than AP-2 alone. HeLa extract is known to contain both of these factors. An additional experiment would be to compare and conttast footprints of recombinant Sp1 and AP-2 when they are simultaneously and separately added to the ATBF1A promoter.

There was also an area that yielded a protection specific to the retinoic acid induced P19 cells which shows up as a strong band in the other lanes (Letter C protected in lane 3). There are some more subtle indications of binding slightly downstream of this site in the RA(+) nuclear extracts(indicated by letters B through E). This would correspond to some of the other AP-2 sites of the ATBF1A

Figure 11 DNase I footprinting of the HindIII-SacI fragment. The lanes are as follows: 1ug RA(+) P19 nuclear extract (1), 5 ug RA(+) P19 nuclear extract (2), 25 ug RA(+) P19 nuclear extract (3), 1 ug RA(-) P19 nuclear extract (4), 5 ug RA(-) P19 nuclear extract (5), 25 ug RA(-) P19 nuclear extract (6), 1 fpu recombinant AP-2 (7), 5 fpu recombinant AP-2 (8), 1ug HeLa extract (9), and 5ug HeLa extract(10). The lower strand was labelled in these assays.


RA(+)
RA(-)
Com AP-2
HeLa Extract
1ug 5ug 25ug 1ug 5ug 25ug
1fpu 5fpu 1ug 5ug
A
B
C
D
E
F
G
H
1 2 3 4 5 6
7 8 9 10

promoter. Therefore the differences with the recombinant footprint and the nuclear extract may be due to the binding of additional factors which in this case may be Sp1. Some other reasons that the footprints differ between the recombinant and the crude extracts may be that the protein may not be present in high enough concentrations to yield as obvious a protection pattern. As the commercial preparation is purified and has been optimized for footprinting it is not surprising that it yields a clear cut pattern in this assay. This indicates that the site is capable of binding AP-2 and suggests that this site participates in the regulation of ATBF1A expression. There are some hypersensitive sites further upstream of the perfect consensus AP-2 site. These may be due to binding of the recombinant AP-2 protein at the less than perfect consensus sequences or at the Sp1 site in the ATBF1A promoter region.

Discussion

The results that have been obtained by a variety of biochemical techniques in this study indicate that AP-2 is involved in regulation of the ATBF1A promoter. However, there is some prior data that suggests that AP-2 peaks at a later stage of P19 development than ATBF1A (Phillipp *et al.*, 1994). However, the induction of AP-2 is 60- fold (Phillipp *et al.*, 1994) which indicates that AP-2 mRNA is present at low levels in uninduced P19 cells whereas ATBF1A transcripts are undetectable prior to induction with retinoic acid (Miura *et al.*, 1995). There also has been a report in which AP-2 activity has been induced without an increase in mRNA expression (Andersson, *et al.*, 1994). Therefore it remains possible that AP-2 may be involved in the upstream regulation of ATBF1A. The AP-2 protein that is present in the P19 cells prior to induction may be inactive and then may be activated and bind the ATBF1a promoter immediately upon induction. It may even be possible that it is in an inactive complex with the retinoic acid receptor which has a precedent within the ligand dependent transcription factor superfamily (Blobel *et al.*, 1995)

It has previously been demonstrated that two to four hours of RA induction is sufficient for commitment of P19 to the neuronal differentiation pathway (McBurney, 1993). It is interesting to note that ATBF1a mRNA is induced within this

time frame (Miura *et al.*, 1995). Since there are no sites for the retinoic acid receptor family (other than a possible half site Figure 1) in the ATBF1A promoter it is unlikely that this factor acts alone or in a direct manner upon ATBF1A expression. If AP-2 is involved in ATBF1A induction then this evidence suggests that there may be multiple waves of AP-2 activity.

The first wave of AP-2 activity may be due to protein activation via some mechanism (eg. phosphorylation or dissociation from an inhibitor) upon the initial induction with retinoic acid. This activates ATBF1A expression and commits the cells to the neuronal pathway within three hours. It would be an interesting future experiment to knock out ATBF1A in P19 cells to observe the effect upon neuronal differentiation or observe the effects of an AP-2 knockout or mutations that block the neuronal differentiation pathway (McBurney, 1993) on ATBF1A expression and neuronal cell development. The second wave of AP-2 induced effects occurs upon the accumulation of additional AP-2 protein which presumably accompanies the later peaks of AP-2 levels which produce sufficient mRNA and protein to carry neuronal differentiation through its course.

One way to test this hypothesis would be to determine whether ATBF1A expression during retinoic acid induction required new protein synthesis. This could be attempted with

induction experiments carried out in the presence of cycloheximide which is an inhibitor of protein synthesis. If ATBF1A RNA levels increased in the presence of cycloheximide then that would indicate that novel protein synthesis is not required. This result would indicate that the low levels of AP-2 that are present in the cell prior to induction with retinoic acid may be involved in the initial activation of ATBF1A. These levels may be sufficient to direct expression of the ATBF1A gene, presumably upon activation through post-translational modification. This is consistent with the results which indicate that AP-2 mRNA is present in uninduced P19 nuclear extracts (Phillipp *et al.*, 1994). AP-2 may act as a factor that potentiates the assembly of other factors onto the ATBF-1A promoter in a similar fashion as the retinoic acid receptor has been reported to act (Dey *et al.*, 1994).

From the gel shift data it appears that the RA(-) nuclear extract contains multiple factors that bind the ATBF1A promoter. These complexes are labile and heterogeneous which may indicate that the specificity of these interactions may not be critical. These factors may serve to silence the promoter in uninduced cells. In contrast the RA(+) extracts form one specific band on the ATBF1A promoter fragments which is not similarly labile. Since ATBF1A is expressed in RA(+) cells and not in RA(-) it could be hypothesized that the factor that binds from RA(+) nuclear extracts is a specific

activator of this promoter. The RA(+) band would be a protein which binds upon RA induction to activate this promoter. The proteins observed in the RA(-) complex would be altered so that they would no longer bind the ATBF1A promoter. Alternatively the RA(-) shift could be an inhibitory arrangement of the promoter.

A set of experiments that could confirm this hypothesis would be to do a time course of nuclear extracts during P19 retinoic acid induced differentiation. These experiments would demonstrate the alterations in binding of the ATBF1A promoter during commitment to a differentiation pathway rather than after commitment has already occurred which is a limitation of the current experiments.

The results that have been obtained during the current studies implicate AP-2 as a factor involved in the neuronal specific expression of the ATBF1A promoter. In summary, the data that support this assertion are gel shift assays which include oligonucleotide competitions as well as gel shifts with commercial AP-2 protein and DNase I footprints, particularly the strong footprint obtained with the commercial AP-2 protein.

However, these assays provide only indirect evidence that the factor from P19 nuclear extracts which binds the ATBF1A promoter is AP-2. Some experiments that would provide more direct evidence of this would be the antibody supershifts,

sequence specific competitive footprints of the observed protection patterns, direct alignment of sequencing reactions of the promoter fragment with the footprints, mutagenesis of the AP-2 sites in the ATBF1a promoter and subsequent CAT assays.

The most obvious experiments that should be done in the near term are CAT assays with mutated promoters. These will give more direct evidence of the importance of the AP-2 sites in the expression of the ATBF1A promoter *in vivo*. It would be interesting to determine whether ATBF1A is induced in the P19 cell line that possesses a mutated RARa gene and does not differentiate into a neuronal cell line upon retinoic acid induction (Pratt *et al.*, 1990). Another set of experiments that could be performed if time and resources were permitting would be *in vivo* footprinting of the ATBF1A gene during RA induction (Dey *et al.*, 1994). The pattern changes observed upon induction of P19 cells with retinoic acid would be quite informative.

As seems to be the case in research, my efforts have produced some tantalizing hints as to the mechanisms underlying the modulation of the ATBF1A promoter. These experiments may serve as a compass to point the way to further investigation of the transcriptional control of tissue specific gene expression during development.

References

Andersson, G., Pahlman, S., Parrow, V., Johansson, I., and Hammerling, U. (1994) Activation of the Human NPY gene during neuroblastoma cell differentiation: Induced transcriptional activities of AP-1 and AP-2 *Cell Growth and Differentiation* 5:27-36.

Andrin, C. and Spencer, C. (1994) The intricacies of beta-globin gene expression. *Biochemistry and Cell Biology* 72: 377-380.

Ausubel,F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1994) Current Protocols in Molecular Biology John Wiley and Sons.

Bacon, E.R., Dalyot, N., Filon, D., Schreiber, L., Rachmilewitz, E.A. and Oppenheim, A. (1995) Hemoglobin switching in humans is accompanied by changes in the ratio of the transcription factors GATA-1 and Sp1. *Molecular Medicine* 1: 297-305.

Bain, G., Ray, W.J., Yao, M. and Gottlieb, D.I. (1994) From embryonal carcinoma cells to neurons: The P19 pathway. *BioEssays* 16: 343-348.

Bain, G., and Gottlieb, D.I. (1994) Expression of retinoid X receptors in P19 embryonal carcinoma cells and embryonic stem cells. *Biochemical and Biophysical Research Communications* 200:1252-1256.

Barton, M.C., Madani, N. and Emerson, B. (1993) The erythroid protein cGATA-1 functions with a stage specific factor to activate transcription of chromatin assembled beta-globin genes. *Genes and Development* 7:1796-1809.

Belikov, S. and Wieslander, L. (1995) Express protocol for generating G+A sequencing ladders. *Nucleic Acids Research* 23: 310.

Berg, R.W. and McBurney, M.W. (1990) Cell density and cell cycle effects on retinoic acid-induced embryonal carcinoma cell differentiation. *Developmental Biology* 138:123-135.

Blobel, G.A., Sieff, C.A., and Orkin, S.H. (1995) Ligand dependent repression of the erythroid transcription factor GATA-1 by the estrogen receptor. *Molecular and Cellular Biology* 15: 3147-3153.

Bouillet, P., Oulad-Abdelghani, M., Vicaire, S., Garnier, J-M., Schuhbaur, B., Dolle, P. and Chambon, P. (1995) Efficient cloning of cDNAs of retinoic acid-responsive genes in P19 embryonal carcinoma cells and characterization of a novel mouse gene, Stra1 (Mouse LERK-2/Eplg2). *Developmental Biology* 170:420-433.

Cao, X., Koski, R.A, Gashler, A., McKiernan, M., Morris, C.F., Gaffney, R., Hay, R.V. and Sukhatme, V.P. (1990) Identification and characterization of the Egr-1 gene product, a DNA binding Zinc Finger protein induced by differentiation and growth signals. *Molecular and Cellular Biology* 10:1931-1939.

Christy, B. and Nathans, D. (1989) DNA binding site of the growth factor inducible protein Zif268. *Proceedings of the National Academy of Sciences* 86: 8737-8741.

Dey, A., Minucci, S. and Ozato, K. (1994) Ligand-dependent occupancy of the retinoic acid receptor beta2 promoter *in vivo*. *Molecular and Cellular Biology* 14:8191-8201.

Doe, C.Q., Smouse, D. and Goodman, C.S. (1988) Control of neuronal fate by the *Drosophila* segmentation gene *even skipped*. *Nature* 333:376-378.

Edwards, M.K.S., Harris, J.F. and McBurney M.W. (1983) Induced muscle differentiation in an embryonal carcinoma cell line. *Molecular and Cellular Biology* 3: 2280-2286.

Edwards, S.A., Darland, T., Sosnowski, R., Samuels, M. and Adamson, E.D. (1991) The transcription factor, Egr-1, is rapidly modulated in response to retinoic acid in P19 embryonal carcinoma cells. *Developmental Biology* 148:165-173.

Engel, J.D. (1993) Developmental regulation of human beta-globin gene transcription: a switch of loyalties. *Trends in Genetics* 9: 304-309.

Evans, R.M. (1988) The steroid and thyroid hormone receptor superfamily. *Science* 240:889-895.

Fisher, K.D., Haese, A., and Nowock, J. (1993) Cooperation of GATA-1 and Sp1 can result in synergistic activation or interference. *The Journal of Biological Chemistry* 268:23915-23923.

Fortini, M. E., Lai, Z., and Rubin, G.M. (1991) The *Drosophila zfh-1* and *zfh-2* genes encode novel proteins containing both

zinc-finger and homeodomain motifs. *Mechanisms of Development* 34: 113-122.

Fraser, P., Pruzina, S., Antoniou, M.T. and Grosveld, F. (1993) Each hypersensitive site of the human Beta-globin locus control region confers a different developmental pattern of expression on the globin genes. *Genes and Development* 7: 106-113.

Fujii, H. and H. Hamada (1993) A CNS-specific POU transcription factor, Brn-2, is required for establishing mammalian neural cell lineages. *Neuron* 11: 1197-1206.

Green, S. and Chambon, P. (1988) Nuclear receptors enhance our understanding of transcription regulation. *Trends in Genetics* 4:309-313.

Hanahan, D. (1983) Studies on Transformation of *Escherichia coli* with Plasmids. *Journal of Molecular Biology* 166:557-580.

Harrrison, P.R. (1990) Molecular mechanisms involved in the regulation of gene expression during cell differentiation and development. *Immunology and Serology* 49:411-464.

Ido, A., Y. Miura and T. Tamaoki (1994) Activation of ATBF1, a multiple-homeodomain zinc-finger gene, during neuronal differentiation of murine embryonal carcinoma cells. *Developmental Biology* 163:184-187.

Jones-Villeneuve, E.M.V., Rudnicki, M.A., Harris, J.F. and McBurney, M.W. (1983) Retinoic acid induced neural differentiation of embryonal carcinoma cells. *Molecular and Cellular Biology* 3:2271-2279.

Kondo, T., Takahashi, N., and Muramatsu, M. (1992) The regulation of the murine Hox-2.5 gene expression during cell differentiation. *Nucleic Acids Research* 20: 5729-5735.

Kostich, W.A. and Sanes, J.R. (1995) Expression of zfh-4, a new member of the Zinc Finger-Homeodomain Family in developing Brain and Muscle *Developmental Dynamics* 202:145-152.

Kozmik, Z., Urbanek, P and Paces, V. (1990) Albumin improves formation and detection of some specific protein-DNA complexes in the mobility shift assay. *Nucleic Acids Research* 18:2198.

Lai, Z, Fortini, M.E., and Rubin, G.M. (1991) The embryonic expression patterns of *zfh-1* and *zfh-2*, two *Drosophila* genes encoding novel zinc-finger homeodomain proteins. *Mechanisms of Development* 34:123-134.

Leid, M., Kastner, P. and Chambon, P. (1992) Multiplicity generates diversity in the retinoic acid signalling pathways. *Trends in Biochemical Sciences* 18: 427-433.

Lemaire, P., Vesque, C., Schmitt, J., Stunnenberg, H., Frank, R., and Charnay, P. (1990) The serum-inducible mouse gene *Krox-24* encodes a sequence-specific transcriptional activator. *Molecular and Cellular Biology* 10:3456-3467.

Li, P., He, X., Gerrero, M.R., Mok, M., Agarwal, A., and Rosenfeld, M.G. (1993) Spacing and orientation of bipartite DNA-binding motifs as potential functional determinants for POU domain factors. *Genes and Development* 7:2483-2496.

Li, L., Suzuki, T., Mori, N. and Greengard, P. (1993) Identification of a functional silencer element involved in neuron-specific expression of the synapsin I gene. *Proceedings of the National Academy of Sciences* 90:1460-1464.

Lundell, M.J. and J. Hirsh (1992) The *zfh-2* gene product is a potential regulator of neuron-specific DOPA decarboxylase gene expression in *Drosophila*. *Developmental Biology* 154: 84-94.

Luscher, B., Mitchell, P.J., Williams, T., and Tjian, R. (1989) Regulation of transcription factor AP-2 by the morphogen retinoic acid and by second messengers. *Genes and Development* 3:1507-1517.

Maden, M and Holder, N. (1992) Retinoic acid and development of the central nervous system. *BioEssays* 14:431-438.

Mader, S., Leroy, P., Chen, J-Y, and Chambon, P. (1993) Multiple Parameters control the selectivity of nuclear receptors for their response elements. *Journal of Biological Chemistry* 268: 591-600.

Manak, J.R. and Scott, M.P. (1994) A class act: conservation of homeodomain functions. *Development-Supplement* 61-77.

McBurney, M.W. (1993) P19 embryonal carcinoma cells. *International Journal of Developmental Biology* 37:135-140.

McBurney, M.W., Reuhl, K.R., Ally, A.I., Nasipuri, S., Bell, J.C. and Craig, J. (1988) Differentiation and maturation of embryonal carcinoma-derived neurons in cell culture. *The Journal of Neuroscience* 8:1063-1073.

Meier, P., Koedood, M., Philipp, J., Fontana, A., and Mitchell, P.J. (1995) Alternative mRNAs encode multiple

isoforms of transcription factor AP-2 during murine embryogenesis *Developmental Biology* 169: 1-14.

Mendel, D.B. and Crabtree, G.R. (1991) HNF-1, a member of a novel class of dimerizing homeodomain proteins. *The Journal of Biological Chemistry* 266: 677-686.

Mitchell, P.J., Timmons, P.M., Hebert, J.M., Rigby, P.W.J., and Tjian, R. (1991) Transcription factor AP-2 is expresssed in neural crest cell lineages during mouse embryogenesis. *Genes and Development* 5: 105-119.

Mitchell, P.J., Wang, C., and Tjian, R. (1987) Positive and negative regulation of transcription *in vitro*: enhancer binding protein AP-2 is inhibited by SV40 T antigen. *Cell* 50: 847-861.

Miura, Y., Tam, T., Ido, A., Morinaga, T., Miki, T., Hashimoto, T. and Tamaoki, T. (1995) Cloning and characterization of an ATBF-1 isoform that expresses in a neuronal differentiation-dependent manner. *The Journal of Biological Chemistry* 270: 26840-26848.

Mori, N., Stein, R., Sigmund, O. and Anderson, D.J. (1990) A cell type-preferred silencer element that controls the neural-specific expression of the SCG10 gene. *Neuron* 4: 583-594.

Mori, N., Schoenherr, C., Vandenbergh, D.J., Anderson, D.J. (1992) A common silencer element in the SCG10 and type II Na+ channel genes binds a factor present in nonneuronal cells but not in neuronal cells. *Neuron* 9: 45-54.

Morinaga, T., Yasuda, H., Hashimoto, T., Higashio, K., and Tamaoki, T. (1991) A human alpha-fetoprotein enhancer-binding protein, ATBF1 contains four homeodomains and seventeen zinc fingers. *Molecular and Cellular Biology* 11:6041-6049.

Mummery, C.L. and Feyen, A. (1990) Characteristics of embryonal stem cell differentiation: a comparison with two embryonal carcinoma cell lines. *Cell Differentiation and Development* 30:195-206.

Muscat, G.E., Downes, M. and Dowhan, D.H. (1995) Regulation of vertebrate muscle differentiation by thyroid homone: the role of the myoD gene family. *BioEssays* 17: 211-218.

Nakai, S. Kamano, H., Yudate, T., Nishi, M., Kuno, T., Naguta, A., Jishage, K., Hamada, H., Fujii, H., Kawamura, K., Shiba, K., and Noda, T. (1995) The POU domain transcription factor

Brn-2 is required for neuronal lineages in the hypothalamus of the mouse. *Genes and Development* 9:3109-3121.

Okamoto, K., Wakamiya, M., Noji, S., Koyama, E., Taniguchi, S., Takemura, R., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Muramatsu, M., and Hamada, H. (1993) A Novel class of murine POU gene predominantly expressed in central nervous system *The Journal of Biological Chemistry* 268: 7449-7457.

Orkin, S.H. (1990) Globin gene regulation and switching: circa 1990. *Cell* 63: 665-672.

Philipp, J., Mitchell, P.J., Malipiero, U. and Fontana, A. (1994) Cell type-specific regulation of expression of transcription factor AP-2 in neuroectodermal cells *Developmental Biology* 165:602-614.

Pratt, M.A.C., Kravlova, J. and McBurney, M.W. (1990) A dominant negative mutation of the alpha retinoic acid receptor gene in a retinoic acid-nonresponsive embryonal carcinoma cell. *Molecular and Cellular Biology* 10:6445-6453.

Pratt, M.A.C, Langstone, A.W., Gudas, L.J. and McBurney, M.W. (1993) Retinoic acid fails to induce expression of Hox genes in differentiation-defective embryonal carcinoma cells carrying a mutant RARa gene. *Differentiation* 53: 105-113.

Rahuel, C., Vinit, M-A., Lemarchandel, V., Cartron, J-P., and Romeo, P-H. Erythroid-specific activity of the glycophorin B (1992) promoter requires GATA-1 mediated displacement of a repressor. *The EMBO Journal* 11:4095-4102.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual Second edition, Cold Spring Harbor Laboratory Press.

Sawadaishi, S., Morinaga, T. and Tamaoki, T. (1988) Interaction of a hepatoma-specific nuclear factor with transcription-regulatory sequences of the human alpha-fetoprotein and albumin genes. *Molecular and Cellular Biology* 8:5179-5187.

Schule, R., Rangarajan, P., Yang, N., Kliewer, S., Ransone, L.J., Bolado, J., Verma, I.M. and Evans, R.M. (1991) Retinoic acid is a negative regulator of AP-1 responsive genes. *Proceedings of the National Academy of Sciences* 88:6092-6096.

Shimazaki, T., Okazawa, Fujii, H., Ikeda, M., Tamai, K., McKay, R.D.G., Muramatsu, M., and Hamada, H. (1993) Hybrid cell extinction and re-expression of *Oct-3* function correlates

with differentiation potential. *The EMBO Journal* 12: 4489-4498.

Stratagene protocol. (1990) Gelshift kit. Stratagene Technical services.

Thiel, G., Schoch, S. and Petersohn, D. (1994) Regulation of Synapsin I Gene Expression by the Zinc Finger Transcription Factor zif268/egr-1 *Journal of Biological Chemistry* 269:15294-15301.

Williams, T. and Tjian, R. (1991) Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. *Genes and Development* 5: 670-682.

Williams, T., Admon, A., Luscher, B. and Tjian, R. (1988) Cloning and expression of AP-2, a cell-type specific transcription factor that activates inducible enhancer elements. *Genes and Development* 2:1557-1569.

Winning, R. S., Shea, L.J., Marcus, S.J., and Sargent, T.D. (1991) Developmental regulation of transcription factor AP-2 during *Xenopus laevis* embryogenesis. *Nucleic Acids Research* 19:3709-3714.

Wuenshell, C. W., Mori, N, and Anderson, D.J. (1990) Analysis of SCG10 gene expression in transgenic mice reveals that neural specificity is achieved through selective derepresssion. *Neuron* 4: 595-602.

Wright, C.V., Cho, K.W., Oliver, G. and De-Robertis, E.M. (1989) Vertebrate homeodomain proteins: families of region-specific transcription factors. *Trends in Biochemical Sciences* 14:52-56.

Yasuda, H., Mizuno, A., Tamaoki, T. and Morinaga, T. (1991) ATBF1, a multiple-homeodomain zinc finger protein selectively down-regulates AT-rich elements of the human alpha-fetoprotein gene. *Genes and Development* 5: 105-119.

Yu, C-Y., Chen, J., Lin, L-I, Tam, M., and Shen, C-K.J. (1990) Cell Type-specific Protein-DNA interactions in the human zeta-globin Upstream Promoter Region: Displacement of Sp1 by the Erythroid Cell-Specific Factor NF-E1. *Molecular and Cellular Biology* 10:282-294.