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Structure and Function of the Mouse *ATBF1* Gene

by

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ABSTRACT

ATBF1 (AT motif Binding Factor 1) protein is a transcription factor containing multiple homeodomains and zinc fingers. Two isoforms of *ATBF1* transcripts have been identified and cloned which are generated by alternative splicing from one *ATBF1* gene. *ATBF1* gene is expressed in the neuronally differentiated P19 mouse embryonal carcinoma cells induced by retinoic acid. Analyses of the gene expression in mouse tissues using Northern hybridizations, ribonuclease protection assays, and *in situ* hybridizations indicate that *ATBF1* gene is expressed mainly in the central nervous system during embryogenesis. In an attempt to understand the function of ATBF1 protein, the mouse *ATBF1* gene was cloned based on its cDNA. The mouse *ATBF1* gene contains 10 exons over 170 kb of gene sequence. The expression of sense- and antisense-*ATBF1* in P19 cells was also carried out. The results indicate that the *ATBF1* gene expression is required for the retinoic acid-induced neuronal differentiation of P19 cells.

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to my parents...

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ATP	adenosine-5′-triphosphate
cDNA	complementary DNA
cpm	counts per minute
CTP	cytidine-5′-triphosphate
dATP	deoxyadenosine-5′-triphosphate
dCTP	deoxycytidine-5′-triphosphate
dGTP	deoxyguanosine-5′-triphosphate
dTTP	deoxythymidine-5′-triphosphate
DNA	deoxyribonucleic acid
g	gram
GTP	guanosine-5′-triphosphate
kb	kilo-base pair
kDa	kilo-Dalton
M	molar
µg	microgram
µM	micromolar
mg	milligram
µl	microliter
ml	milliliter

mM	millimolar
mRNA	message RNA
OD	optical density
PIPES	piperazine-N,N'-bis(2-ethanesulfolnic acid)
RNA	ribonucleic acid
rpm	revolutions per minute
Tris	tris (hydroxymethyl) aminomethane
UTP	uridine-5'-triphosphate
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER I: INTRODUCTION

α-Fetoprotein (AFP) is an onco-fetal protein which is expressed at high levels in embryonic and neoplastic tissues. *AFP* expression is low in the serum and liver of adult mammals so far studied. During a study of the regulation of human *AFP* gene expression, our laboratory identified a protein, termed ATBF1 (AT motif Binding Factor 1), which could down-regulate the human *AFP* gene expression (Morinaga, *et al.*, 1991; Yasuda, *et al.*, 1994).

ATBF1 protein is a transcription factor which contains multiple-homeodomains and zinc fingers. There are two *ATBF1* mRNA isoforms generated by alternative splicing from a single *ATBF1* gene (Miura *et al.*, 1995). The human *ATBF1-B* cDNA was first isolated based on the ability of its product to bind to a human *AFP* gene enhancer (Morinaga *et al.*, 1991). Later, our laboratory cloned another human *ATBF1* cDNA, termed *ATBF1-A* cDNA (Miura *et al.*, 1995). The mouse homolog of human *ATBF1-A* cDNA was also cloned in our laboratory (Ido *et al.*, 1996). The deduced amino acid sequences from the nucleotide sequences of *ATBF1-A* and *ATBF1-B* cDNAs show that the ATBF1-A protein extends 920 more amino acids at the N-terminus than ATBF1-B protein. For the rest part, ATBF1-A and ATBF1-B proteins are the same (Morinaga *et al.*, 1991; Miura *et al.*, 1995).

Although ATBF1 protein was first identified in a human hepatoma cell line, *ATBF1* gene is also expressed in many nonhepatic tissues (Morinaga *et al.*, 1991). *ATBF1* gene is preferentially expressed during embryogenesis and the

ATBF1 transcripts are found at high levels in embryonic and neonatal brains (Miura *et al.*, 1995; Ido *et al.*, 1996; Watanabe *et al.*, 1996). *ATBF1* expression is also activated in P19 mouse embryonal carcinoma cells and NT2/D1 human embryonal carcinoma cells when these cells are induced into neuronal differentiation by retinoic acid. In addition, the human *ATBF1-A* promoter is activated in the neuronally differentiated mouse P19 cells (Ido *et al.*, 1994; Miura *et al.*, 1995). These data indicate that ATBF1 protein may play roles during the embryonic development of the nervous system.

In this report, I carried out the molecular cloning of the mouse *ATBF1* gene. The *ATBF1* gene itself is significant in that it may represent a member of a gene family with similar gene structure and biological function in embryonic development and differentiation. In addition, the characterized gene provides us with an approach to study the function of the *ATBF1* gene in the mouse. To investigate the function of the *ATBF1* gene expression in P19 cells during the retinoic acid-induced neuronal differentiation, I established and analyzed a cell line with enforced expression of antisense-*ATBF1*. P19 cells transfected with sense-*ATBF1* expression vector were also analyzed. The results indicate the association of *ATBF1* expression and the neuronal differentiation.

CHAPTER II: BACKGROUND

2.1 ATBF1 Proteins and Potential Functional Domains

ATBF1 is a multiple-homeodomain-zinc finger containing protein. The ATBF1 protein was first identified in the HuH-7 human hepatoma cells by its ability to bind to an AT-rich motif in the human *AFP* gene enhancer. This *ATBF1* cDNA is 8.6 kb and termed *ATBF1-B* (Morinaga *et al.*, 1991). The deduced ATBF1-B protein contains four homeodomains and eighteen zinc fingers (including one pseudo-zinc finger) with a large molecular weight of 306 kDa. A second *ATBF1* cDNA, 12 kb in length, termed *ATBF1-A*, was isolated from a human fibroblast cDNA library (Miura *et al.*, 1995). The deduced ATBF1-A protein is 404 kDa in molecular weight and differs from ATBF1-B by a 920 amino acid extension at the N-terminus which includes five zinc fingers. The deduced ATBF1 proteins contain a number of segments rich in glutamine, proline, and acidic amino acids, which are potentially involved in transcriptional regulation (Figure 1). ATBF1 proteins also contain an ATP-binding motif with two lysine residues (Kamps *et al.*, 1984), of which the ATP-binding and ATPase activity have been confirmed using purified glutathione S-transferase (GST) fusion protein by fusing the ATP-binding region with GST (Kawaguchi and Tamaoki, personal communications).

Figure 1. Potential Functional Domains of ATBF1 Proteins

Analysis of the deduced amino acid sequence from *ATBF1* cDNAs show several potential functional domains as indicated (Miura *et al.*, 1995). The ATP-binding site has been confirmed in our laboratory (Kawaguchi and Tamaoki, personal communications).

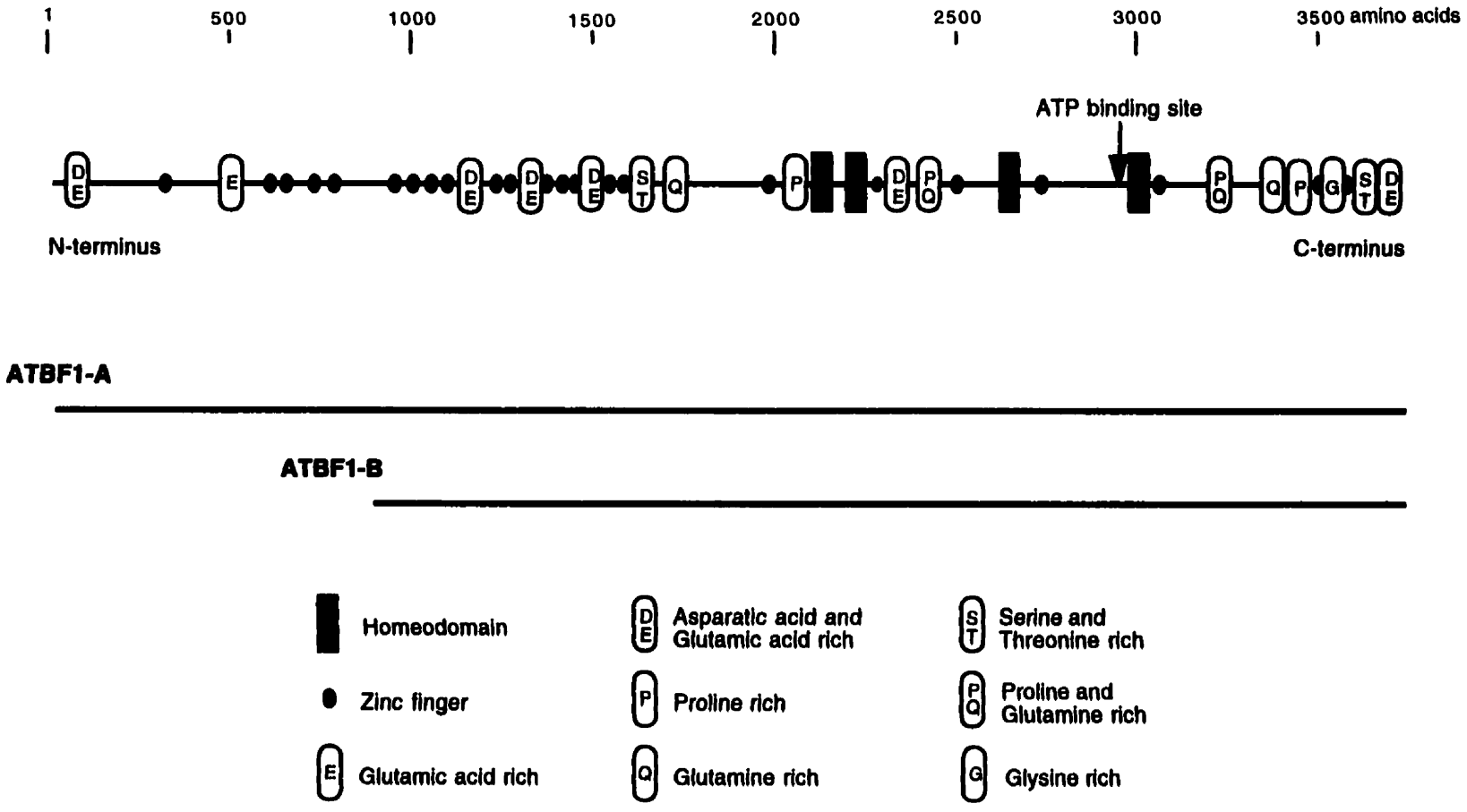

Figure 1. Potential Functional Domains of ATBF1 Proteins.

2.2 Alternative Splicing Generating *ATBF1-A* and *ATBF1-B*

Two human *ATBF1* transcripts, *ATBF1-A* and *ATBF1-B*, were identified in our laboratory (Morinaga *et al.*, 1991; Miura *et al.*, 1995). The difference between *ATBF1-A* and *ATBF1-B* transcripts is at the 5'-termini. Our laboratory showed that *ATBF1-A* and *ATBF1-B* mRNA had distinct 5'-end exons. The possible mechanism of the alternative splicing to generate *ATBF1-A* and *ATBF1-B* mRNA is shown in Figure 2. Exon 1 specifies most of the 5'-noncoding sequence of *ATBF1-B* mRNA, and exon 2 specifies that of *ATBF1-A* mRNA. It indicates that two different promoters may be used for the transcription of *ATBF1-A* and *ATBF1-B* mRNAs (Miura *et al.*, 1995). Northern blot analyses and ribonuclease protection assays in tissues and cell lines in which the *ATBF1* gene was expressed showed that the majority of *ATBF1* transcripts was *ATBF1-A*, whereas *ATBF1-B* was expressed at a very low level (Ido *et al.*, 1996; Miura *et al.*, 1995). For the human *ATBF1* gene, transient transfection assays showed that the 5'-flanking region of the *ATBF1-A*-specific exon 2 supported chloramphenicol acetyltransferase (CAT) reporter gene expression (Miura *et al.*, 1995). It suggests that *ATBF1-A* and *ATBF1-B* mRNAs are transcribed from the same *ATBF1* gene using independent promoters combined with alternative splicing at the 5'-end of the *ATBF1* primary transcripts. This alternative splicing is an example of the mode of alternative promoters/alternative 5'-splice sites (Smith *et al.*, 1989; McKeown, 1992).

Figure 2. Alternative Splicing to Generate the *ATBF1-A* and *ATBF1-B* mRNAs

ATBF1-A and *ATBF1-B* mRNAs are transcribed from the same *ATBF1* gene. These two isoforms of *ATBF1* mRNA are generated by the mechanism of alternative promoters and alternative splicing at the 5′-region of the *ATBF1* primary transcripts. The first five exons are shown in boxes with the numbers on top and coding regions filled.

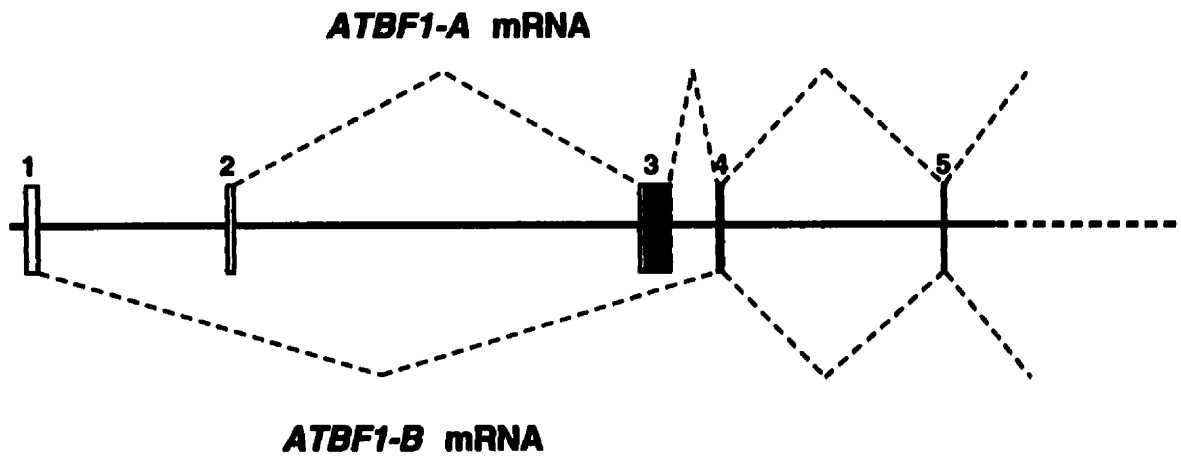

Figure 2. Alternative Splicing to Generate the *ATBF1-A* and *ATBF1-B* mRNAs.

2.3 ATBF1 is a Transcription Factor

ATBF1 protein contains multiple-homeodomains and zinc fingers. Homeodomains and zinc fingers in transcription factors play roles in the specific sequence recognition (Mitchell and Tjian, 1989; Johnson and McKnight, 1989; Pabo and Sauer, 1992; Gehring *et al.*, 1994). The transcriptional-regulatory function of ATBF1 was assessed in transient transfection assays in the HuH-7 human hepatoma cells. The results showed that ATBF1 suppressed the activity of the human *AFP* gene enhancer AT-rich motif through the specific interaction between homeodomain IV of the ATBF1 protein and the AT-rich motif of the human *AFP* gene (Morinaga *et al.*, 1991; Yasuda *et al.*, 1994). However, the AT-rich element in the enhancer of the human *AFP* gene is not conserved in those of the mouse and the rat genes (Godbout *et al.*, 1988). *ATBF1* transcripts were found in various human hepatic and nonhepatic cell lines (Morinaga *et al.*, 1991), and a second *ATBF1* cDNA, *ATBF1-A*, was isolated from a human fibroblast cDNA library (Miura *et al.*, 1995). Furthermore, it was found that the mouse homolog of the human *ATBF1* gene was expressed in several different mouse tissues and mouse cell lines. The mouse *ATBF1-A* cDNA, 12,068 base pairs in length, was cloned and the deduced protein sequence contains four homeodomains and twenty-three zinc fingers including one pseudo-zinc finger (Ido *et al.*, 1994; Ido *et al.*, 1996). It suggests that ATBF1 protein may play roles in other cells, not only in human hepatic cells.

2.4 The Homeodomain-Zinc Finger Family

The mouse and the human ATBF1 proteins share extremely high similarities. Mouse ATBF1-A protein shows overall 94% identity to the human ATBF1-A protein. The amino acid sequences of all four homeodomains of mouse ATBF1-A are 100% identical to those of human ATBF1-A, although identities at the nucleotide level are 88, 89, 88 and 95% for the four homeodomains. Sequence identities between corresponding zinc fingers in mouse ATBF1-A and human ATBF1-A are from 94 to 100% at the amino acid level and from 85 to 98% at the nucleotide level (Ido *et al.*, 1996).

Besides ATBF1, there are other transcription factors which also contain both homeodomains and zinc fingers. *Drosophila* ZFH-1 contains one homeodomain and nine zinc fingers (Fortini *et al.*, 1991). The *ZFH-1* gene is expressed during embryogenesis in the mesoderm and in the central nervous system (Lai *et al.*, 1991). The loss-of-function ZFH-1 mutant embryos showed various degree of local errors in cell fate or position (Lai *et al.*, 1993). *Drosophila* ZFH-2 contains three homeodomains and sixteen zinc fingers (Fortini *et al.*, 1991). The *ZFH-2* gene is also expressed during embryogenesis, but in a more limited pattern, largely restricted to the central nervous systems of late embryos (Lai *et al.*, 1991). It was shown that ZFH-2 bound to an opsin regulatory element through homeodomain III and activated the DOPA decarboxylase gene through homeodomain II (Fortini *et al.*, 1991; Lundell and Hirsh, 1992). Kostich and Sanes (1995) cloned a small proportion of a putative

mouse transcription factor termed ZFH-4, which contains two homeodomains and two zinc fingers. The protein organization of ZFH-4 at that region is analogous to that of ATBF1 and ZFH-2. The *ZFH-4* gene is expressed prominently in developing mouse brain and muscle during embryogenesis, whereas the expression is decreased to a barely detectable level in adult tissues.

Amino acid sequence analyses showed (Figure 3) that the homeodomains in ATBF1 and in ZFH-2 shared a high percentage of identities. Thirteen zinc fingers in ATBF1 protein and in ZFH-2 protein show 22 to 89% identities. All these homologous domains are colinearly arranged in the ATBF1 and ZFH-2 molecules. The homeodomain in ZFH-1 also shows a high percentage of identity to its according homeodomain in ATBF1. Homeodomain identities between ZFH-1 and ZFH-2 proteins are less than 39%, indicating that they are more similar to ATBF1 homeodomains than between themselves (Hashimoto *et al.*, 1992; Lundell and Hirsh, 1992). The two cloned homeodomains in ZFH-4 have a very high percentage of identities to their analogous homeodomains in ATBF1. Therefore, the mouse ZFH-4 is more related to the mammalian ATBF1 than to the *Drosophila* ZFH-2. ATBF1, ZFH-1, ZFH-2 and ZFH-4 are members of the homeodomain-zinc finger family.

Figure 3. The Homeodomain-Zinc Finger Family

The percentages shown here are the amino acid sequence identities of potential homeodomains and other domains between ATBF1 protein and other members of the homeodomain-zinc finger family.

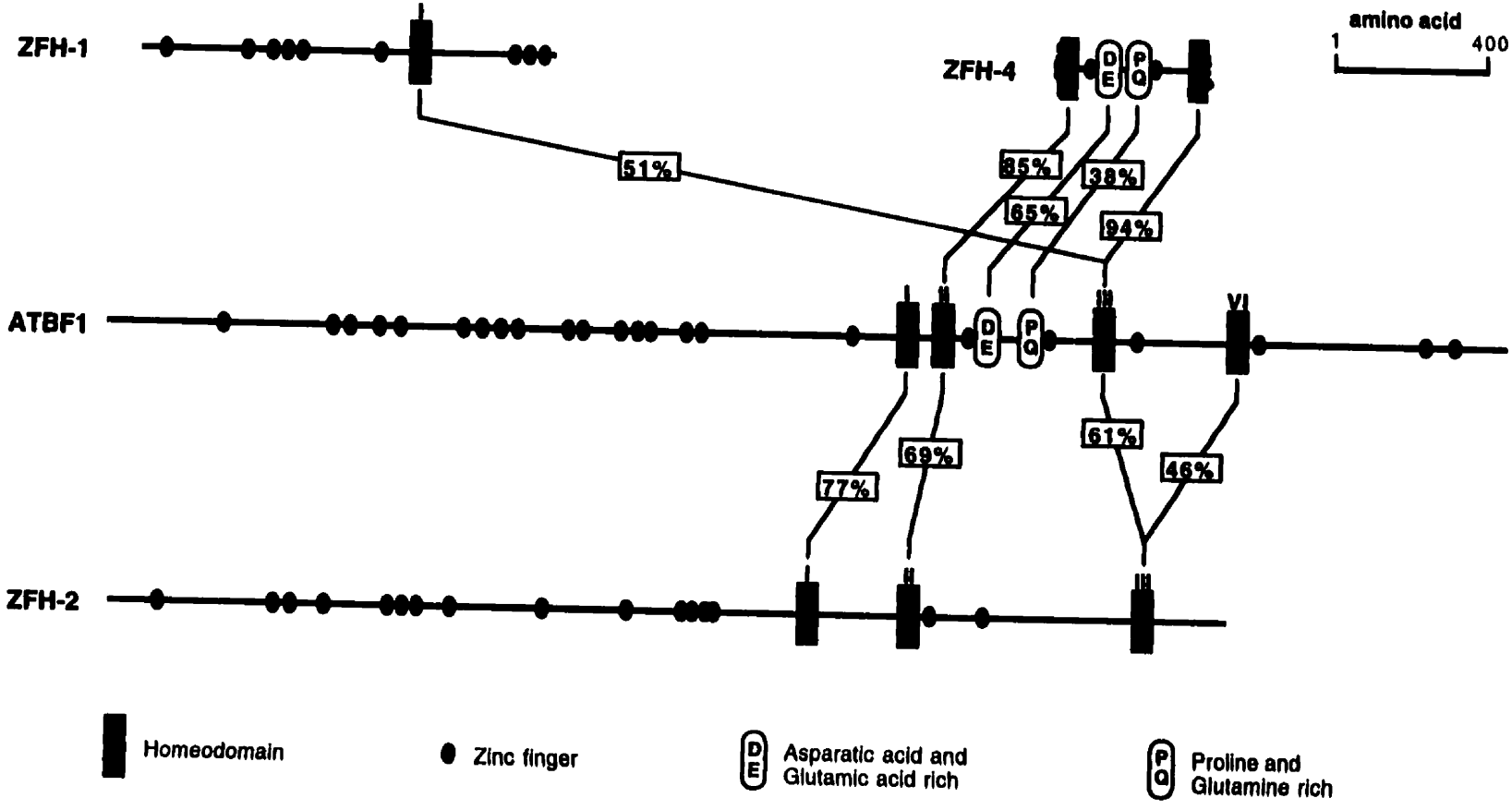

Figure 3. The Homeodomain-Zinc Finger Family.

2.5 Possible Roles of ATBF1 Proteins in Neural Development

Since ATBF1 protein as a member of the homeodomain-zinc finger family shares a large percentage of protein sequence identities with other members, it may also possess similar functional roles as other members. *Drosophila* ZFH-1 and ZFH-2, and mouse ZFH-4 are expressed during embryogenesis, predominantly in the embryo central nervous systems (Lai *et al.*, 1991; Kostich and Sanes, 1995). It suggests the possibility that ATBF1 proteins also play roles during the development of mammalian central nervous systems.

ATBF1 transcripts were found to be expressed at low levels in various adult mouse tissues but at high levels in embryonic and neonatal brains. *In situ* hybridization analyses of *ATBF1* transcripts showed a wide distribution of hybrid signals in several regions of the embryonic and neonatal mouse brains. These data suggest a role of the *ATBF1* gene in the formation of specific cell populations in developing central nervous systems (Ido *et al.*, 1996; Watanabe, *et al.*, 1996). In addition, *ATBF1* expression is activated in P19 mouse embryonal carcinoma cells and in NT2/D1 human embryonal carcinoma cells after these cells are induced into neuronal differentiation by retinoic acid, whereas the *ATBF1* expression in undifferentiated cells is undetectable (Ido *et al.*, 1994; Miura *et al.*, 1995). Furthermore, the promoter activity of human *ATBF1-A* is shown in the retinoic acid-induced, neuronally differentiated

mouse P19 cells, but not in the undifferentiated P19 cells (Miura *et al.*, 1995). These results are consistent with a role of *ATBF1* in neural development.

2.6 P19 Embryonal Carcinoma Cells

The P19 cell line was initially established by McBurney and Rogers (1982). It was derived from a teratocarcinoma in C3H/He mice, produced by transplantation of an embryo at 7.5 days of gestation into the testes of an adult male mouse. The resulting tumor called embryonal carcinoma was excised, dissociated, and plated for growth in tissue culture. A clonal cell line was selected which possessed the typical morphology of embryonal carcinoma cells and was designated P19.

P19 cells are a line of pluripotent embryonal carcinoma able to grow continuously in serum-supplemented media. The differentiation of these cells can be controlled by certain drugs. The drugs most effective in inducing differentiation of P19 cells are retinoic acid and dimethyl sulfoxide (Jones-Villeneuve *et al.*, 1982; McBurney *et al.*, 1982). Exposure of aggregated P19 cells to retinoic acid leads to the formation of neurons, astroglia and microglia which were normally derived from the neuroectoderm. Alternatively, treatment of aggregated P19 cells with dimethyl sulfoxide results in their differentiation into endodermal and mesodermal derivatives including cardiac and skeletal muscle. Both drugs are not demonstrably toxic to P19 cells at the doses effective in inducing differentiation, indicating that their effects

are true inductions but not selection of differentiated cells pre-exiting in the cultures.

CHAPTER III: OBJECTIVES

3.1 Isolation and Characterization of the Mouse *ATBF1* Gene

In general, the *ATBF1* gene is preferentially expressed during embryogenesis and the *ATBF1* transcripts are found at high levels in embryonic and neonatal brains. To understand the function of ATBF1 protein, I cloned the mouse *ATBF1* gene. The *ATBF1* gene may represent a member of a gene family with similar gene structure and biological function in embryonic development and differentiation. The characterized mouse gene provides us with an approach to study the function of the *ATBF1* gene in the mouse and, to study the spatial and temporal expression pattern of this gene *in vivo*.

3.2 Transfection of P19 Cells with Sense- and Antisense-*ATBF1* Expression Vectors

ATBF1 gene expression is activated in a mouse embryonal carcinoma cell line, P19, during the neuronal differentiation induced by retinoic acid. To study the function of *ATBF1*, especially along the neuronal differentiation pathway of P19 cells, I established and analyzed a P19 cell line with enforced expression of antisense-*ATBF1*. P19 cells transfected with a sense-*ATBF1* expression vector were also analyzed.

CHAPTER IV: MATERIALS AND METHODS

Most of the manipulations were carried out using established and standard protocols (Sambrook *et al.*, 1989; Ausubel *et al.*, 1996). For those manipulations using specific reagents or kits, the manufacturers' instructions were followed. All standard primers for the plasmid vector pBluescript II KS (+), such as M13 forward and M13 reverse primers, were prepared in the Regional DNA Synthesis Laboratory, University of Calgary. All custom-made primers which represented sequences in the mouse *ATBF1* gene were synthesized either by the Regional DNA Synthesis Laboratory, University of Calgary or by Life Technologies (Gaithersburg, MD, USA).

4.1 Preparation of DNA Fragments Used as Probes for Library Screening

DNA fragments were obtained by restriction endonuclease digestion of the inserts of plasmid clones which contained both the mouse *ATBF1-A* cDNA (cloned in our laboratory, Ido *et al.*, 1996) and the mouse *ATBF1* gene intron regions (cloned by myself). The restriction fragments were separated on agarose gels by electrophoresis and then recovered from the gels by electroelution. These DNA fragments were used as probes for the mouse genomic library screening.

4.2 DNA Probe Labeling

All DNA probes were ^{32}P-dCTP labeled using the random priming kit purchased either from Pharmacia (^{T7}QuickPrime Kit) or from Beohringer Mannheim (Random Primed DNA Labeling Kit). The labeling procedures were performed following the manufacturers' instructions. For each labeling reaction, approximately 50 ng of DNA fragment were denatured in boiling water for two minutes, and chilled on ice, then the reagent mix (random hexamers of oligonucleotides, dATP, dGTP, dTTP), 50 μCi of α-^{32}P-dCTP and T7 (or Klenow) DNA polymerase were added. The above mixture was incubated at 37°C for 15 to 60 minutes. The unincorporated ^{32}P-dCTP was separated from the labeled probe using Sephadex G-50 (Pharmacia) spin columns as previously described (Sambrook *et al.*, 1989). One μl of labeled probe was used to determine the counts using a Beckman LS 5000CE scintillation counter. The specific activity of the above labeled DNA probe was about 10^9 dpm/μg.

4.3 Library Screening Using Nucleic Acid Hybridization

A 129SV mouse genomic library in λFIX II vector (Stratagene) was screened to isolate the mouse *ATBF1* genomic clones as described below.

4.3.1 Preparation of the Host Bacteria for the Transfection of λFIX II Phage

E. coli XL1-Blue MRA (P2) (Stratagene) strain was used as the host bacteria for λFIX II phage. The bacteria were grown overnight in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) with 10 mM magnesium sulfate and 0.2% maltose at 37°C with shaking. The culture was then diluted at the ratio of 1:50 in the same medium, and incubated at 37°C with shaking until the OD_{600} reached 0.6. The bacteria were collected by centrifugation and the bacteria pellet was resuspended in 10 mM magnesium sulfate to make the OD_{600} to 0.5 and used as the bacteria solution for the transfection of λFIX II phage.

4.3.2 Infection of *E. coli* XL1-Blue MRA (P2) with λFIX II Phage

λFIX II phage were incubated in 0.6 ml of the *E. coli* XL1-Blue MRA (P2) bacteria solution at 37°C for 15 minutes. Then, 6.5 ml of warm (48°C) NZCYM medium (Sambrook *et al.*, 1989) containing 0.7 % agarose were added to the above culture. The mixture was plated onto 150-mm NZCYM plates.

For library screening, the plates were incubated at 37°C for 16 hours. For preparation of λFIX II phage DNA, the plates were incubated at 39°C for 6 to 8 hours.

4.3.3 Titration of λFIX II Phage

λFIX II phage were diluted in SM buffer (0.1 M sodium chloride, 10 m M magnesium sulfate, 50 mM Tris-Cl pH 7.5, 0.01% gelatin) in a series and, the phage were grown on plates as described in Section 4.3.2. The plates were incubated at 37°C for 16 hours. Plaques on plates were counted and the phage titer was determined.

4.3.4 Library Screening

For the primary screening, approximately $1x10^6$ phage were plated onto 150-mm plates, with about $5x10^4$ phage for each plate. For the secondary and the tertiary screening, 50 to 200 phage were plated onto each 85-mm plate. The plates were cooled down at 4°C for at least two hours. Hybond-N nylon membranes (Amersham) were used for transferring nucleic acid from plaques on plates. Duplicate membranes were lifted from each plate. Waterproof ink was used to mark the nylon membranes and plates for the orientation. The membranes were denatured for 3 minutes on the Whatman 3MM paper pre-soaked with 0.5 M sodium hydroxide, 1.5 M sodium chloride, neutralized for 3 minutes on 3MM paper pre-soaked with 0.5 M Tris-Cl pH 8.0, 1.5 M sodium chloride, submerged in 2x SSC (20x SSC: 3 M sodium chloride, 0.3 M tri-sodium citrate, pH 7.0) for 15 minutes with gentle shaking and, baked overnight (16 hours) at 80°C. The membranes were hybridized with labeled probes as described in Section 4.7.

4.4 Preparation of λFIX II Phage DNA

The λFIX II phage were grown on 150-mm NZCYM plates as described in Section 4.3.2. About 15 ml of SM buffer were added onto the plate and, the plate was incubated overnight at 4°C with gentle shaking. The recovered phage solution from the plate was centrifuged and, the supernatant was used to prepare the λFIX II phage DNA using the Wizard Lambda Prep Kit (Promega) following the manufacturer's instructions. Briefly, 10 ml of the above supernatant were treated with nuclease mix and, the phage were precipitated with phage precipitant which was supplied with the kit. Then, the pellet was dissolved in phage buffer, and the purification resin was added. The mixture was applied onto a minicolumn which was supplied with the kit, and washed with 80% isopropanol. The phage DNA was eluted from the resin with 100 µl of TE buffer pH 8.0 (10 mM Tris-Cl pH 8.0, 1 mM disodium ethylenediaminetetra-acetate [EDTA] pH 8.0).

4.5 Restriction Mapping of the Insert DNA in λFIX II Phage Clones

The λFIX II phage DNA was digested with *Not* I restriction endonuclease to show the insert sizes, since *Not* I released the inserts. The inserts of λFIX II phage clones were flanked by bacteriophage T3 and T7 promoters. In order to choose proper enzymes for the mapping of the genomic clones, the *Not* I digested λFIX II phage DNA was further digested with several

different restriction endonucleases individually. The restriction mapping of insert DNA in λFIX II phage clones was carried out using the FLASH® Nonradioactive Gene Mapping Kit (Stratagene) and following the manufacture's instruction. Briefly, the *Not* I digested λFIX II phage DNA was partially digested with appropriate restriction endonucleases and analyzed on agarose gels. Those gels were blotted onto Hybond-N nylon membranes (Amersham). The membranes were hybridized respectively with T3 and T7 alkaline phosphatase-conjugated probes which were supplied with the kit, and incubated with the chemiluminescent substrate. The membranes were then exposed to X-ray films. The restriction maps of the inserts of λFIX II phage clones were determined by the partial and the complete digestion ladders on the autoradiograms.

4.6 Subcloning of the Insert DNA in λFIX II Phage Clones

The restriction fragments of the insert DNA in λFIX II phage clones were gel purified and cloned into the multiple cloning site of the plasmid vector pBluescript II KS (+) (Stratagene). The *E. coli* XL1-Blue (Stratagene) strain was used for plasmid transformation. All manipulations including ligation, competent cell preparation, transformation and plasmid DNA isolation were performed using standard procedures (Sambrook *et al.* 1989).

4.7 Southern Blotting and Hybridization Analyses

The agarose gels with DNA fragments separated by electrophoresis were soaked in 0.5 M sodium hydroxide, 1.5 M sodium chloride for 30 minutes. Then, those DNA fragments were transferred to Hybond-N nylon membranes (Amersham) overnight using 0.25 M sodium hydroxide, 1.5 M sodium chloride as transfer solution. The membranes were UV-cross-linked on an UV transilluminator (UVP, Inc.) for 5 minutes. The membranes carrying immobilized DNA fragments were prehybridized in the solution of 5x SSPE (20x SSPE: 3 M sodium chloride, 0.2 M sodium dihydrogen orthophosphate, 20 mM EDTA, pH 7.4), 50% formamide, 5x Denhardts' solution (50x Denhardts' solution: in 500 ml containing 5 g of Ficoll, 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin), 0.5% sodium dodecyl sulfate (SDS), 7.5% dextran sulfate, and 50 μg/ml of salmon sperm DNA at 42°C for 2 to 3 hours. Hybridization was then carried out in the same solution with ^{32}P-labeled DNA probes overnight at 42°C. The membranes were washed once in 1x SSPE, 0.1% SDS at 50°C for 10 minutes, and then, several times in 0.1x SSPE, 0.1% SDS from 50°C to a maximum of 65°C. The membranes were exposed to X-ray films with intensifying screens at -70°C from several hours to overnight or even longer times depending on the counts on the membranes.

4.8 DNA Sequencing

The DNA templates used for sequencing were plasmids which were the subclones of mouse *ATBF1* gene in pBluescript II KS (+) plasmid vector.

4.8.1 Automated Sequencing

Nucleotide sequences of exon and exon-intron junctions were determined using the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) with standard sequencing primers and custom-made primers following the manufacturer's instruction and using a DNA Thermal Cycler (Perkin-Elmer Cetus). Briefly, the *Taq* DNA polymerase was used to elongate DNA along the templates at 60°C, and the dye-labeled dideoxynucleotides were used to terminate the elongation reactions. The extension products were purified through Centri-Sep spin columns (Princeton Separation). The samples were analyzed using an Applied Biosystems 373A DNA Sequencer at the University Core DNA Services, University of Calgary.

4.8.2 Manual Sequencing

Manual sequencing was performed in the primer extension experiment using the ^{T7}Sequencing™ Kit (Pharmacia) following the manufacture's instruction. The genomic clones containing exon 1 (specific for *ATBF1-B* transcript) and exon 2 (specific for *ATBF1-A* transcript) along with their 5'-flanking regions were used as templates. T7 DNA polymerase was used to

elongate DNA along the templates with the custom-made primers (the same primers used in the primer extension experiment) in the presence of α-^{35}S-dATP. The reaction mixture was divided into four and terminated by adding dideoxynucleotides. The chain-terminated fragments were separated on a 9% sequencing gel along with the primer extension products as described in Section 4.11.2. The gel was dried and exposed to X-ray film.

4.9 Polymerase Chain Reaction

The polymerase chain reaction (PCR) was performed in a DNA Thermal Cycler (Perkin-Elmer Cetus). For each reaction, 4 ng of plasmid, 100 ng of each specific primer, and 1.5 U of *Taq* DNA polymerase were added to a final volume of 50 µl containing 200 µM dNTP, 50 mM potassium chloride, 10 mM Tris-Cl pH 8.3, and 1.5 mM magnesium chloride. Usually, 20 cycles were carried out at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes. The PCR products were analyzed on agarose gels.

4.10 DNA Size Marker Labeling

The ^{32}P-labeled DNA size marker was used in "Section 4.11 Primer Extension" and "Section 4.12 Ribonuclease Protection Assay" to estimate the sizes of DNA (primer extension products) and the sizes of RNA (ribonuclease protection assay products). Fifty ng of *Hpa* II digested pBluescript II KS (+) were added to a final volume of each 10 µl reaction containing 90 mM HEPES pH

6.6, 10 mM magnesium chloride, 5 mM dithiothreitol (DTT), 0.5 mM (dATP, dGTP, dTTP) mix, 20 μCi α-^{32}P-dCTP, and 1 U of Klenow DNA polymerase. The labeling reactions were carried out at 30°C for 30 minutes.

4.11 Primer Extension

The primer extensions were performed using combined protocols, partly as described by Ausubel *et al.* (1996) for primer labeling and, partly as described in the manufacturer's instruction (Life Technologies) for using its reverse transcriptase in the primer extension reaction.

4.11.1 Primer Labeling

The primer-labeling reaction was performed as described by Ausubel *et al.* (1996). Approximately 100 ng synthetic primer were end-labeled with 30 μCi γ-^{32}P-ATP and 15 U T4 polynucleotide kinase in 10 μl reaction mix containing 50 mM Tris-Cl pH 7.5, 10 mM magnesium chloride, 15 mM DTT, and 0.1 mM spermidine. The reaction was incubated for 1 hour at 37°C and the labeled primer was purified using Centri-Sep spin columns (Princeton Separations). The total counts of the labeled primer were determined to be approximately 6 x 10^6 cpm using a Beckman LS 5000CE scintillation counter.

4.11.2 Primer Extension Reaction

The Life Technologies' reverse transcriptase was used in the primer extension reaction and the manufacturer's instructions were followed. Five µg of total cellular RNA and 0.2 pmoles of a labeled specific primer were mixed and heated to 70°C for 10 minutes. Then, the mixture was quickly chilled on ice, and the following reagents were added: 4 µl of 5x RT buffer (Life Technologies), 2 µl of 0.1 M DTT, 1 µl of 10 mM dNTP, and 1 µl (20 to 40 U) of rRNasin® ribonuclease inhibitor (Promega). The final volume was adjusted to 19 µl with distilled water. The mixture was incubated at 42°C for 2 minutes. Then, 1 µl of 200 U/µl Super Script RNase H^- Reverse Transcriptase (Life Technologies) was added and the reaction mixture was incubated at 42°C for 30 minutes. Then, 130 µl of TE buffer pH 8.0 and 15 µl of 3 M sodium acetate pH 5.2 were added, and the mixture was extracted with 150 µl of phenol/chloroform/isoamyl alcohol (v/v/v: 25/24/1). The DNA was precipitated by adding 300 µl of 100% ethanol, washed with 70% ethanol, and resuspended in 5 µl loading dye (0.05% bromphenol blue, 0.05% xylene cyanol, and 20 mM EDTA pH 8.0 in deionized formamide). Samples were heated at 65°C for 5 minutes, chilled on ice, and then subjected to electrophoresis on a 9% sequencing gel along with the manual sequencing products as described in Section 4.8.2. Gels were dried and exposed to X-ray film overnight.

4.12 Ribonuclease Protection Assay

The ribonuclease protection assays were performed as described by Ausubel *et al.* (1996).

4.12.1 Template DNA Preparation for RNA Probe Generation

For the detection of antisense-*ATBF1* RNA expression in transfected P19 cells by ribonuclease protection assays, pα-ATBF1-346 recombinant plasmid was constructed and used as a DNA template to generate the RNA probe (Figure 4). For the detection of endogenous *ATBF1* expression in P19 cells upon the induction with retinoic acid, pATBF1-151 recombinant plasmid previously constructed in our laboratory (Miura *et al.*, 1995) was used as a DNA template to generate the RNA probe (Figure 5). To normalize the amount of RNA prepared from different cell samples, pGAP150 recombinant plasmid containing mouse *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) cDNA was constructed and used as DNA template to generate the RNA probe (Figure 6). The expression of *MASH1* (mammalian achaete-scute homolog 1) and *GAD67* (glutamic acid decarboxylase with molecular weight of 67,000 Dalton) genes are used as molecular markers during the neuronal differentiation of P19 cells. The recombinant plasmids pMASH1-227 (Figure 7) and pGAD67-257 (Figure 8) were used as DNA templates to generate RNA probes. pMASH1-227 and pGAD67-257 contain *MASH1* and *GAD67* cDNA, respectively, and were kindly provided by Dr. Gerard Bain.

Figure 4. pα-ATBF1-346

A 346-base pair human *ATBF1* cDNA fragment from nucleotides 3263-3608 was cloned into the multiple cloning site (MCS) of pBluescript II KS (+) (Stratagene). Digestion with *Bsa* AI would produce a linear template for the generation of a sense probe using T3 RNA polymerase. This RNA probe consists with 346 nucleotides of the human *ATBF1* sequence and 424 nucleotides of the vector sequence. The RNA probe was used to determine the antisense-*ATBF1* RNA expression (a 346-nucleotide product) in the ribonuclease protection assay. The arrows in the figure indicate the orientations of the DNA sequences.

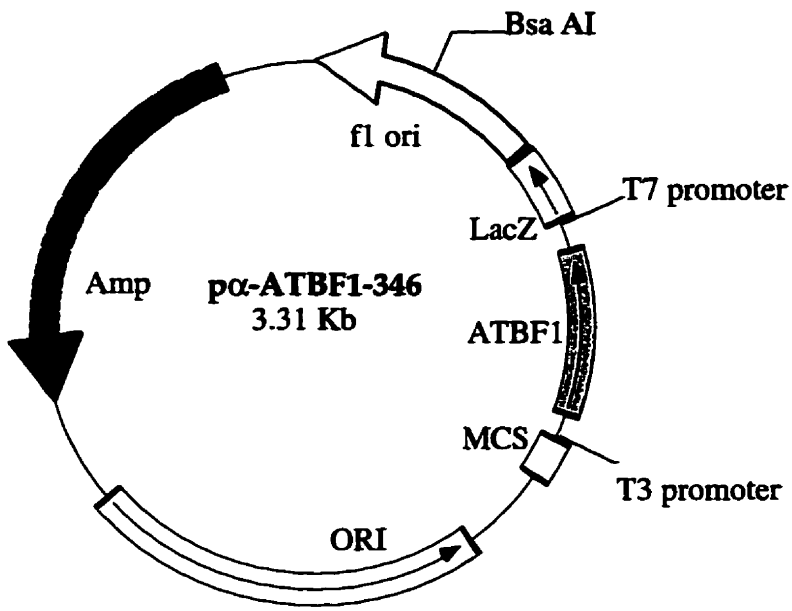

Figure 4. pα-ATBF1-346.

Figure 5. pATBF1-151

A 151-base pair mouse *ATBF1* cDNA fragment from nucleotides 3413-3563 was cloned into the multiple cloning site (MCS) of pBluescript II KS (+) (Miura *et al.*, 1996). This cDNA fragment is located at the *ATBF1-A* and *ATBF1-B* alternative splicing region. Digestion with *Bam* HI produces a template for the generation of an antisense probe using T3 RNA polymerase. This RNA probe consists with 151 nucleotides of the mouse *ATBF1* sequence and 77 nucleotides of the vector sequence. The RNA probe was used to determine the *ATBF1-A* mRNA expression (a 151 nucleotide product), and the *ATBF1-B* mRNA expression (a 111-nucleotide product) in ribonuclease protection assays. The arrows in the figure indicate the orientations of the DNA sequences.

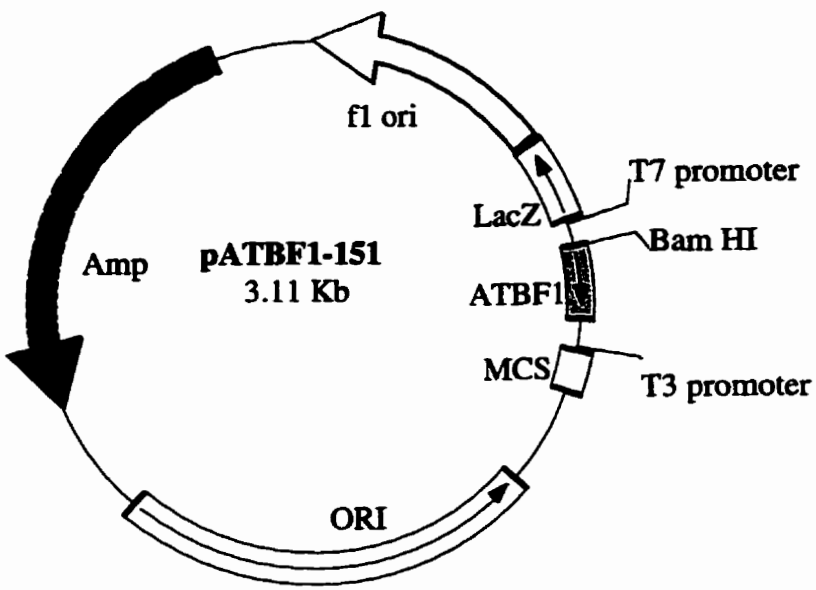

Figure 5. pATBF1-151.

Figure 6. pGAP150

A 150-base pair mouse *GAPDH* cDNA fragment from nucleotides 88-237 was cloned into the multiple cloning site (MCS) of pBluescript II KS (+). Digestion with *Hind* III would produce a template for the generation of an antisense probe using T7 RNA polymerase. This RNA probe consists with 150 nucleotides of the mouse *GAPDH* sequence and 200 nucleotides of the vector sequence and the linker sequence which is at the 5'-end of the *GAPDH* cDNA. The RNA probe was used to normalize the amount of RNA prepared from different cell samples by determining the *GAPDH* mRNA expression (a 150-nucleotide product) in the ribonuclease protection assay. The arrows in the figure indicate the orientations of the DNA sequences.

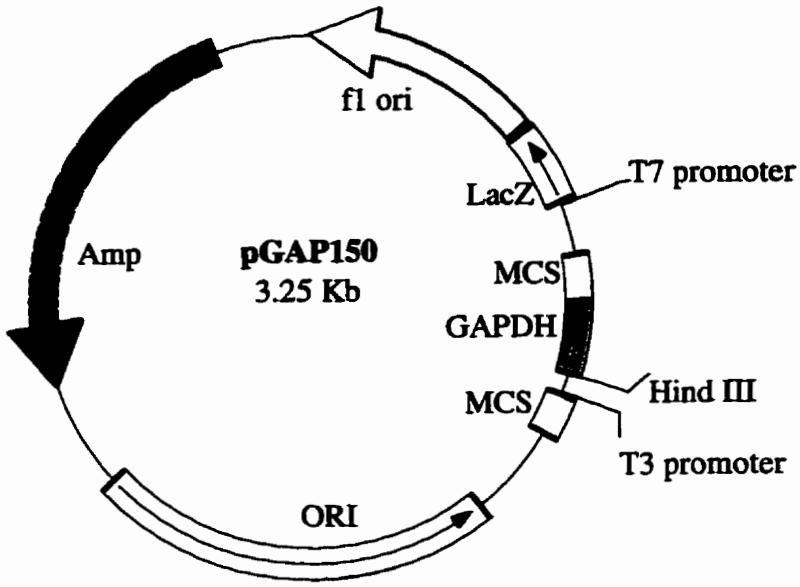

Figure 6. pGAP150.

Figure 7. pMASH1-277

A 277-base pair mouse *MASH1* cDNA fragment from nucleotides 1175-1451 was cloned into the multiple cloning site (MCS) of pBluescript II KS (+). This recombinant plasmid was kindly provided by Dr. Gerard Bain. Digestion with *Eco* RI would produce a template for the generation of an antisense probe using T3 RNA polymerase. This RNA probe consists with 277 nucleotides of the mouse *MASH1* sequence and 85 nucleotides of the vector sequence. The RNA probe was used to determine the *MASH1* mRNA expression (a 277-nucleotide product) in the ribonuclease protection assay. The arrows in the figure indicate the orientations of the DNA sequences.

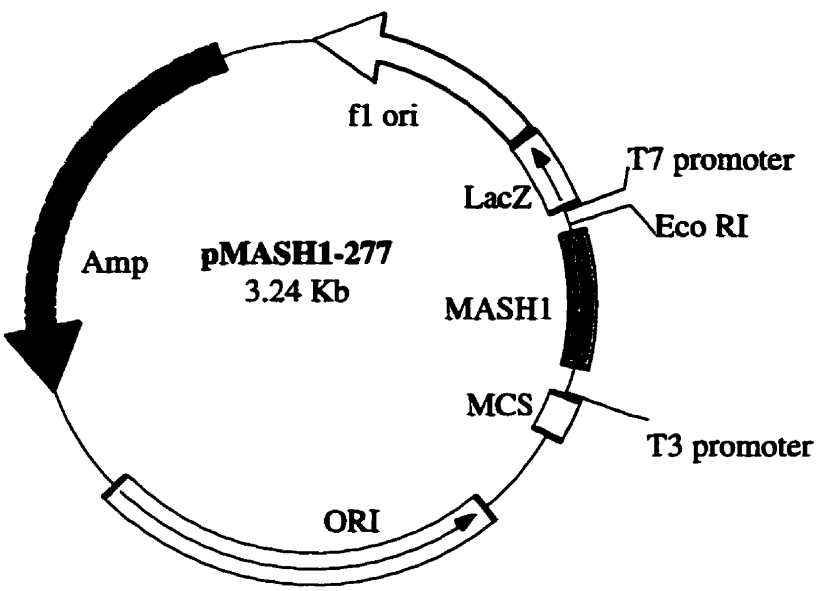

Figure 7. pMASH1-277.

Figure 8. pGAD67-257

A 257-base pair mouse *GAD67* cDNA fragment from nucleotides 163-419 was cloned into the multiple cloning site (MCS) of pBluescript II KS (+). This recombinant plasmid was kindly provided by Dr. Gerard Bain. Digestion with *Bam* H1 produces a template for the generation of an antisense probe using T7 RNA polymerase. This RNA probe consists with 257 nucleotides of the mouse *GAD67* sequence and 70 nucleotides of the vector sequence. The RNA probe was used to determine the *GAD67* mRNA expression (a 257-nucleotide product) in the ribonuclease protection assay. The arrows in the figure indicate the orientations of the DNA sequences.

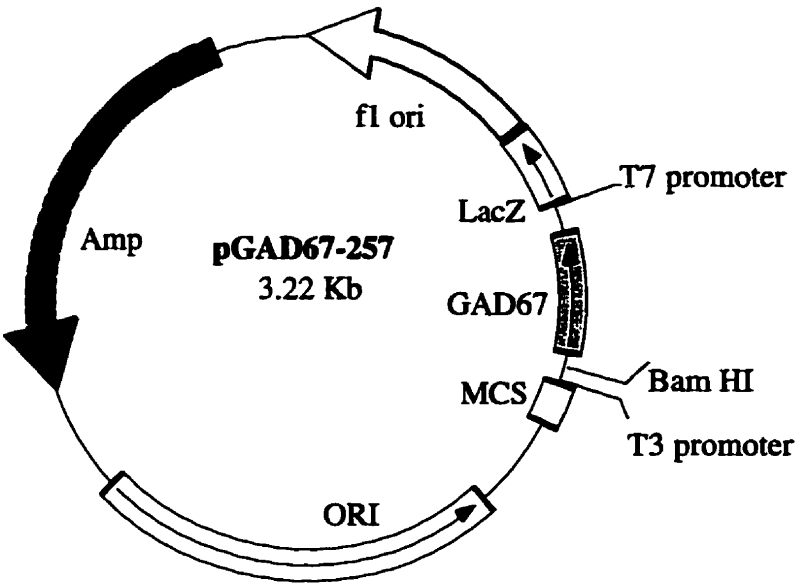

Figure 8. pGAD67-257.

4.12.2 Riboprobe Generation

For each reaction, the following reagents were mixed sequentially: 4 µl of 5x transcription buffer (200 mM Tris-Cl pH 8.0, 40 mM magnesium chloride, 10 mM spermidine, 250 mM sodium chloride), 1 µl of 200 mM DTT, 2 µl of 4 mM ATP, GTP, and UTP mix, 10 µl of α-^{32}P-CTP (10 mCi/ml, 400 to 800 Ci/mmole), 1 µl (20 to 40 U) of rRNasin® ribonuclease inhibitor (Promega), 1 µl of 0.5 mg/ml template DNA, and 1 µl (5 to 10 U) bacteriophage T3 or T7 RNA polymerase (Boehringer Mannheim). The reaction mixture was incubated at 37°C for 30 to 60 minutes. Five µg (10 U) RNase-free DNase I was added and, the mixture was incubated at 37°C for 15 minutes. Two µl of 10 mg/ml yeast tRNA (Boehringer Mannheim) was added and the final volume was adjusted by adding water to 50 µl. The reaction was extracted with phenol/chloroform/isoamyl alcohol (v/v/v: 25/24/1) and then with chloroform/isoamyl alcohol (v/v: 24/1). The aqueous phase was applied onto a Sephadex G-50 (Pharmacia) spin column and the total counts of the flowthrough was about $5x10^7$ cpm. The RNA probe was precipitated with 100% ethanol, washed with 70% ethanol, and redissolved in hybridization buffer (80% formamide containing 40 mM PIPES pH 6.4, 0.4 M sodium chloride, 1 mM EDTA pH 8.0) to make the final concentration of the probe to $5x10^5$ cpm/µl.

4.12.3 Ribonuclease Protection Assay Reaction

Ten µg of total cellular RNA in 29 µl of hybridization buffer and 1 µl of probe RNA ($5x10^5$ cpm) were mixed, denatured at 85°C for 5 minutes, rapidly transferred to 45°C, and hybridized overnight. Then, 350 µl of ribonuclease digestion buffer (10 mM Tris-Cl pH 7.5, 300 mM sodium chloride, 5 mM EDTA pH 8.0, 40 µg/ml of ribonuclease A, 700 U/ml of ribonuclease T1) was added to the above reaction mixture and incubated at 30°C for 30 to 60 minutes. Ten µl of 20% SDS and 2.5 µl of 20 mg/ml proteinase K were added and the mixture was incubated at 37°C for 15 minutes. The mixture was extracted with phenol/chloroform/isoamyl alcohol (v/v/v: 25/24/1) and the RNA was precipitated by adding 1 µl of 10 mg/ml yeast tRNA and 1 ml of 100% ethanol to the aqueous phase. The RNA was collected by centrifugation, redissolved in 5 µl of RNA loading buffer (80% formamide containing 1 mM EDTA pH 8.0, 0.1% bromphenol blue, 0.1% xylene cyanol) and denatured at 85°C for 3 minutes. Samples were subjected to electrophoresis on a 6% sequencing gel along with the DNA size marker as described in Section 4.10. The gel was dried and exposed to X-ray film overnight.

4.13 Cell Culture

XBF mouse teratoma cells (Gierthy and Crane, 1985) were grown in D-MEM (Dulbecco's modified Eagle medium, Life Technologies) with 20% fetal bovine serum, 100 units/ml penicillin G sodium, and 100 µg/ml streptomycin

sulfate. F9 mouse embryonal carcinoma cells were grown in D-MEM with 15% fetal bovine serum. P19 mouse embryonal carcinoma cells (kindly provided by Dr. M.W. McBurney, Department of Medicine, University of Ottawa) were grown in α-MEM (minimum essential medium α medium, Life Technologies) with 10% fetal bovine serum. Cells were grown at 37°C in a 5% CO_2 incubator with humidity. The two-step procedure was used to induce the neuronal differentiation of P19 cells. P19 cells were first allowed to form aggregates in bacteriological grade Petri dishes for 4 days in the presence of 0.5 μM retinoic acid and then transferred to tissue culture grade Petri dishes without retinoic acid (Jones-Villeneuve *et al.*, 1982). The cell aggregates attach to the surface of the dishes and then initiate the neuronal differentiation of P19 cells.

4.14 Construction of Plasmid Expression Vectors for Transfection

pPOP expression vector (McBurney *et al.*, 1994a) was used for the P19 cell transfection (kindly provided by Dr. M.W. McBurney). The pPOP expression vector contains several regions of the *pgk-1* (phosphoglycerate kinase) gene and a multiple cloning site (Figure 9). I obtained the full lengths of *ATBF1-A* and *ATBF1-B* cDNA by releasing the inserts from two expression vectors previously constructed in our laboratory. The full length of *ATBF1-A* cDNA was released from the expression vector pTSME (Miura and Tamaoki, unpublished) by *Sal* I restriction endonuclease digestion. The full length of *ATBF1-B* cDNA was released from the expression vector pcATBF1-B (Yasuda

Figure 9. pPOP Expression Vector

pPOP vector contains the promoter (within the region of PGK 5'), intragenic regions (within the regions of PGK 5' and PGK 3'), and the 3'-end (within the region of PGK 3') of the *pgk-1* gene (McBurney *et al.*, 1994b). This vector was kindly provided by Dr. M.W. McBurney. The pPOP expression vector was used to express sense-*ATBF1-A*, sense-*ATBF1-B*, and antisense-*ATBF1* in P19 cells.

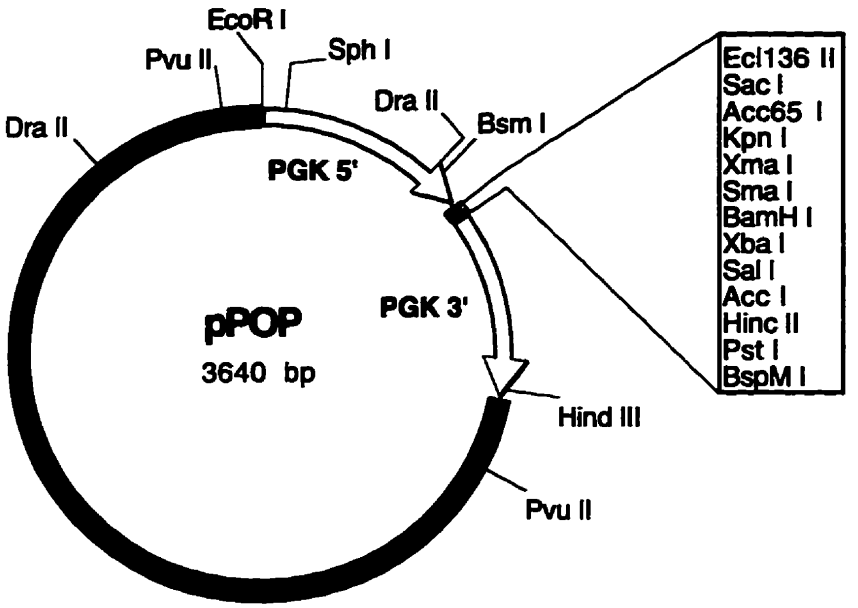

Figure 9. pPOP Expression Vector.

et al., 1994) by *Hind* III restriction endonuclease digestion. They were respectively cloned into the *Sal* I site (*ATBF1-A*) and *Hind* III site (*ATBF1-B*) of the pPOP expression vector. Three expression vectors were constructed, pATBF1-A (expressing sense-*ATBF1-A*), pATBF1-B (expressing sense-*ATBF1-B*), and pα-ATBF1 (expressing antisense-*ATBF1-A*).

Bacteria transformed with the above recombinant pPOP expression vectors containing *ATBF1-A* and *ATBF1-B* inserts were screened using colony hybridization techniques. The Hybond-N nylon membranes (Amersham) were used to lift the bacteria colonies from plates. The colonies on membranes were then lysed on the Whatman 3MM paper pre-soaked with 10% SDS for 3 minutes, denatured on 3MM paper pre-soaked with 0.5 M sodium hydroxide, 1.5 M sodium chloride for 5 minutes, and neutralized on 3MM paper pre-soaked with 0.5 M Tris-Cl pH 7.4, 1.5 M sodium chloride for 5 minutes. Finally, the membranes were submerged in 2x SSC with gentle shaking for 15 minutes. The membranes were baked overnight at 80°C. Before hybridization, the membranes were incubated at 50°C for 30 minutes in prewashing solution containing 5x SSC, 0.5% SDS, and 1 mM EDTA pH 8.0. The membranes were then hybridized with ^{32}P-labeled *ATBF1* cDNA as described in Section 4.2 and in Section 4.7.

The orientations of the inserts of these recombinant expression vectors were determined by restriction endonuclease digestion. The pPOP vectors with the sense-*ATBF1-A* and *ATBF1-B* cDNA inserts (according to the *pgk-1*

promoter in pPOP) were named pATBF1-A (expressing sense-*ATBF1-A*) and pATBF1-B (expressing sense-*ATBF1-B*) respectively. The pPOP vector with the antisense-*ATBF1-A* cDNA insert (according to the *pgk-1* promoter in pPOP) was named pα-ATBF1 (expressing antisense-*ATBF1*).

4.15 Preparation of Expression Vectors for Transfection

The bacteria carrying expression vectors were grown in 500 ml of Terrific broth (Sambrook *et al.*, 1989) with 50 μg/ml of ampicillin overnight at 37°C with shaking. The bacterial pellets were collected by centrifugation at 3,000g for 10 minutes and were resuspended in 10 ml of GTE buffer (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0). Fifteen ml of freshly prepared 0.2 M sodium hydroxide/1% SDS were added and, the mixture was incubated on ice for 5 minutes. Then, 15 ml of 3.0 M potassium acetate pH 4.8 were added, mixed, and incubated on ice for 5 minutes. The cellular debris were removed by centrifugation at 10,000g, room temperature for 10 minutes. The supernatant was extracted with phenol/chloroform/isoamyl alcohol (v/v/v: 25/24/1), and with chloroform/isoamyl alcohol (v/v/v: 24/1). An equal volume of isopropanol was added and the DNA was centrifuged at 10,000g, room temperature for 15 minutes. The pellet was washed with 70% ethanol, resuspended in 6 ml of water, 1.5 ml of 4 M sodium chloride, and 7.5 ml of 13% polyethylene glycol-8000 (PEG-8000), and incubated on ice for 20 minutes. The DNA was collected by centrifugation at 10,000g, 4°C for 15 minutes. The pellet

was resuspended in 7.6 ml of TE buffer pH 8.0 containing 8.46 g of cesium chloride. The solution was loaded into an ultracentrifuge tube with 200 µl of 10 mg/ml ethidium bromide solution. The tubes were placed in a Beckman NVT65 rotor and centrifuged at 342,317g (60,000 rpm), 18°C for 18 hours using a Beckman L8-80M ultracentrifuge. The lower band containing the supercoiled plasmid was recovered using a 18 G needle. The plasmid DNA was ultracentrifuged twice. The DNA solution was extracted with an equal volume of water-saturated-1-butanol to remove ethidium bromide, and diluted in 2 volumes of water. Then, the DNA was precipitated by adding 2 volumes of ethanol and incubating at 4°C for 15 minutes. The DNA was centrifuged at 10,000g, 4°C for 15 minutes and washed with 70% ethanol. The DNA pellet was resuspended in TE buffer pH 8.0 and quantitated using UV spectrophotometer (1 OD_{260} = 50 µg DNA/ml) (Sambrook *et al.*, 1989).

4.16 Transfection of P19 Cells

The transfection of P19 cells was performed using the BES (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid) buffered calcium phosphate procedure as described by Chen and Okayama (1987) with some modifications (McBurney *et al.*, 1994a). Approximately, 10^6 cells were plated onto an 85-mm tissue culture dish with 10 ml of α-MEM medium containing 10% fetal bovine serum and incubated at 37°C with 5% CO_2 for 16 hours. The following components, 1 ml of 2x BES-buffered saline (pH 6.88), 1 ml of 0.25 M calcium

chloride, 20 μg of expression vector (pATBF1-A, pATBF1-B, pα-ATBF1, or pPOP), and 4 μg of expression vector pMC1neoPolyA (carrying the neomycin-resistance coding sequence, Stratagene), were added to the above cultured cells. The DNA-calcium phosphate co-precipitate was incubated with the cells at 35°C with 3% CO_2 for 8 hours and the medium was replaced with a fresh medium without the DNA-calcium phosphate. The incubation was continued at 37°C with 5% CO_2 for 40 hours, and then, the cells were harvested by trypsinization. An aliquot of 10^6 cells from the culture was plated onto an 85-mm culture dish in 10 ml of the medium supplemented with 300 μg/ml of G418 (Life Technologies) and incubated at 37°C with 5% CO_2 for 4 days. The cultures were then incubated in the medium supplemented with 150 μg/ml of G418 for about 7 days to allow colony formation. Colonies were picked up using small pieces of Whatman 3MM papers pre-soaked with trypsin-EDTA (Life Technologies).

4.17 Total Cellular RNA Preparation from Cultured Mammalian Cells

The total RNA from cell cultures was prepared using the guanidinium method with a cesium chloride step gradient to separate the RNA from other cellular macromolecules. The RNA preparation was essentially performed as described by Sambrook *et al.* (1989). For one preparation, about 10^8 cells harvested from culture dishes were washed with phosphate-buffered saline (PBS) (Sambrook *et al.*, 1989) and homogenized in 4 M guanidinium

thiocyanate solution with 1% β-mercaptoethanol using a Polytron homogenizer (Kinematica AG). N-Lauroyl sarcosine was added to the homogenate to a final concentration of 0.5%. Cesium chloride was added to the homogenate and the mixture was centrifuged at 210,053g (35,000 rpm), 18°C for 16 hours in a Beckman SW41TI rotor using a Beckman L8-80M ultracentrifuge. RNA pellets were dissolved, extracted with phenol/chloroform/isoamyl alcohol (v/v/v: 25/24/1) and then with chloroform/isoamyl alcohol (v/v: 24/1), and precipitated with 100% ethanol. The RNA samples were dissolved in water and quantitated using UV spectrophotometer (1 OD_{260} = 40 µg RNA/ml) (Sambrook *et al.*, 1989).

4.18 Immunoperoxidase Cell Staining

The imunostaining was carried out using the mouse ABC Immunostain™ System kit (Santa Cruz Biotechnology) following the manufacturer's instructions. Cells were grown in 24-well plates for 24 hours before staining. All staining procedures were done at room temperature. Cells were fixed sequentially in 3.7% formaldehyde/PBS, 0.5% Triton X-100/PBS, and 0.05% Twin 20/PBS. Then, 0.1% hydrogen peroxide/PBS was added to quench endogenous peroxides activity, and 1.5% normal blocking serum/PBS was added to suppress non-specific binding of IgG. The primary antibody, Rat-401 monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa), was added to the cells and incubated at room temperature

for 30 minutes. This monoclonal antibody obtained in the format of the hybridoma culture supernatant was diluted twenty times in 1.0% blocking serum/PBS. The cells were then incubated at room temperature for 30 minutes sequentially with the biotin-conjugated secondary antibody and the avidin biotin enzyme reagent. Finally, the cells on plates were incubated in DAB (diaminobenzidene) solution, and examined under an Olympus IM inverted microscope (100x magnification) until the desired stain intensity developed.

4.19 Determination of the Transient Transfection Efficiency of P19 Cells

The expression vector pCH110 (Pharmacia) was used to determine the transient transfection efficiency of P19 cells. The pCH110 contains the *E. coli* *LacZ* gene which encodes the β-galactosidase. The transfection was performed as described in Section 4.16. After being cultured for 24 hours following the adding of DNA, P19 cells were fixed in PBS containing 2.0% formaldehyde and 0.2% glutaraldehyde, and were permeabilized in 0.5% Triton X-100/PBS. The cells were then incubated at 37°C in the staining solution (PBS solution containing 1 mg/ml of X-gal, 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, and 2 mM magnesium chloride), and examined under the Olympus IM inverted microscope (100x magnification) until desired stain intensity developed. The stained and unstained cells on plates were counted, and the transient transfection efficiency was determined.

CHAPTER V: RESULTS

5.1 Isolation and Characterization of the Mouse *ATBF1* Gene

The mouse *ATBF1-A* cDNA was cloned in our laboratory (Ido *et al.*, 1996). The *ATBF1* gene was not cloned. Using the human *ATBF1* cDNAs as probes, several 5′ exons of the human *ATBF1* gene have been identified (Miura *et al.*, 1995), but other exons have not been analyzed. The exact number of exons or the size of the gene are not known. In order to further investigate the structure and function of *ATBF1*, I cloned the mouse *ATBF1* gene. With the knowledge of the mouse *ATBF1* gene, we can generate *ATBF1* deficient mice through homologous recombination to study the function of the *ATBF1* gene *in vivo*. Furthermore, after identifying the mouse *ATBF1* gene promoters, we can fuse the promoters to reporter genes to generate transgenic mice to study the expression patterns of the *ATBF1* gene *in vivo*.

5.1.1 cDNA Probe Preparation

Six cDNA clones covering the full length of the mouse *ATBF1-A* cDNA are available in our laboratory (Ido *et al.*, 1996). The restriction endonuclease fragments of the insert DNAs of the mouse *ATBF1-A* cDNA clones were used as probes in the library screening (Figure 10).

Figure 10. Map of the Mouse *ATBF1-A* cDNA Restriction Fragments Used as Probes

Six cDNA clones covering the full length of the mouse *ATBF1-A* cDNA are shown in the figure (Ido *et al.*, 1996). The names of those clones are indicated along with the inserts starting and end points with respect to the mouse *ATBF1-A* cDNA. The inserts of the mouse *ATBF1-A* cDNA clones were digested with restriction endonucleases to generate cDNA fragments used as probes for screening a genomic library. The digestion sites on six mouse *ATBF1-A* cDNA clones along with the names of restriction endonucleases are indicated. The following is the list of abbreviations of those restriction endonuclease names used in this figure.

A: *Acc* I; B1: *Bsm* I; B2: *Bgl* I; B3: *Bst* NI; C: *Cla* I; E: *Eco* RI; H1: *Hind* III; H2: *Hae* II; H3: *Hinc* II; S1: *Stu* I; S2: *Sal* I; T: *Taq* I.

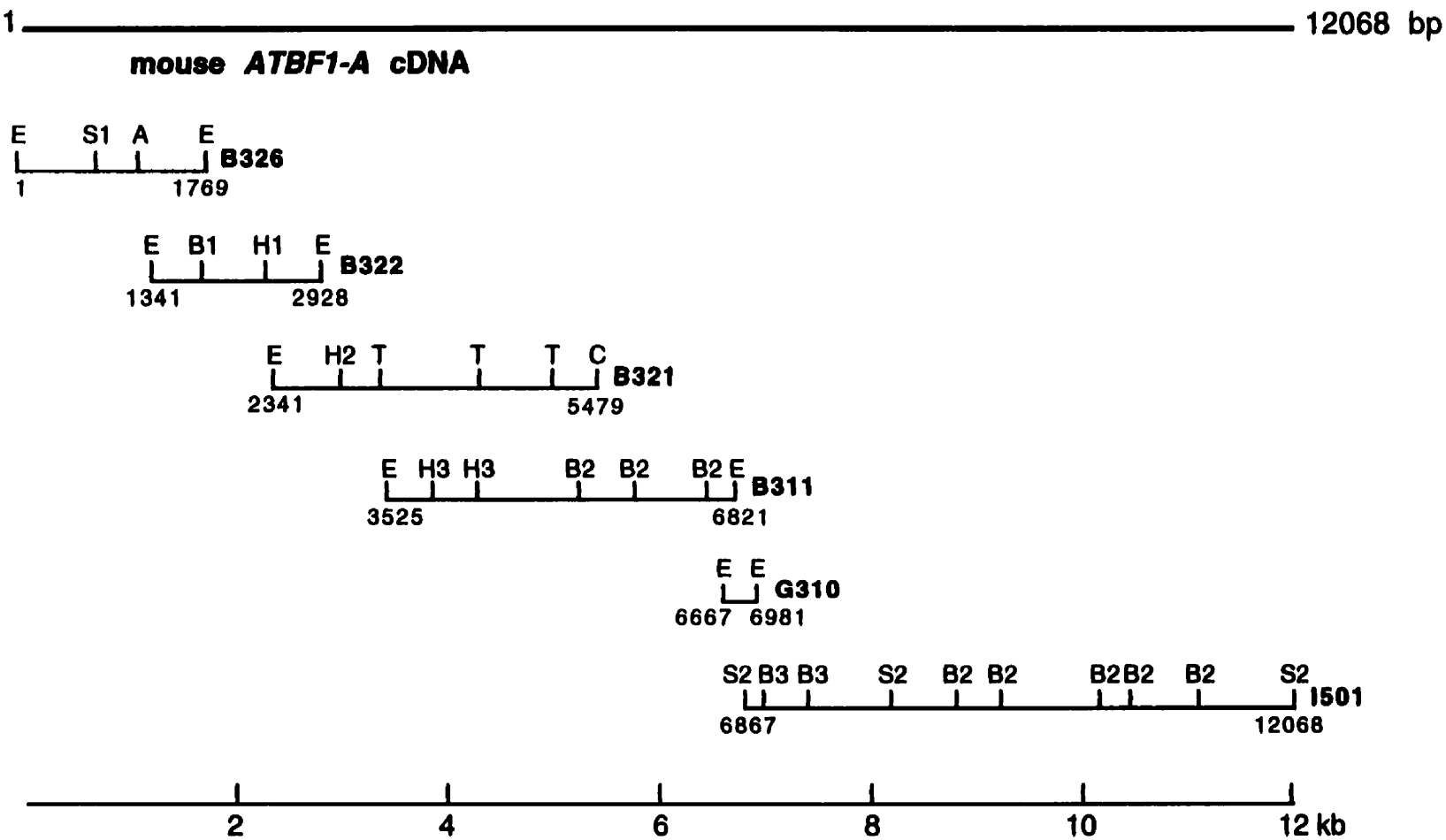

Figure 10. Map of the Mouse *ATBF1-A* cDNA Restriction Fragments Used as Probes.

5.1.2 Genomic DNA Probe Preparation

For screening those genomic clones covering only the intron regions of the mouse *ATBF1* gene, the inserts of the intron subclones were used as probes (as described below, Section 5.1.3) or the inserts were further subcloned if they were too long.

5.1.3 Library Screening and Genomic Clone Characterization

A 129SV mouse genomic library in λFIX II vector (Stratagene) was screened using the ^{32}P-labeled DNA probes as described above. More than 100 positive clones were isolated from the genomic library and were characterized as described below. Those clones were first analyzed by Southern hybridization using different probes to estimate their physical positions in the mouse *ATBF1* gene. The candidate clones, which were located in the pertinent genomic regions, were subcloned and further characterized by Southern hybridization, polymerase chain reaction and DNA sequencing. Here, I took one genomic clone named λ5 as an example to illustrate the cloning and characterization of the mouse *ATBF1* gene.

The clone λ5 was isolated from the mouse genomic library using the cDNA probe located at the 5'-end of the mouse *ATBF1-A* cDNA, that is, the 5'-end of the E-S1-fragment of the cDNA clone B326 insert (Figure 10 shows the detailed location of this cDNA probe.). The phage DNA of clone λ5 was

prepared and the insert of clone λ5 was released by digestion with *Not* I restriction endonuclease. The insert was flanked by bacteriophage T3 and T7 promoters and, these promoter sites could be hybridized with T3 and T7 probes and thus could be used to map the insert. The insert was first digested with several restriction endonucleases to identify appropriate enzymes for subcloning. *Bam* HI was found to release four fragments from the λ5 insert. The partially *Bam* HI digested fragments of clone λ5 insert were separated on agarose gels and blotted onto nylon membranes (Hybond-N, Amersham). The duplicate membranes were then hybridized with T3 and T7 probes, respectively. The sizes of the partially digested fragments with respect to T3 or T7 site were used to determine the *Bam* HI sites in the λ5 insert, as shown and described in Figure 11. Each of the four completely *Bam* HI digested fragments of the λ5 insert was cloned into the multiple cloning site of the plasmid vector pBluescript II KS (+) (Stratagene). To identify and to localize the exon-intron boundaries in λ5 subclones, Southern hybridization was carried out to identify the exon-containing subclones (Figure 12, panel A and B). Two subclones which could hybridize to the cDNA probe were sequenced to identify exon and exon-intron junction regions. As shown in Figure 12 panel C, one exon of the mouse *ATBF1* gene was identified in the clone λ5 insert and it contains the 5′-end sequence of the mouse *ATBF1-A* cDNA which is split into two λ5

Figure 11. Mapping the Insert of Clone λ5

(A) Mapping

The FLASH® Nonradioactive Gene Mapping Kit (Stratagene) was used for the mapping. Two blots of the *Bam* HI partial digested fragments of the clone λ5 insert were hybridized with T3 and &T7 alkaline phosphatase-conjugated probes, respectively. Then, the chemiluminescent substrate was added to the membranes and the membranes were exposed to X-ray films. The exposed films are shown here. Those bands shown on the films with different sizes represented the partially digested fragment started from the T3 site and from the T7 site. The size of each band is indicated.

(B) Physical Map

There were three *Bam* HI sites within the clone λ5 insert. The *Bam* HI restriction map of the clone λ5 insert was obtained based on the mapping result.

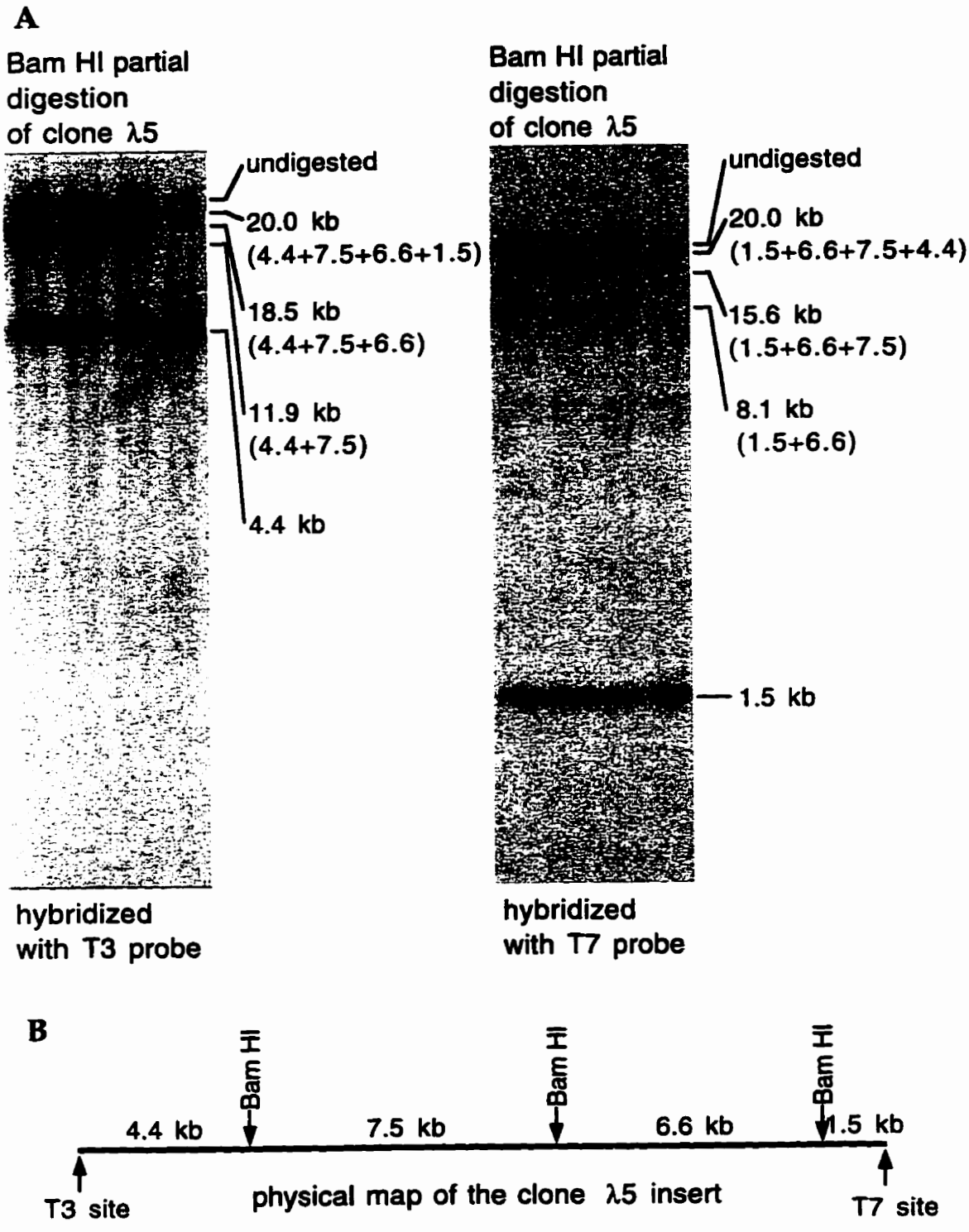

Figure 11. Mapping the Insert of Clone λ5.

Figure 12. Identification and Localization of an Exon in the Subclones of Clone λ5

(A) The λ5 Subclones

The *Bam* HI restriction fragments of the clone λ5 insert were cloned into the multiple cloning site of the plasmid vector pBluescript II KS (+). The inserts of the four subclones were released and were separated on an agarose gel. The agarose gel picture is shown in panel A.

(B) Southern Hybridization

The blot of the above gel was hybridized with a ^{32}P-labeled cDNA probe located at the 5′-end of the mouse *ATBF1-A* cDNA which was used to isolate clone λ5 from the mouse genomic library. The autoradiogram is shown in panel B. The subclone pλ5-4.4 and subclone pλ5-7.5 could hybridize to that cDNA probe. Those two subclones were probably the exon-containing subclones.

(C) Identification of Exon(s) in the Subclones

A *Bam* HI site located between subclone pλ5-4.4 and subclone pλ5-7.5 is probably the *Bam* HI site at the 5′-end of the mouse *ATBF1-A* cDNA. The exon(s) in subclone pλ5-4.4 and subclone pλ5-7.5 detected by Southern

hybridization is probably located at the junction of subclone pλ5-4.4 and subclone pλ5-7.5. The DNA sequencing using the M13 forward and M13 reverse primers on the plasmid vector confirmed the Southern hybridization result and revealed the precise location of that exon containing the 5'-end sequence of the mouse *ATBF1-A* cDNA.

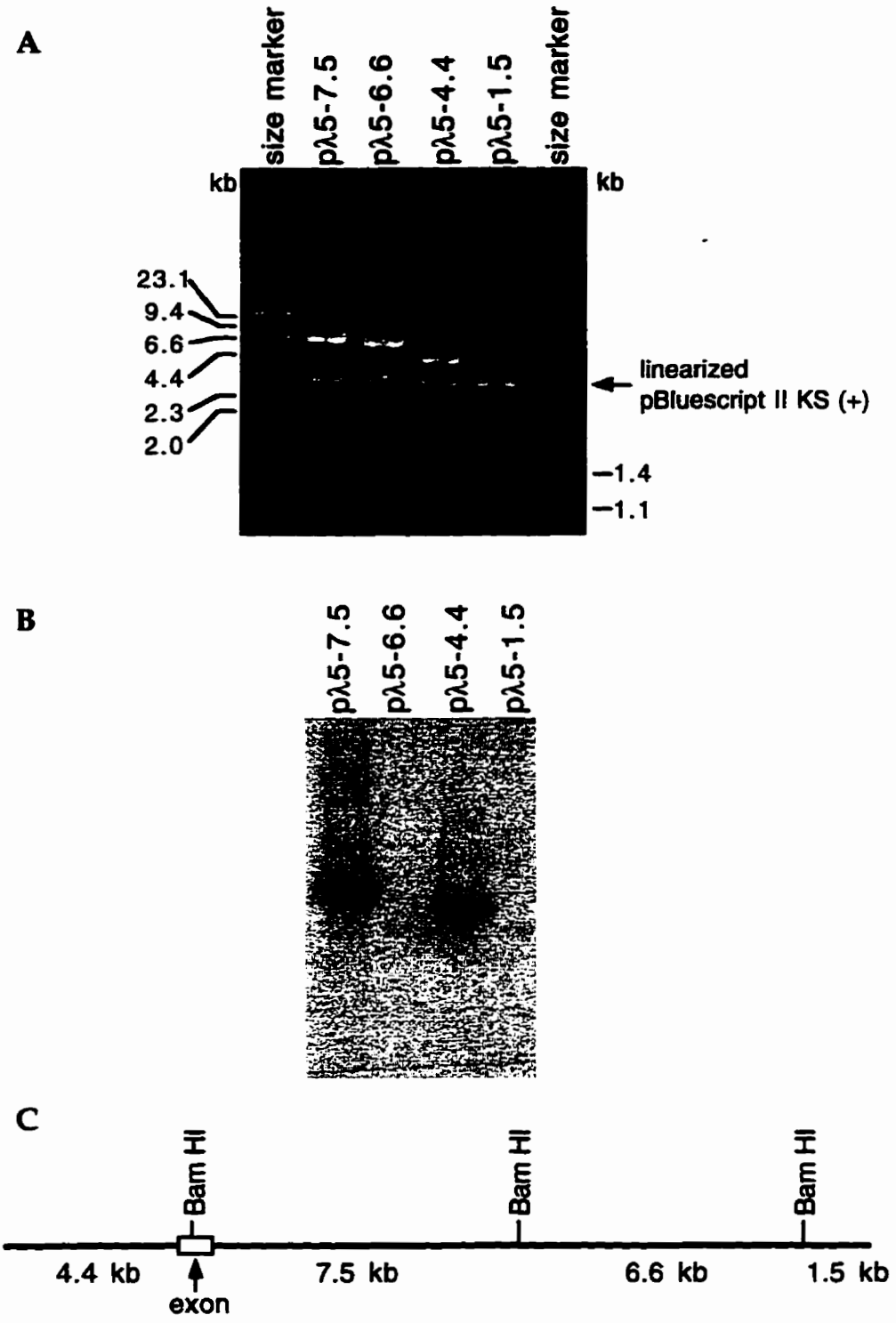

Figure 12. Identification and Localization of Exon-Intron in the Subclones of Clone λ5.

subclones. In the human *ATBF1* gene, exon 2 was determined previously and was specific for the *ATBF1-A* transcript (Miura *et al.*, 1995). The alternative splicing to generate *ATBF1-A* and *ATBF1-B* transcripts was also observed in mouse (Miura *et al.*, 1995). Therefore, according to the sequence, the exon identified in the λ5 insert was a mouse counterpart of the human exon 2. Southern hybridization using the cDNA probe (S1-A-fragment of the cDNA clone B326 insert, as described in Figure 10) which corresponds to the region downstream of exon 2 showed that no subclones of clone λ5 could hybridize to the cDNA probe. Therefore, there is no other exon further downstream of exon 2 within the clone λ5 insert.

To further isolate the mouse *ATBF1* genomic clones downstream of clone λ5, a cDNA fragment (S1-A-fragment of the cDNA clone B326 insert, as described in Figure 10) located downstream of the characterized exon 2 was used as probe together with another probe, the insert of the λ5 subclone pλ5-1.5, representing the 3'-end intron fragment, to screen the genomic library. The best expected result would be the isolation of a new genomic fragment that could hybridize with both probes. Therefore, the new clone started from the 3'-end of clone λ5, extended further towards the 3'-region of the mouse *ATBF1* gene, and contained exon 3. The insert of pλ5-1.5 was first tested for the presence of the repetitive sequences which often exist in mammalian introns.

The result showed that there was no repetitive sequence in the insert of pλ5-1.5 (Figure 13). However, the library screening result showed that no genomic clone could hybridize to both probes. Clone λ108 was isolated from the mouse genomic library using the insert of pλ5-1.5 as probe. Clone λ108 was characterized and aligned to clone λ5 by *Bam* HI mapping and Southern hybridization as described in Figure 14.

For those subclones' inserts with exons flanked by introns, the custom-made primers located within the exons were used to obtain the exon-intron junction sequences through sequencing. To locate the exons flanked by introns in those subclones, polymerase chain reaction was carried out using one specific primer within the exon and the other on the plasmid vector to determine the intron sizes. In some cases, if subclones containing exons were too big to be characterized properly, further subcloning needed to be done.

The mouse *ATBF1-A* cDNA was cloned previously (Ido *et al.*, 1996). However, the cDNA for the exon 1 of the mouse *ATBF1* gene, which was specific for the *ATBF1-B* transcript (Figure 15), was not available. Since the mouse *ATBF1-A* cDNA showed a high percentage of identity to the human *ATBF1-A* cDNA (Ido *et al.*, 1996), the first exon of the *ATBF1* gene (specific for the *ATBF1-B* transcript) might also be highly conserved between mouse and human. To clone the first exon of the mouse *ATBF1* gene, I used a human

Figure 13. Test of pλ5-1.5 Genomic Probe for Repetitive Sequences

The genomic DNA from a mouse cell line, P19, was digested with restriction endonuclease *Eco* RI and *Hind* III, separated on an agarose gel and, the gel was blotted onto a nylon membrane. The blot was hybridized with the ^{32}P-labeled pλ5-1.5 insert and exposed to X-ray film. There were several specific bands on the film. Therefore, there was no repetitive sequence in this intron fragment and it could be used as probe to screen the mouse genomic library.

Figure 13. Test of pλ5-1.5 Genomic Probe for Repetitive Sequences.

Figure 14. Characterization and Alignment of Clone λ108 to Clone λ5

(A) Physical Map of Clone λ108

There were two *Bam* HI sites within the clone λ108 insert. The *Bam* HI restriction map of clone λ108 insert was obtained using the method described for the mapping of clone λ5.

(B) Southern Hybridization

Three *Bam* HI restriction fragments of the clone λ108 insert were cloned into the multiple cloning site of the plasmid vector pBluescript II KS (+). The inserts of these subclones were analyzed by Southern hybridization with the ^{32}P-labeled subclone pλ5-1.5 insert. The insert of subclone pλ108-7.0 could hybridize to that genomic probe as shown on the autoradiogram.

(C) Alignment of Clone λ108 to Clone λ5

On the basis of the hybridization result shown in panel B and the *Bam* HI restriction map shown in panel A, the clone λ108 was aligned to the clone λ5.

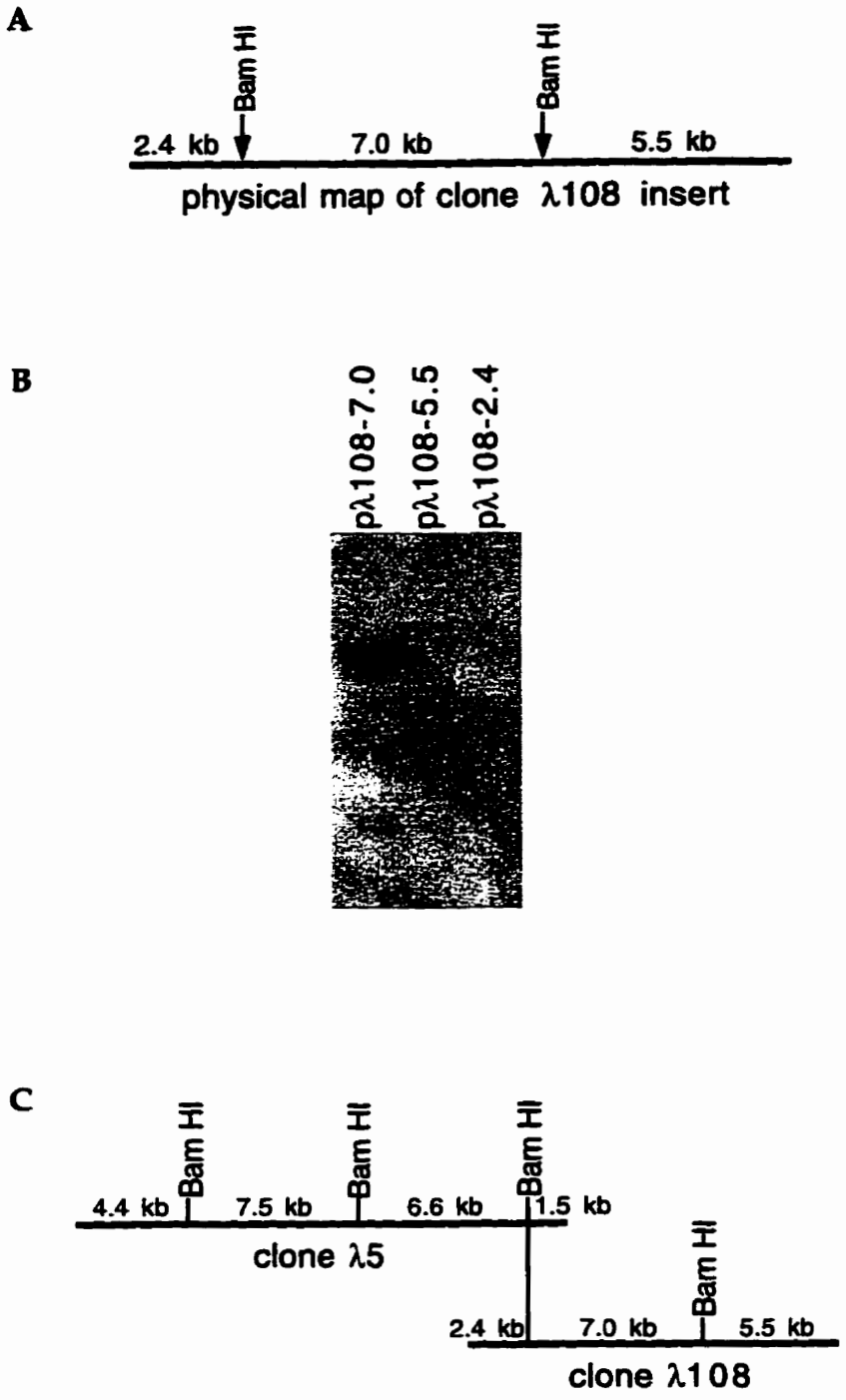

Figure 14. Characterization and Alignment of Clone λ108 to Clone λ5.

Figure 15. Alternative Splicing to Generate the Mouse *ATBF1-A* and *ATBF1-B* mRNAs

ATBF1-A and *ATBF1-B* mRNAs are transcribed from the same *ATBF1* gene. These two isoforms of *ATBF1* mRNA are generated by the mechanism of alternative promoters and alternative splicing at the 5′-region of the *ATBF1* gene. The exons are shown in boxes with the numbers on top and coding regions filled. *ATBF1-A* and *ATBF1-B* mRNA have distinct 5'-end exons. Two different promoters may be used for the transcription of *ATBF1-A* and *ATBF1-B* mRNA.

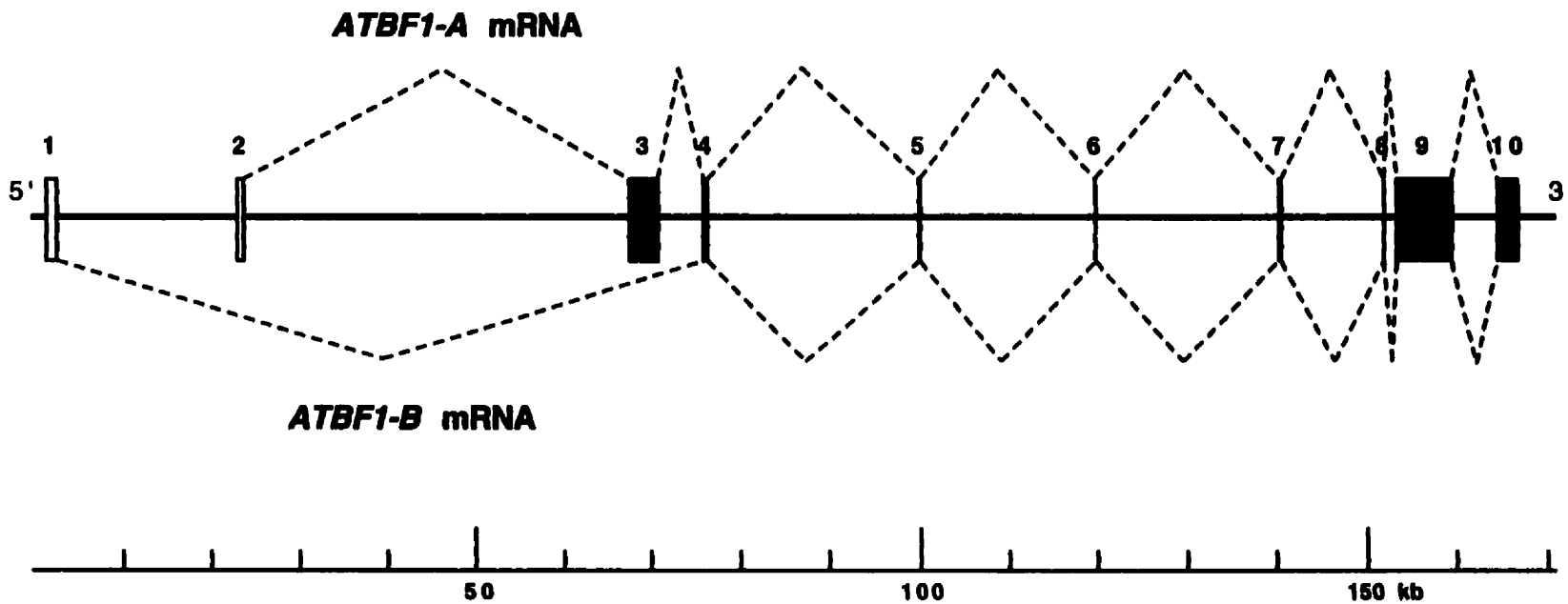

Figure 15. Alternative Splicing to Generate the Mouse *ATBF1-A* and *ATBF1-B* mRNAs.

ATBF1 genomic subclone insert containing the 5'-end of exon 1 and some 5'-flanking region (cloned in our laboratory, Miura *et al.*, 1995) as probes, together with a 2.9-kb mouse genomic subclone insert located at the 5'-end of the clone λ21 (Figure 16) as probe to screen the mouse genomic library. The clone λ89 (Figure 16) was identified which hybridized with both probes. This indicates that the clone λ89 was linked to the clone λ21 and might contain the first exon of the mouse *ATBF1* gene. Endonuclease digestion, Southern hybridization, and sequence analysis revealed the putative exon 1 of the mouse *ATBF1* gene which was about 60% identical to exon 1 of the human *ATBF1* gene. The existence and the location of exon 1 in the clone λ21 was confirmed by the primer extension analysis (see below, Section 5.1.4).

The molecular cloning of the mouse *ATBF1* gene was carried out basically using the methods and the strategies described above. The mouse *ATBF1* gene contains 10 exons. The size of the gene is over 170 kb including 1.5 kb of 5′-flanking and 3.6 kb of 3′-flanking regions. There are 21 overlapping genomic clones covering the mouse *ATBF1* gene (Figure 16). The mouse *ATBF1* gene exon-intron junction sequences, and the sizes of exons and introns are summarized in Table 1. The exon-intron junction sequences are similar to the consensus sequence proposed by Breathnach and Chambon (1981), that is, with the presence of the consensus splice donor (GT) sites and acceptor (AG) sites. The distribution of homeodomains and zinc fingers in

Figure 16. The Mouse *ATBF1* Gene

The schematic structure of the mouse *ATBF1* gene is shown with the exons indicated in filled boxes and the exon numbers at the bottom. There are 21 genomic overlapping clones covering the mouse *ATBF1* gene. The mouse *ATBF1* gene contains 10 exons and, the size of the gene is just over 170 kb which includes 1.5 kb of 5′-flanking and 3.6 kb of 3′-flanking regions. The exons are black-filled. The restriction endonuclease sites indicated are not exhaustive. The following is the list of abbreviations of those restriction endonuclease names used in this figure.

B: *Bam* HI; E: *Eco* RI; H: *Hind* III; N: *Not* I; P: *Pst* I; S1: *Sma* I; S2: *Sac* I; X: *Xho* I.

Mouse *ATBF1* Gene

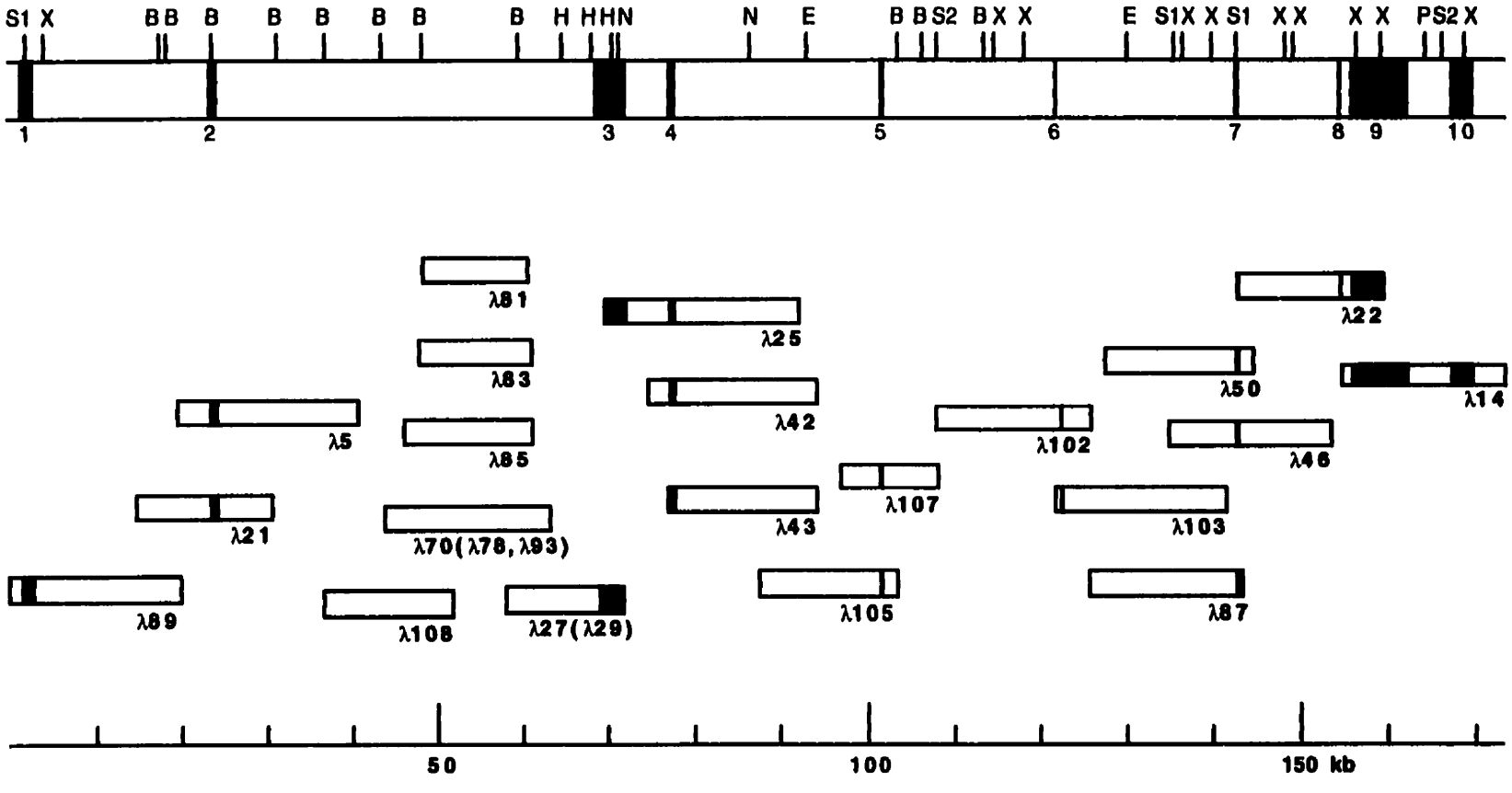

Figure 16. The Mouse *ATBF1* Gene.

Table 1. Summary of the Mouse *ATBF1* Gene

The sizes of exons and introns together with the exon-intron junction sequences of the mouse *ATBF1* gene are listed in this table. The size of exon 1 of the mouse *ATBF1* gene is the estimated size according to the human counterpart.

Table 1. Summary of the Mouse *ATBF1* Gene.

Exon			Intron			Exon	
No.	Size	3′ Junction	5′ Junction	Size	3′ Junction	5′ Junction	No.
					CTCCCAGATG	CAGGAATTTA	1
1	1200			21300	TTGGTGCAGG	CCAGGGCAGC	2
2	745	TGTGTGCCAG	GTGAGTGCAG	43600	CGCCCTTCAG	GTCCGATCCG	3
3	2768	CCTGCTCTAG	GTGAAGGTTA	4900	TTCCCTACAG	TGGGCGGTGA	4
4	497	GCTGTACAAG	GTGAGGTCGG	23500	GTCCCTGCAG	CAGCTGCAGC	5
5	232	ACTGACCCAG	GTGAGACCCG	20700	TGCATTTCAG	AGGAAGCCGT	6
6	108	AAGAGGGCAG	GTACTGTACA	20500	TTTCTTTCAG	CGTCATCCAG	7
7	335	CATTATGACG	GTAAGGCAGC	11200	CTCCCCTCAG	GTGACCGCTC	8
8	103	AAGCAACCTG	GTCAGTGTCG	1400	TTTTTCACAG	AAGCGTCAGA	9
9	5454	GCCAACACAG	GTGAGTCGCC	4800	TTTTTTTTAG	CTTTAACGTC	10
10	1887	AAAAAAAAAC	AAAAGAAAAA				

their coding exons of the mouse *ATBF1* gene is shown in Figure 17. All the homeodomains and zinc fingers are within single exons, that is, not disrupted by introns. Exon 9 of the mouse *ATBF1* gene, which is the largest exon, encodes 4 homeodomains and 10 zinc fingers.

5.1.4 The 5'-Boundaries of the Mouse *ATBF1-A* and *ATBF1-B* Transcripts

Primer extension experiments were carried out to determine the 5' ends of exon 1 (specific for the *ATBF1-B* transcript) and exon 2 (specific for the *ATBF1-A* transcript). Two 30-mer primers were synthesized, one near the 5'-end of the cloned mouse *ATBF1-A* cDNA which corresponds to exon 2 and the other near the putative 5'-end of the exon 1 of the mouse *ATBF1* gene which I cloned.

Our laboratory previously showed that the *ATBF1* gene was expressed in XBF (a mouse teratoma cell line) and retinoic acid-treated P19 (a mouse embryonal carcinoma cell line) cells (Ido *at al.*, 1994; Ido *et al.*, 1996). Total cellular RNAs prepared from XBF and P19 cells were used for the primer extension.

In the primer extension analyses of exon 2 (for the *ATBF1-A* transcript), a single major band was obtained in both RNA samples from XBF and retinoic acid-treated P19 cells. Two bands showed the same size. The genomic DNA sequence of the corresponding region was accompanied with the primer extension products and the transcription initiation site of *ATBF1-A* was

Figure 17. The Distribution of Homeodomains and Zinc Fingers in the Exons of the Mouse *ATBF1* Gene

The exons of the mouse *ATBF1* gene are shown in boxes with the numbers at the bottom and coding regions filled. The allocation of homeodomains (HD) and zinc fingers (ZF) are shown on top of their according exons. Exon 9, the largest exon, encodes 4 homeodomains and 10 zinc fingers.

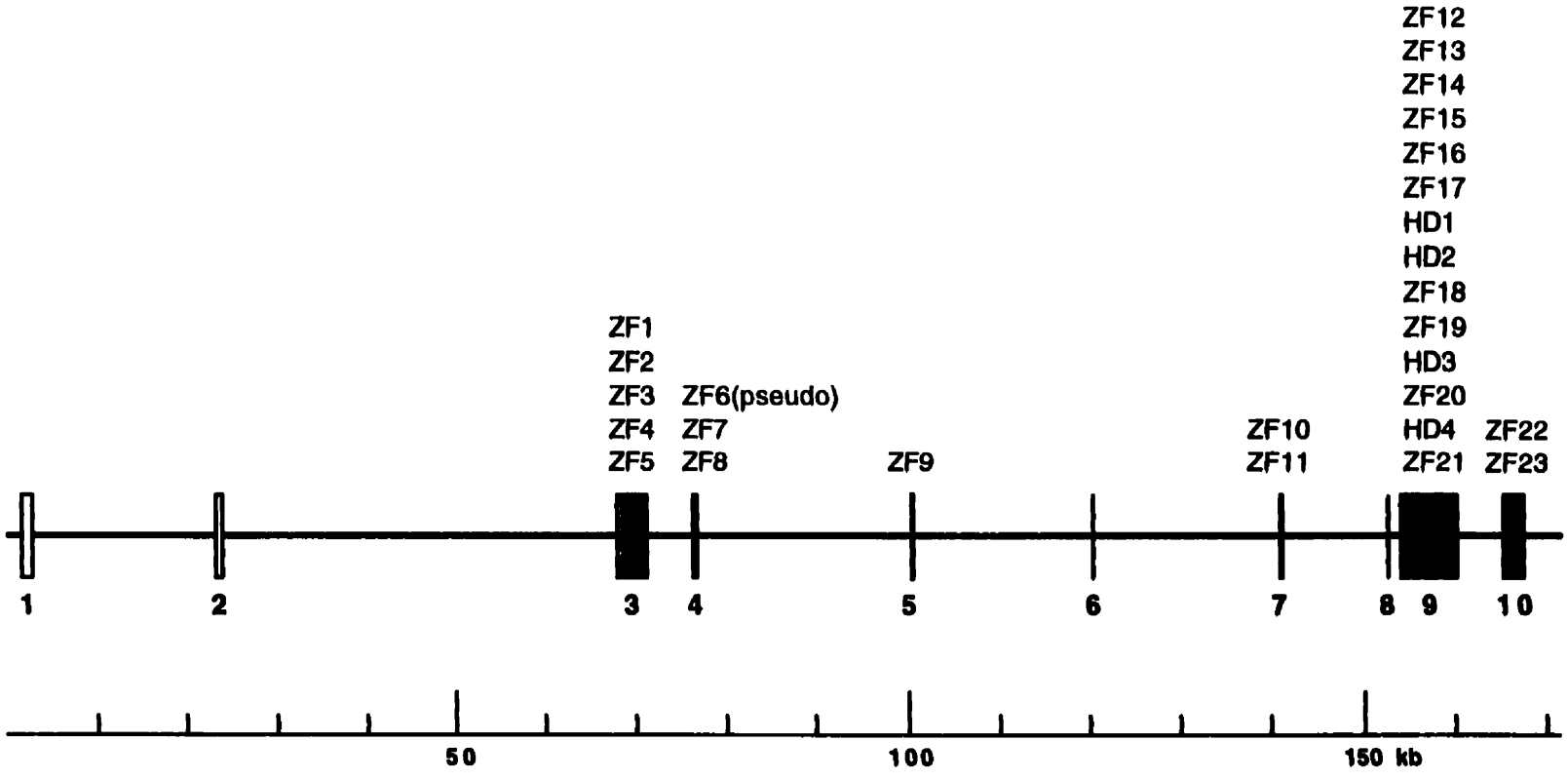

Figure 17. The Distribution of Homeodomains and Zinc Fingers in the Exons of the Mouse *ATBF1* Gene.

precisely identified. Figure 18 shows the primer extension results for the *ATBF1-A* transcript. As shown in Figure 19, the 5'-boundary of exon 2 was identified at the 61-nucleotides upstream of the 5'-end of the mouse *ATBF1-A* cDNA (Ido *et al.*, 1996).

In the primer extension analyses of exon 1 (for the *ATBF1-B* transcript), a single major band was obtained in both RNA samples from XBF and retinoic acid-treated P19 cells. These two bands showed the same size. The genomic DNA sequence of the corresponding region was accompanied with the primer extension products and the transcription initiation site of *ATBF1-B* was precisely identified (Figure 20). Since the cDNA for exon 1 of the mouse *ATBF1* gene, which was specific for the *ATBF1-B* transcript (Figure 15), was not available, the exon 1 of the mouse *ATBF1* gene was cloned and located to the clone λ89 (Figure 16) using the human counterpart as probe. The primer extension analysis of the mouse *ATBF1-B* transcript further confirmed the presence and the location of exon 1 of the mouse *ATBF1* gene in the clone λ89 (Figure 16).

5.1.5 Sequence Comparison between the Human and the Mouse *ATBF1-A* Promoter Region

In the promoter assay of the human *ATBF1-A* transcript, a 5.5-kb fragment of the 5'-flanking region of exon 2 (specific for the *ATBF1-A* transcript) showed promoter activity in neuronally differentiated P19 cells

Figure 18. Primer Extension Analyses of the Transcription Initiation Site of the Mouse *ATBF1-A* (5'-Boundary of Exon 2)

A single major band of the primer extension product is shown in both RNA samples from XBF (as marked in this figure) and retinoic acid-treated P19 (marked as P19-RA$^+$ in this figure) cells. The genomic DNA of the corresponding region was sequenced using the same primer as that used in the primer extension analysis. The primer extension and the genomic DNA sequencing products were run together on a sequencing gel. Those lanes marked with G, A, T, and C were the sequencing reactions terminated by each of the four dideoxynucleotides, respectively. The precise transcription initiation site of the mouse *ATBF1-A* transcript is indicated by an arrow.

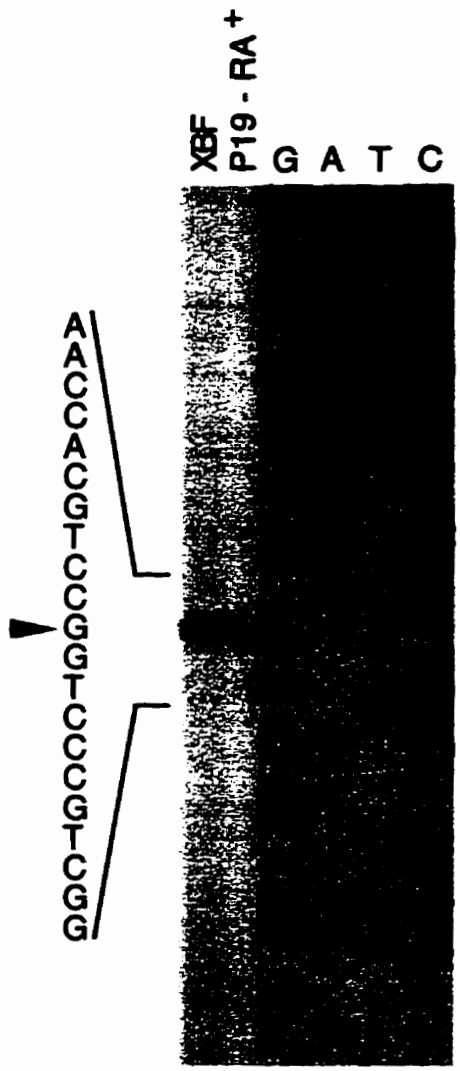

Figure 18. Primer Extension Analyses of the Transcription Initiation Site of the Mouse *ATBF1-A* (5'-Boundary of Exon 2).

Figure 19. The 5′-End Sequence of Exon 2 of the Mouse *ATBF1* Gene

The 5′-boundary of exon 2 of the mouse *ATBF1* gene, which is the transcription initiation site of mouse *ATBF1-A*, was determined by the primer extension analysis. The transcription starts at 61-nucleotides upstream of the 5′-end of the mouse *ATBF1-A* cDNA cloned earlier (Ido *et al.*, 1996).

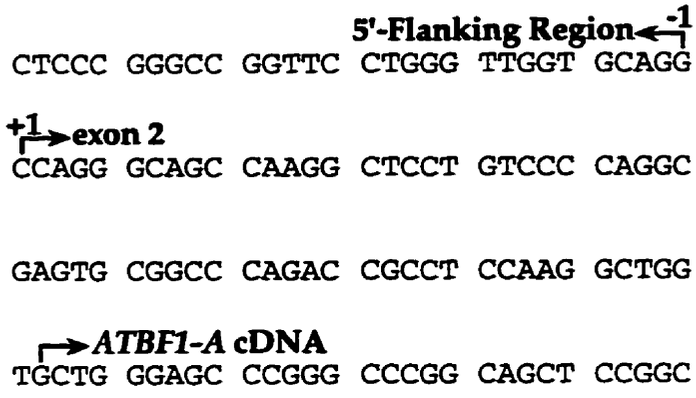

Figure 19. The 5′-End Sequence of Exon 2 of the Mouse *ATBF1* Gene.

Figure 20. Primer Extension Analyses of the 5′-Boundary of Exon 1 of the Mouse *ATBF1* Gene

A single major band from the primer extension products is shown in each RNA samples from XBF (as marked in this figure) and retinoic acid-treated P19 (marked as P19-RA$^+$ in this figure) cells. The genomic DNA of the corresponding region was sequenced using the same primer which was used in the primer extension analysis. The primer extension and the genomic DNA sequencing products were run together on a sequencing gel. Those lanes marked with G, A, T, and C were the sequencing reactions terminated by each of four dideoxynucleotides, respectively. The precise transcription initiation site of the mouse *ATBF1-B* transcript is indicated by an arrow.

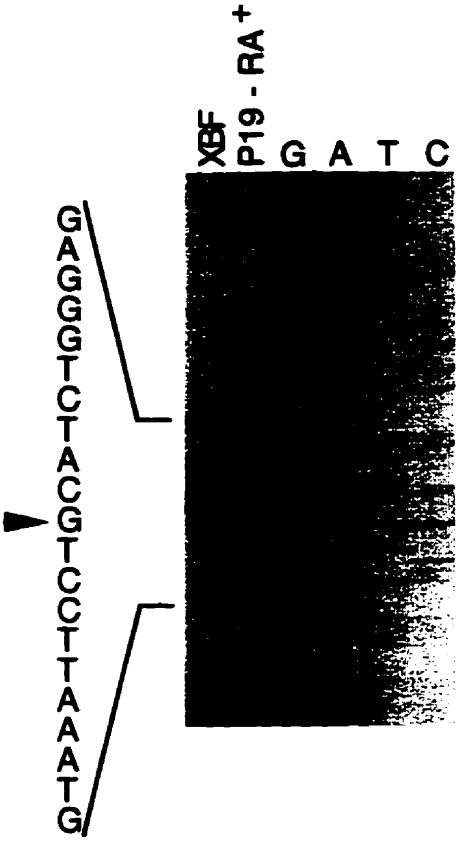

Figure 20. Primer Extension Analyses of the 5'-Boundary of Exon 1 of the Mouse *ATBF1* Gene.

(Miura *et al.*, 1995). The promoter region (within about 200-base pairs upstream of exon 2, the first exon for the *ATBF1-A* transcript) contains several potential transcription factor binding sites, such as AP-2 and SP1 sites, by computer analysis of the consensus sequences (Miura and Tamaoki, personal communications).

The mouse *ATBF1* gene is expressed in the neuronally differentiated P19 mouse embryonal carcinoma cells (Ido *et al.*, 1994), and the human *ATBF1* gene is also expressed in the neuronally differentiated NT2/D1 human embryonal carcinoma cells (Miura *et al.*, 1995). Furthermore, the 5'-flanking region of the human *ATBF1* gene for the human *ATBF1-A* transcript showed the promoter activity in neuronally differentiated P19 cells (Miura *et al.*, 1995). Based on these results, the 5'-flanking region of the mouse and the human *ATBF1-A* may contain similar transcription factor binding sites.

The sequences of the mouse and the human, 249 nucleotides upstream of exon 2, were amalyzed. Unexpectedly, the result showed that the 5'-flanking region of exon 2 of the mouse *ATBF1* gene had about 46% overall identity with its human counterpart, and there were different potential transcription factor binding sites in the mouse sequence than those identified in the human sequence (Figure 21). In the human sequences, there are three potential AP-2 binding sites and one potential SP1 binding site. However, in the mouse sequences, there are three potential MZF-1 binding sites, one potential CDP

Figure 21. Sequence Comparison of the 5′-Flanking Regions of Exon 2 between the Human and the Mouse *ATBF1* Genes

The 5′-flanking sequences of exon 2 of the mouse and the human *ATBF1* genes were compared and the "*" indicates the same nucleotide between mouse and human. The overall identity of the nucleotide sequence at this region between mouse and human is about 46%. The potential transcription factor binding sites are also indicated in both sequences. One potiential MZF-1 site with lower strand indicates that the consensus sequence of the MZF-1 site is located at the antisense-strand. Those sites in the human sequence were previously identified in our laboratory (Miura and Tamaoki, personal communications). Those sites in the mouse sequence were identified by transcription-factor-binding-site database analysis.

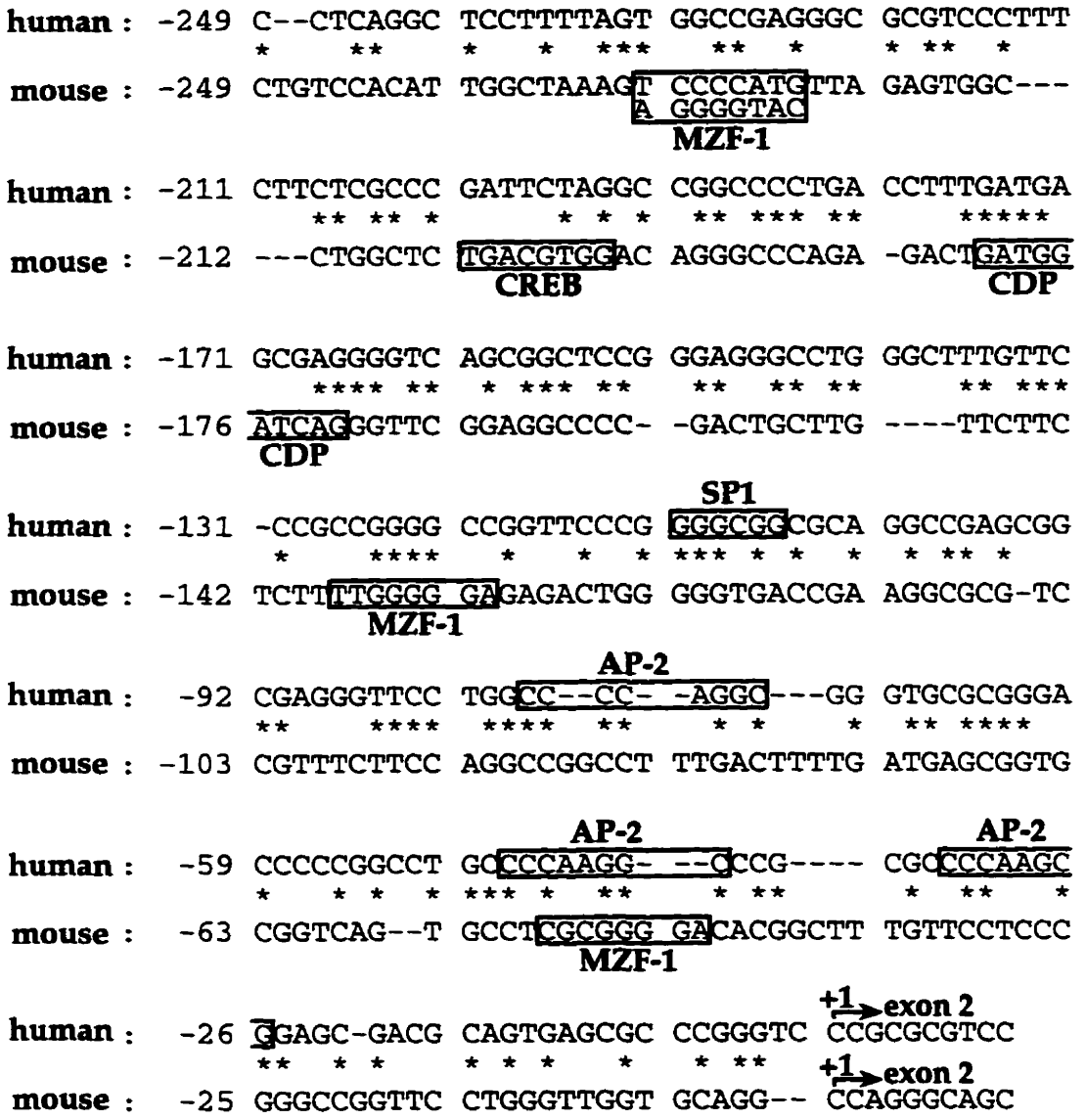

Figure 21. Sequence Comparison of the 5'-Flanking Regions of Exon 2 betwee the Human and the Mouse *ATBF1* Genes.

binding site, and one potential CREB binding site. The possible reasons for the differences will be discussed in Section 6.1.

Transcription-factor-binding-site database analysis showed that there were several potential transcription factor binding sites in the 5'-flanking region of exon 2 of mouse *ATBF1* gene, such as MZF-1, CDP, and CREB sites. MZF-1 is a multiple zinc finger protein and *MZF-1* gene expression is retinoic acid-responsive (Hromas *et al.*, 1991). CDP is a homeodomain protein and is implicated in the regulation of mammalian development (Neufeld *et al.*, 1992). The cAMP-response-element-binding protein (CREB) binds directly to cAMP-inducible promoters and has been shown to mediate response to cAMP in intact cells and animals. The *CREB* gene is expressed in many types of cells (Lee and Masson, 1993).

5.2 Enforced Expression of Sense- and Antisense-*ATBF1* in P19 Cells

Our laboratory previously showed that the *ATBF1* gene was expressed in the neuronally differentiated but not in the undifferentiated P19 cells (Ido *et al.*, 1994). Therefore, the enforced expression of sense- and antisense-*ATBF1* in undifferentiated P19 cells can be used for the functional analysis of the *ATBF1* gene, especially for the function of the *ATBF1* gene product along the neuronal differentiation pathways in P19 cells.

As shown by McBurney *et al.* (1994a,b), plasmid DNA can be effectively transfected into P19 cells. However, it is difficult to isolate clones of cells stably

expressing genes present on the transfected plasmids. Even in an isolated colony which has transfected plasmid DNA, the introduced gene is expressed only in some but not all cells. The vector used to express sense- and antisense-*ATBF1* in P19 cells is pPOP (described in Section 4.14). The pPOP vector contains intragenic regions of the murine *pgk-1* locus which can enhance the extent of ligation of all cotransfected plasmids as well as that of integration into the P19 cell genome, although the mechanism is unclear (McBurney *et al.* 1994a). Therefore, pPOP can be used to express genes in P19 cells with improved stable transfection efficiency.

5.2.1 Expression of Antisense-*ATBF1* in P19 Cells

Seven clones of stable P19 cell transfectants with the pα-ATBF1 expression vector (antisense-*ATBF1*) were established using the BES-buffered calcium phosphate transfection procedure as described in Section 4.16. The clones of the antisense-*ATBF1* transfectants were analyzed for the morphological changes and gene expression in P19 cells following treatment with retinoic acid to induce neuronal differentiation.

5.2.1.1 Analyses of the Morphological Changes in the Antisense-*ATBF1* Transfectant P19 Cells Treated with Retinoic Acid

The morphological changes during the neuronal differentiation of P19 cells induced by retinoic acid has been well characterized (Jones-Villeneuve *et*

al., 1982).

Seven clones of antisense-*ATBF1* transfectants and the control transfectant (transfected with the empty expression vector) did not show any morphological changes as compared to the untransfected cells when they were cultured in medium without retinoic acid. The antisense-*ATBF1* and the control transfectants were then treated with retinoic acid (described in Section 4.13). Eight days following the start of retinoic acid treatment, the untransfected and the control transfectant P19 cells showed the morphological changes of neuronal differentiation. However, seven clones of the antisense-*ATBF1* transfectant basically showed little neuronal differentiation in terms of morphological changes. The results are shown in Figure 22. The morphological changes shown in panel C and D were quantitated in terms of the number of neuron-like cells and the length of neurite-like extensions. In the field of panel D (antisense-*ATBF1*), there are about two neuron-like cells and, each has two neurite-like extensions of about one body length. While in the field of panel C (control), there are about 35 neuron-like cells and, each has two or more neurites of about 10 to 20 cell body lengths. These observations indicate that the antisense-*ATBF1* blocks the neuronal differentiation of P19 cells induced by retinoic acid in terms of morphological changes.

Figure 22. The Morphology of P19 Cells Transfected with Antisense-*ATBF1* Following Retinoic Acid Treatment

All panels show the morphologies of P19 cells and were photographed by phase contrast. Panel A shows the morphology of untreated P19 cells. Panel B, C, and D show the morphology of P19 cells at 8 days following the start of retinoic acid treatment.

The cells shown in Panel A and B were untransfected P19 cells. Retinoic acid treatment could induce the neuronal differentiation as shown in panel B.

Panel C and D show the morphology of control and antisense-*ATBF1* P19 cell transfectants. Control P19 cells could be induced into neuronal differentiation (panel C). Antisense-*ATBF1* transfectants show few of the morphological changes corresponding to the neuronal differentiation.

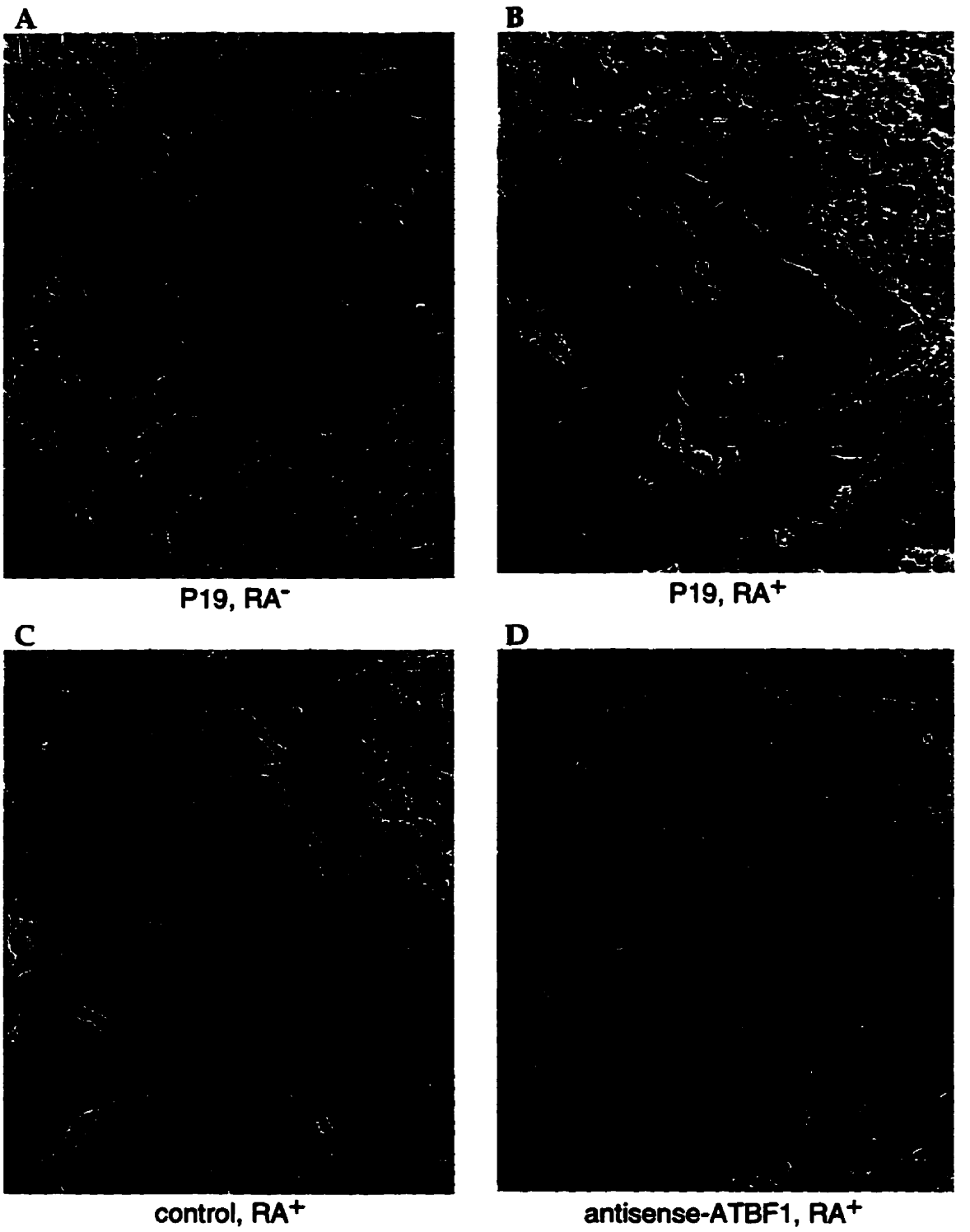

Figure 22. The Morphology of P19 Cells Transfected with Antisense-*ATBF1* Following Retinoic Acid Treatment.

5.2.1.2 Analyses of Gene Expression in the Antisense-*ATBF1* Transfectant P19 Cells Treated with Retinoic Acid

To analyze the gene expression in the antisense-*ATBF1* transfectant P19 cells, the ribonuclease protection assay was performed using total cellular RNA (described in Section 4.12). The total cellular RNA was prepared from the untransfected and the transfectant P19 cells which were cultured for 8 days following the start of retinoic acid treatment. The total cellular RNA from the untreated wild type P19 cells was also prepared (described in Section 4.17).

First, all those RNA samples were analyzed for *GAPDH* mRNA to normalize the amount of RNA prepared from different cell samples. Figure 23 shows that the *GAPDH* mRNA was present in all the RNA samples with relatively the same amount.

The antisense-*ATBF1* expression in the transfectants was analyzed and the result is shown in Figure 24. The antisense-*ATBF1* RNA was expressed in all seven clones of the transfectant.

Our laboratory previously showed that *ATBF1* mRNA expression was inducible in P19 cells by retinoic acid treatment (Ido *et al.*, 1994). Analysis of the RNA samples showed that the endogenous *ATBF1* mRNA was expressed in all seven clones of antisense-*ATBF1* transfectant which were treated by retinoic acid. As shown in Figure 25, the *ATBF1* mRNA levels of clone 1, 3, 4 and 6 of the antisense-*ATBF1* transfectant were lower compared to that of the control. In clone 2, 5, and 7 of the antisense-*ATBF1* transfectant, the *ATBF1*

Figure 23. The *GAPDH* mRNA Expression in the Transfected P19 Cells

The ribonuclease protection assay was conducted using the probe as described in Section 4.12.1. The RNA samples from seven clones (as marked in this figure) of antisense-*ATBF1* transfectant and from one control (as marked in this figure) transfectant (transfected with empty expression vector) were analyzed for the presence of *GAPDH* mRNA to normalize the amount of RNA prepared from different cell samples. Two RNA samples from the untransfected P19 cells as controls (marked as untransfected in this figure) were also analyzed for the presence of *GAPDH* mRNA. The RNA samples from the retinoic acid-treated cells are marked with RA^+ and, the RNA sample from the untreated cells is marked with RA^-. The ribonuclease protection assay products are indicated by an arrow.

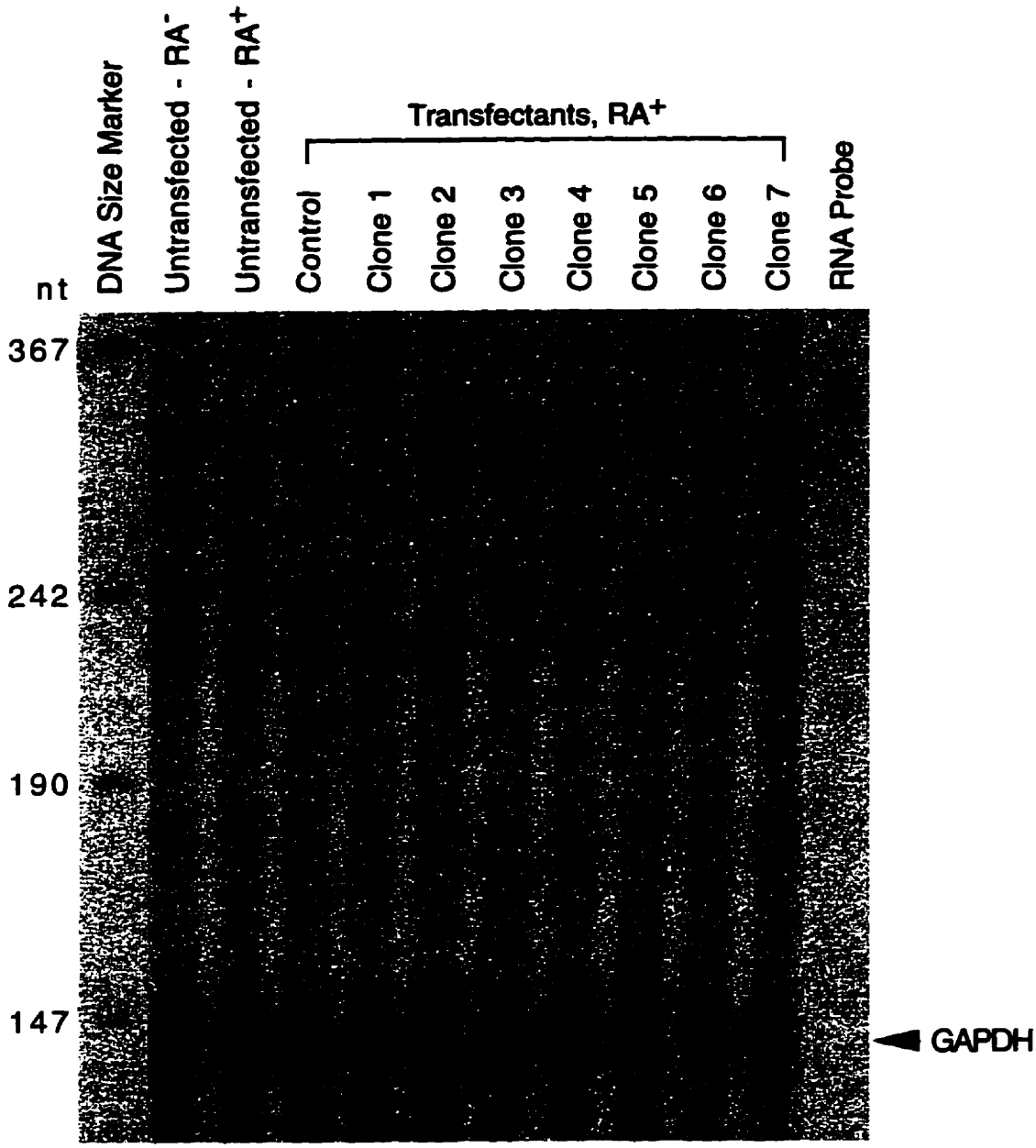

Figure 23. The *GAPDH* mRNA Expression in the Transfected P19 Cells.

Figure 24. Antisense-*ATBF1* RNA Expression in the Transfected P19 Cells

The ribonuclease protection assay was conducted using the probe as described in Section 4.12.1. The RNA samples from seven clones (as marked in this figure) of antisense-*ATBF1* transfectants and from one control (as marked in this figure) transfectant (transfected with empty expression vector) were analyzed for the expression of antisense-*ATBF1* RNA. The ribonuclease protection assay products are indicated by an arrow.

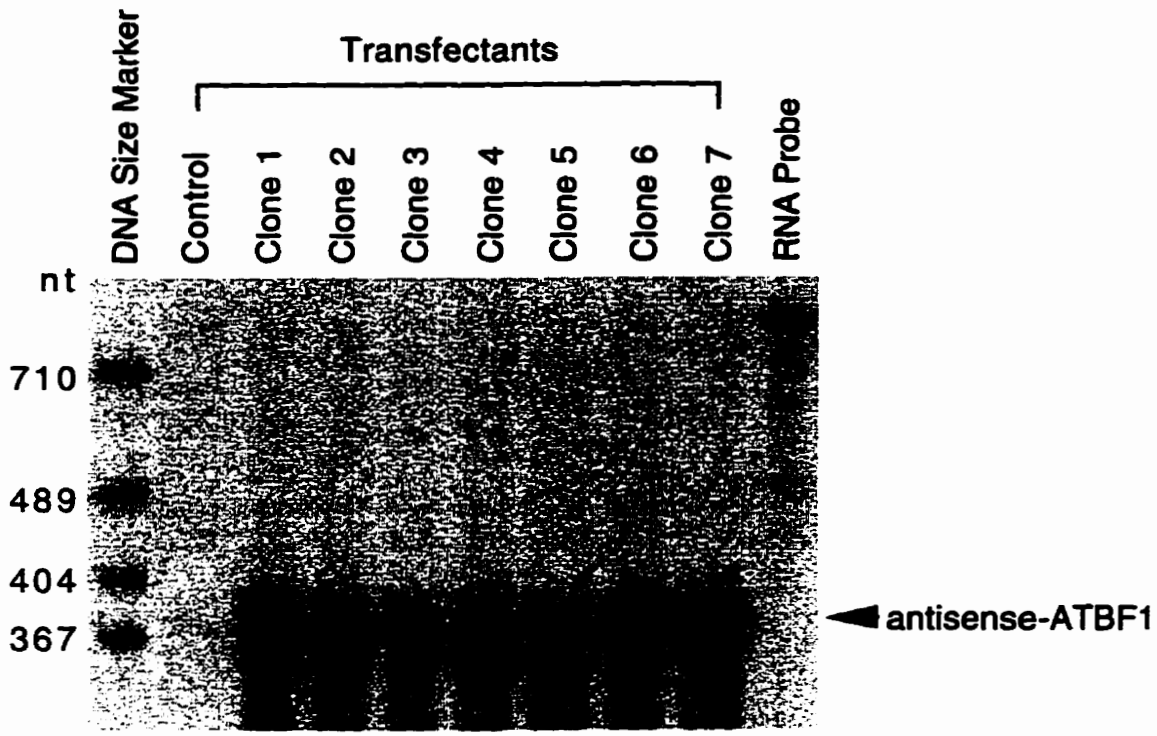

Figure 24. Antisense-*ATBF1* RNA Expression in the Transfected P19 Cells.

Figure 25. Endogenous *ATBF1* mRNA Expression in the Transfected P19 Cells upon Retinoic Acid Treatment

The ribonuclease protection assay was conducted using the probe as described in Section 4.12.1. The RNA samples from seven clones (as marked in this figure) of antisense-*ATBF1* transfectants and from one control (as marked in this figure) transfectant (transfected with empty expression vector) were analyzed for the expression of *ATBF1* mRNA. Two RNA samples from the untransfected P19 cells as controls (marked as untransfected in this figure) were also analyzed for the expression of *ATBF1* mRNA. The RNA samples from the retinoic acid-treated cells are marked with RA^+ and, the RNA sample from the untreated cells is marked with RA^-. The ribonuclease protection assay products are indicated by two arrows.

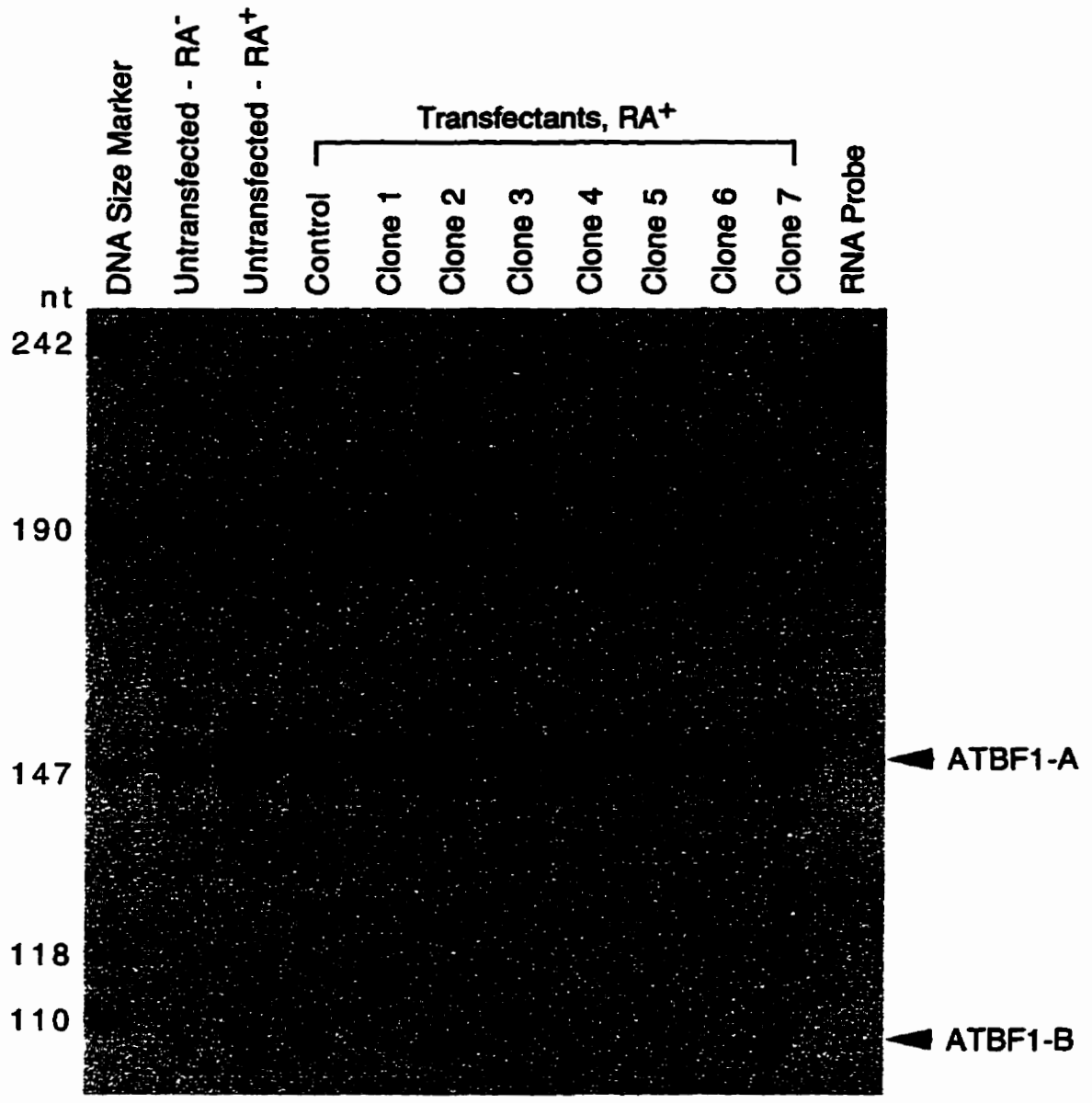

Figure 25. Endogenous *ATBF1* mRNA Expression in the Transfected P19 Cells upon the Retinoic Acid Treatment.

mRNA levels were higher than those in clone 1, 3, 4 and 6, but were still lower than that of the control. The relative *ATBF1* mRNA levels in the above clones were quantitated to be 57, 86, 73, 61, 87, 52, and 97% (100% for the level in the control) using an NIH Image computer program.

The clones of antisense-*ATBF1* transfectant were further analyzed for the expression of marker genes during neuronal differentiation to confirm the effect of antisense-*ATBF1* on the blockage of neuronal differentiation.

The MASH1 (mammalian achaete-scute homolog 1) protein is a member of the basic helix-loop-helix family of transcription factors. The *MASH1* gene is transiently expressed in the early development of the nervous system (Lo *et al.*, 1991). The *MASH1* mRNA level in P19 cells has been shown to dramatically increase about 2 days after retinoic acid treatment (Johnson *et al.*, 1992). The *MASH1* mRNA expression was analyzed using ribonuclease protection assays and the results are shown in Figure 26. The *MASH1* mRNA was expressed in untransfected cells and cells transfected with empty expression vector (control) after retinoic acid treatment, whereas the *MASH1* mRNA was undetectable in untransfected cells without retinoic acid treatment. In the clones of antisense-*ATBF1* transfectant, the *MASH1* mRNA expression level decreased markedly, although the expression levels varied from clone to clone. The decreased expression of *MASH1* mRNA in the clones of the antisense-*ATBF1* transfectants was correlated with the morphological

Figure 26. The *MASH1* mRNA Expression in the Transfected P19 Cells upon Retinoic Acid Treatment

The ribonuclease protection assay was conducted using the probe as described in Section 4.12.1. The RNA samples from seven clones (as marked in this figure) of antisense-*ATBF1* transfectant and from one control (as marked in this figure) transfectant (transfected with empty expression vector) were analyzed for the expression of *MASH1* mRNA. Two RNA samples from the untransfected P19 cells as controls (marked as untransfected in this figure) were also analyzed for the expression of *MASH1* mRNA. The RNA samples from the retinoic acid-treated cells are marked with RA^+ and, the RNA sample from the untreated cells is marked with RA^-. The ribonuclease protection assay products are indicated by an arrow.

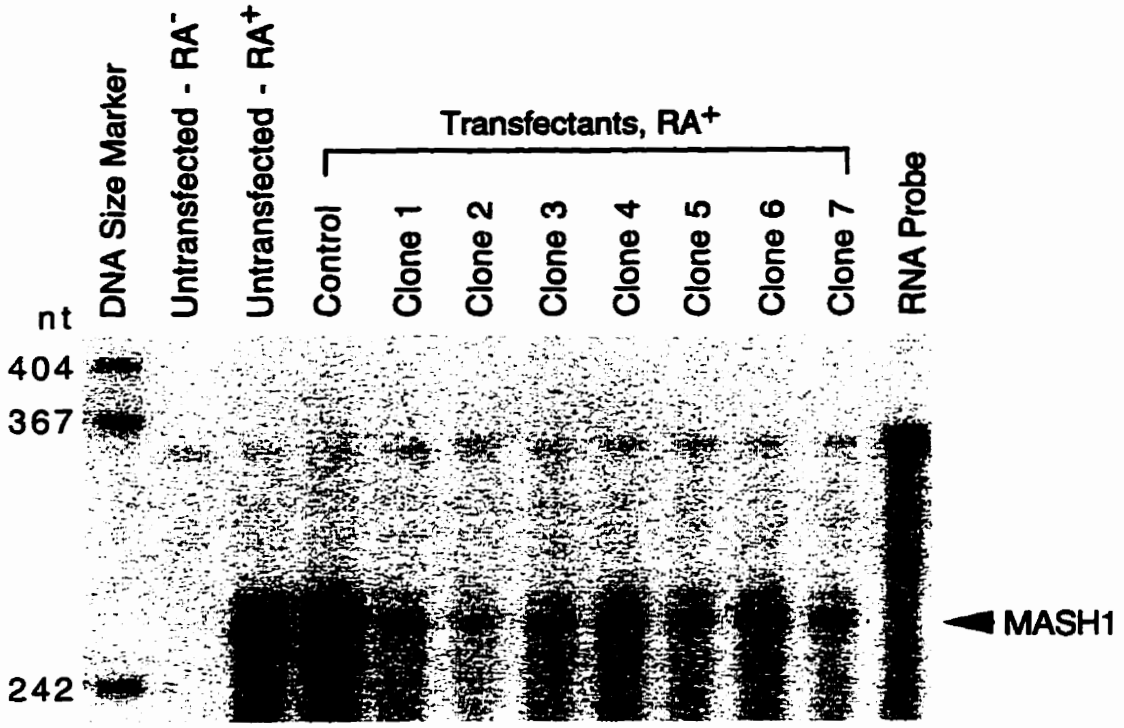

Figure 26. *MASH1* mRNA Expression in the Transfected P19 Cells upon the Treatment of Retinoic Acid.

characteristics of very little neuronal differentiation (described in Section 5.2.1.1).

GAD67 is a glutamic acid decarboxylase (GAD) with molecular weight of 67,000 Dalton. GAD catalyzes the production of γ-aminobutyric acid, a major inhibitory neurotransmitter of the vertebrate retina. GAD67 is expressed in the mammalian brain (Kaufman *et al.*, 1991). *GAD67* mRNA expression is dramatically increased in P19 cells at 8 to 10 days following the start of retinoic acid treatment (Bain *et al.*, 1993). *GAD67* mRNA expression detected by ribonuclease protection assays is shown in Figure 27. The expression level of *GAD67* mRNA decreased dramatically in the antisense-*ATBF1* transfectant cells, which resembled the expression pattern of *MASH1* mRNA. These results support each other. Since the RNA samples were prepared at the time point when *GAD67* mRNA expression reached its peak level, the expression pattern of *GAD67* mRNA more clearly demonstrates the significantly decreased expression of the neuronal differentiation marker gene in the clones of the antisense-*ATBF1* transfectants upon retinoic acid treatment.

The results from the analysis of the morphological changes are consistent with the results from the analysis of the gene expression in the antisense-*ATBF1* transfectant P19 cells treated with retinoic acid. It indicates that the neuronal differentiation of P19 cells induced by retinoic acid is blocked by the expression of the antisense-*ATBF1* RNA.

Figure 27. The *GAD67* mRNA Expression in the Transfected P19 Cells upon Retinoic Acid Treatment

The ribonuclease protection assay was conducted using the probe as described in Section 4.12.1. The RNA samples from seven clones (as marked in this figure) of antisense-*ATBF1* transfectants and from one control (as marked in this figure) transfectant (transfected with empty expression vector) were analyzed for the expression of *GAD67* mRNA. Two RNA samples from the untransfected P19 cells as controls (marked as untransfected in this figure) were also analyzed for the expression of *GAD67* mRNA. The RNA samples from the retinoic acid-treated cells are marked with RA^+ and, the RNA sample from the untreated cells is marked with RA^-. The ribonuclease protection assay products are indicated by an arrow.

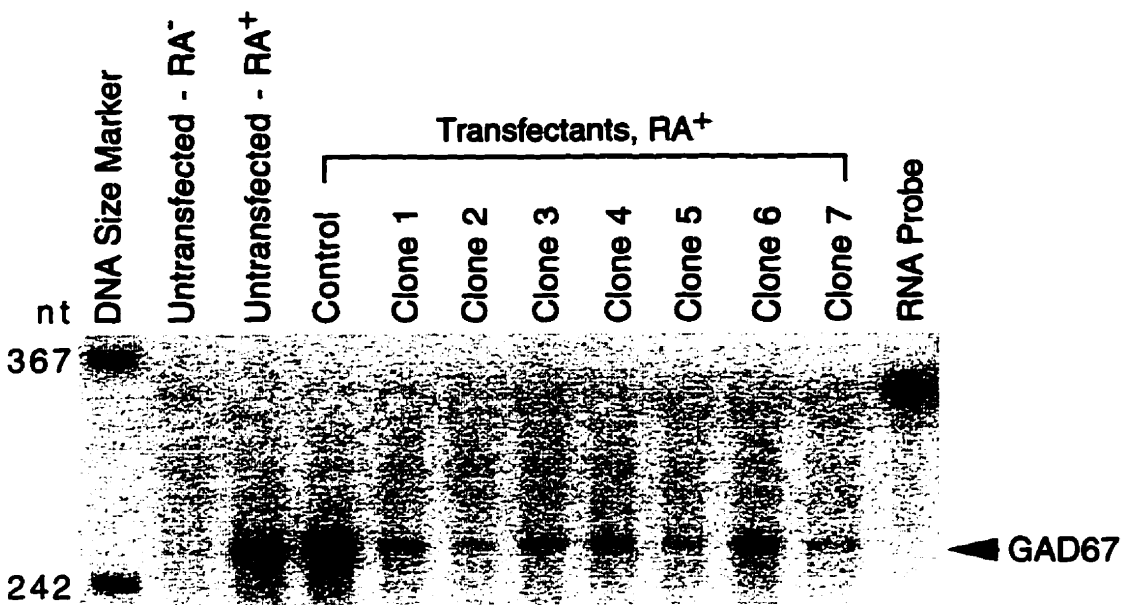

Figure 27. *GAD67* mRNA Expression in the Transfected P19 Cells upon the Retinoic Acid Treatment.

5.2.2 Expression of Sense-*ATBF1* in P19 Cells

The sense-*ATBF1-A* and sense-*ATBF1-B* expression vectors were transfected into P19 cells (described in Section 4.16). During the drug G418 selection, there were no colonies formed on the plates in which cells were transfected either with sense-*ATBF1-A* or with sense-*ATBF1-B* expression vectors, whereas colony formation was observed on the plates in which cells were transfected either with empty expression vector (control) or with antisense-*ATBF1* expression vector.

These results indicate that the expression of sense-*ATBF1* in P19 cells may either cause cell differentiation (no cell proliferation) or cause cell death. Further analyses on the effects of sense-*ATBF1* expression in P19 cells was carried out.

5.2.2.1 Immunostaining of P19 Cells Transfected with Sense-*ATBF1-A* Expression Vector

Immunostaining was carried out using Rat-401 monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa) as the primary antibody to recognize an intermediate filament protein called nestin expressed during the early neural development. Nestin is named because it is specifically expressed in neuroepithelial stem cells (Hockfield and McKay, 1985; Lendahl *et al.*, 1990). Lin *et al.*(1996) showed that nestin was transiently expressed in P19 cells during the first 5 days after exposure to retinoic acid.

Therefore, the expression of nestin protein can be used as an early marker of neuronal differentiation for the analysis of the effects of sense-*ATBF1* expression in P19 cells.

First, the Rat-401 antibody was tested using P19 cells cultured for 5 days (two days on bacterial plates with retinoic acid and three days on cell culture plates without retinoic acid). Figure 28 shows the positive staining of nestin in the retinoic acid-treated cells but not in the untreated cells.

P19 cells transfected with sense-*ATBF1-A* expression vector and empty expression (control) were immunostained with Rat-401 antibody. After 72-hour cultured in the medium containing 10% fetal bovine serum following the transfection of expression vectors, the P19 cells transfected with sense-*ATBF1-A* expression vector showed no specific staining of nestin compared to the control cells transfected with empty expression vector. This might be due to the rapidly dividing P19 cells of the untransfected population during the 72-hour culturing period. The untransfected population of P19 cells might override the transfected population of P19 cells since there was no colony formation observed from the sense-*ATBF1* expression vector transfected P19 cells.

To suppress the growth of the untransfected population of P19 cells, the cells were cultured in the serum-free medium after transfection, as described in Figure 29. Figure 30 shows the result of immunostaining of nestin in P19 cells transfected with sense-*ATBF1-A* expression vector. In the 24-hour serum

Figure 28. Immunostaining of Nestin in P19 Cells Treated with Retinoic Acid

The immunostaining was performed as described in Section 4.18. Panel B shows the positive staining of nestin in P19 cells cultured for 5 days following the start of retinoic acid treatment. There was no positive staining of untreated cells as shown in panel A.

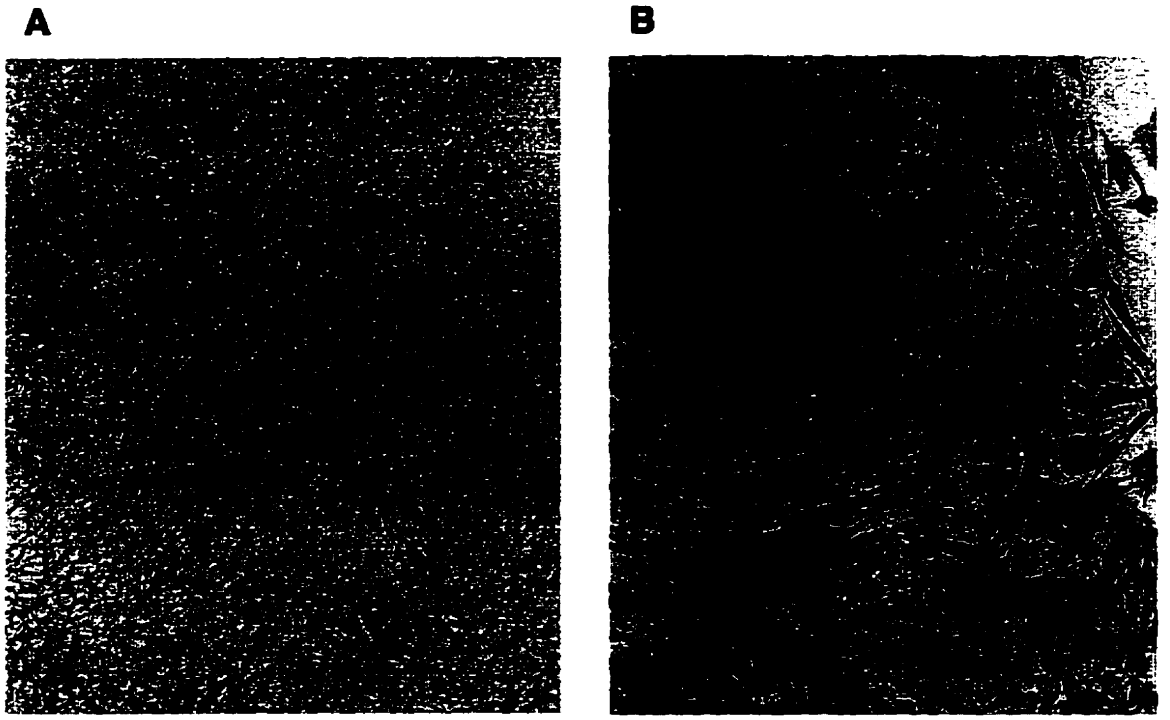

Figure 28. Immunostaining of P19 Cells Treated with Retinoic Acid.

Figure 29. Scheme of Analysis of P19 Cells Transfected with the Sense-*ATBF1-A* Expression Vector

The P19 cells were transfected with sense-*ATBF1-A* expression vector and with empty expression vector (pPOP, as control). Those cells were also co-transfected with the neomycin resistance gene carrying expression vector, pMC1neoPolyA. The transfection was performed as described in Section 4.16. After being cultured 24 hours in the 10% FBS (fetal bovine serum) medium, the cells were then cultured in serum-free medium for up to 48 hours. Before immunostaining of the nestin protein, cells were grown on 24-well plates for 24 hours in 10% FBS medium. To count the surviving cells after serum starvation, cells were stained with Trypan Blue Stain 0.4% and the unstained viable cells were counted.

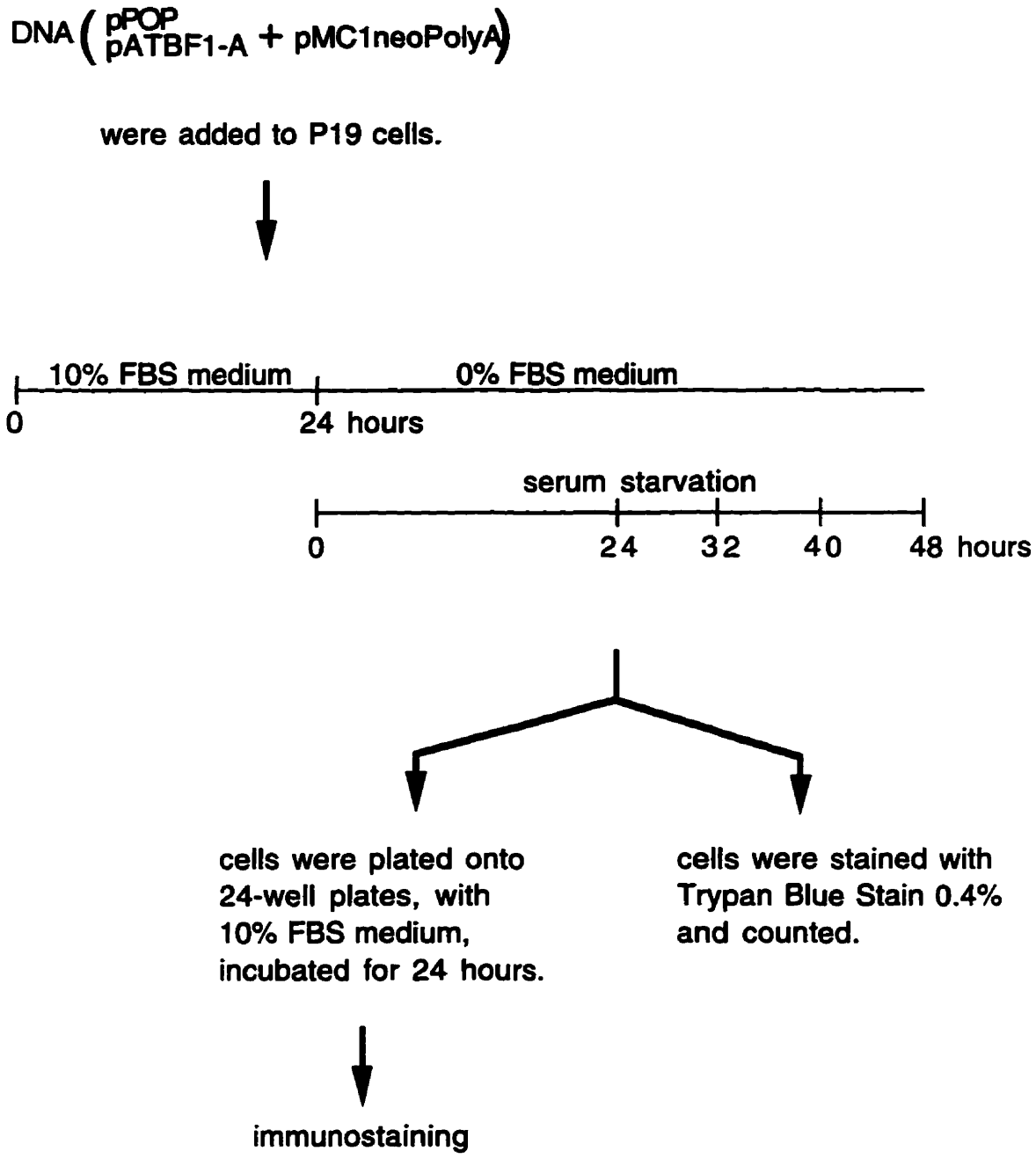

Figure 29. Scheme of Analysis of P19 Cells Transfected with the Sense-*ATBF1-A* Expression Vector.

Figure 30. Immunostaining of Nestin in P19 Cells Transfected with the Sense-*ATBF1-A* Expression Vector

The immunostaining was performed as described in Section 4.18. The cells shown in both panel A and B were at the time point of 24-hour serum starvation as described in Figure 29. The cells shown in panel B were transfected with sense-*ATBF1-A* expression vector and, some of those cells were stained. There was no such staining in the control cells shown in panel A which were transfected with empty expression vector.

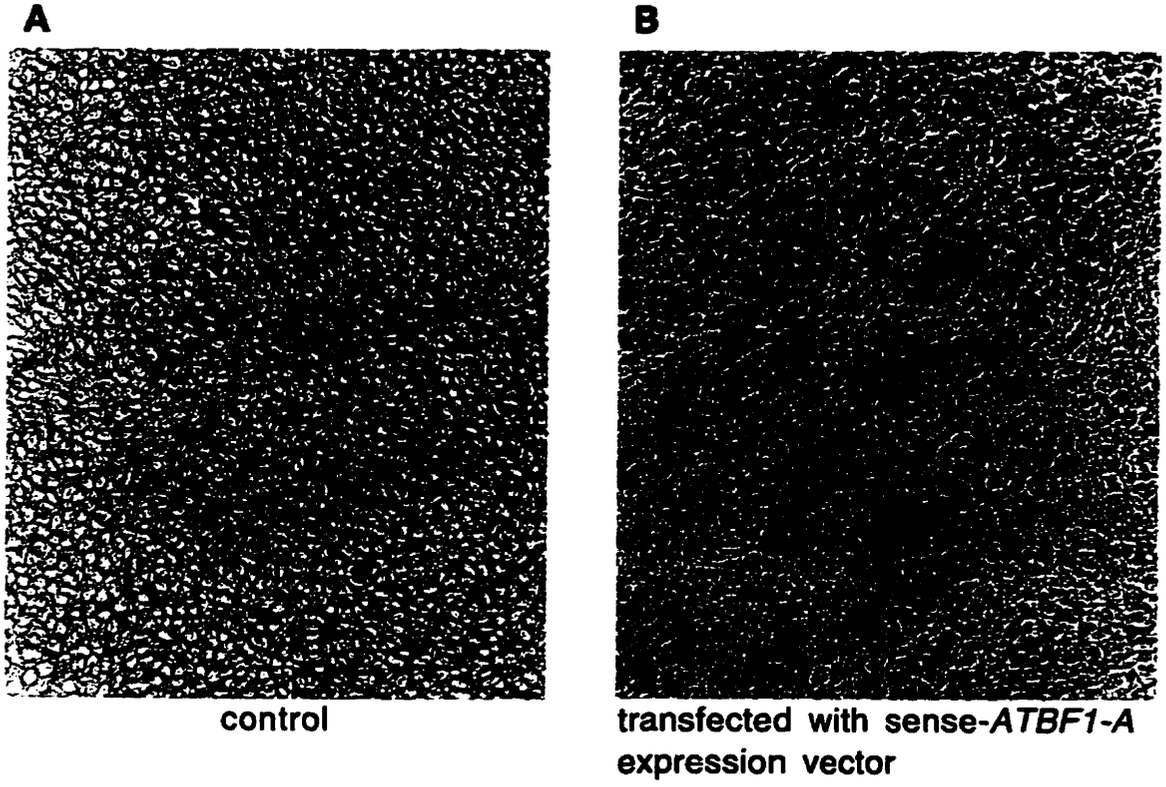

Figure 30. Immunostaining of P19 Cells Transfected with the Sense-*ATBF1-A* Expression Vector.

starved cells, there was more staining in the sense-*ATBF1-A* expression vector transfected cells than in the control cells. After 48-hour cultured in the serum-free medium, the cells transfected with sense-*ATBF1-A* expression vector showed relatively more staining (with the same pattern as shown in 24-hour serum starvation treatment) than that of 24-hour serum starvation treatment (data not shown).

Two more controls for the immunostaining of nestin were carried out. One was P19 cells transfected with the antisense-*ATBF1* expression vector. The other was F9 cells transfected with the sense-*ATBF1-A* expression vector. The F9 cell is a line of mouse embryonal carcinoma cells. Upon retinoic acid treatment, F9 cells differentiate into endoderm-like cells (Linder *et al.*, 1981) in which the *ATBF1* gene is not expressed (Ido *et al.*, 1994). The transfectants of those P19 and F9 cells underwent the same procedures of serum starvation and staining as did to the P19 cells transfected with the sense-*ATBF1-A* expression vector. The results showed that there was no nestin staining in these two controls (Figure 31).

All the results from the immunostaining of nestin suggest that P19 cells transfected with sense-*ATBF1-A* expression vector show a certain degree of differentiation in terms of the nestin protein expression.

Figure 31. Controls of the Immunostaining of Nestin

P19 and F9 cells were transfected with antisense-*ATBF1* and sense-*ATBF1-A* expression vectors respectively and, those cells underwent the same serum starvation and staining as did to the P19 cells transfected with sense-*ATBF1-A* expression vector. There was no specific staining of nestin as shown in panel B (P19 cells) and panel D (F9 cells). Panel A and C showed the control cells which were transfected with empty expression vector.

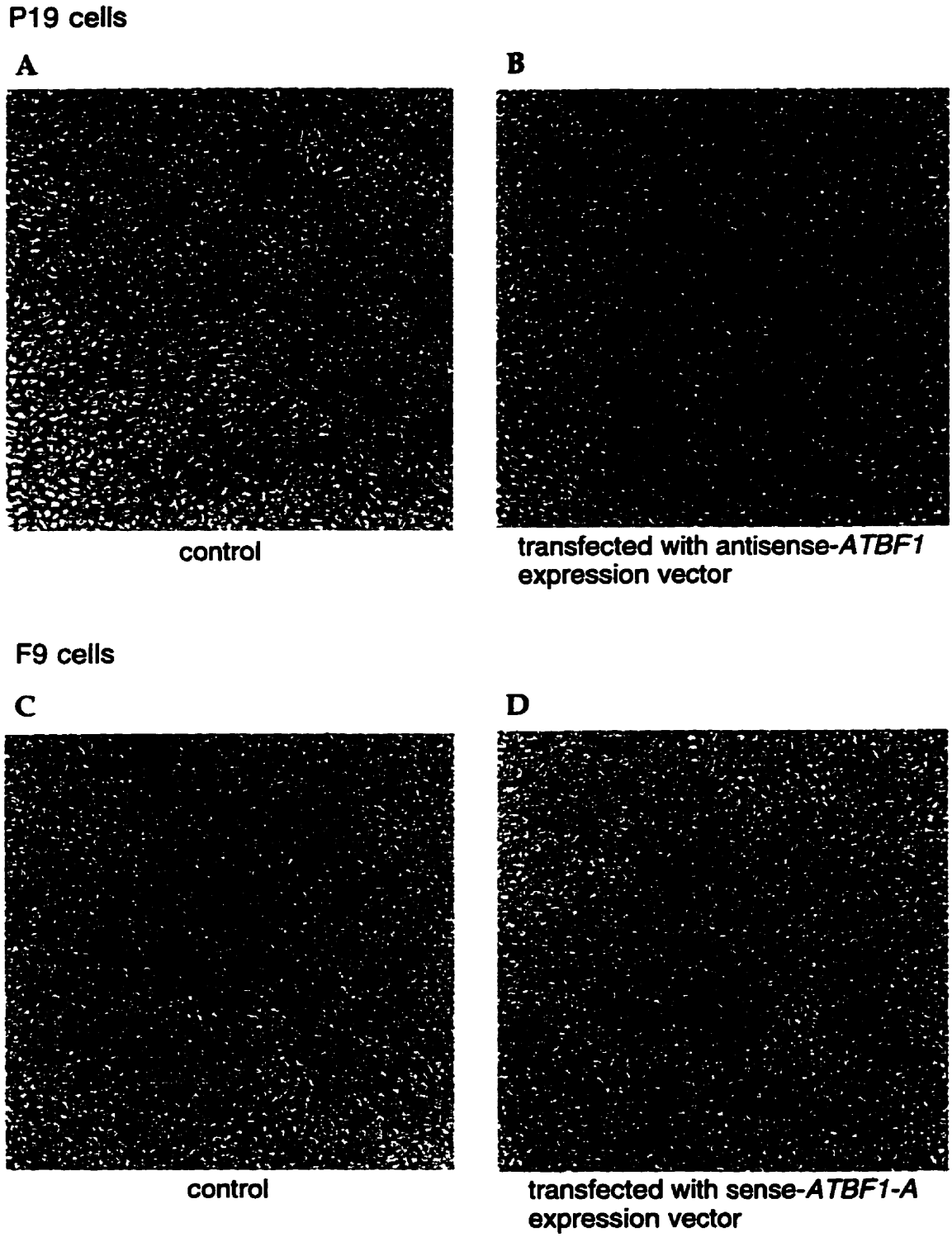

Figure 31. Controls of the Immunostaining.

5.2.2.2 Serum Starvation Resistance of P19 Cells Transfected with the Sense-*ATBF1* Expression Vector

An interesting observation obtained during the course of these experiments was that P19 cells appeared to be more resistant to the serum starvation compared to the control cells. To confirm this possiblity, the quantitation of cells was done according to the time course as described in Figure 29. The cells were stained with Trypan Blue Stain 0.4% (Life Technologies) and the unstained viable cells were counted under the microscope using a hemacytometer (Figure 32). The result confirmed that P19 cells transfected with the sense-*ATBF1-A* expression vector were more resistant to serum starvation than the control cells.

To determine the transient transfection efficiency of P19 cells, the cells were transfected with a *LacZ* gene expression vector (described in Section 4.19) and, the efficiency turned out to be about 15% as shown in Figure 33. This is consistent with the margin of the cell survival rate between the sense-*ATBF1-A* transfection and the control as shown in Figure 32.

All these results suggest that P19 cells transfected with the sense-*ATBF1-A* expression vector no longer proliferate and undergo a certain degree of differentiation as revealed by the nestin protein expression and the serum starvation resistance.

Figure 32. Serum Starvation Resistance of P19 Cells Transfected with the Sense-*ATBF1-A* Expression Vector

P19 cells transfected with the sense-*ATBF1-A* expression vector and the empty expression vector (control) showed different survival rates following serum starvation. The aliquots of cells recovered from four plates were counted at each time point. The mean of the cell number from four plates was used to draw the survival curves. The standard deviations are indicated on the curves.

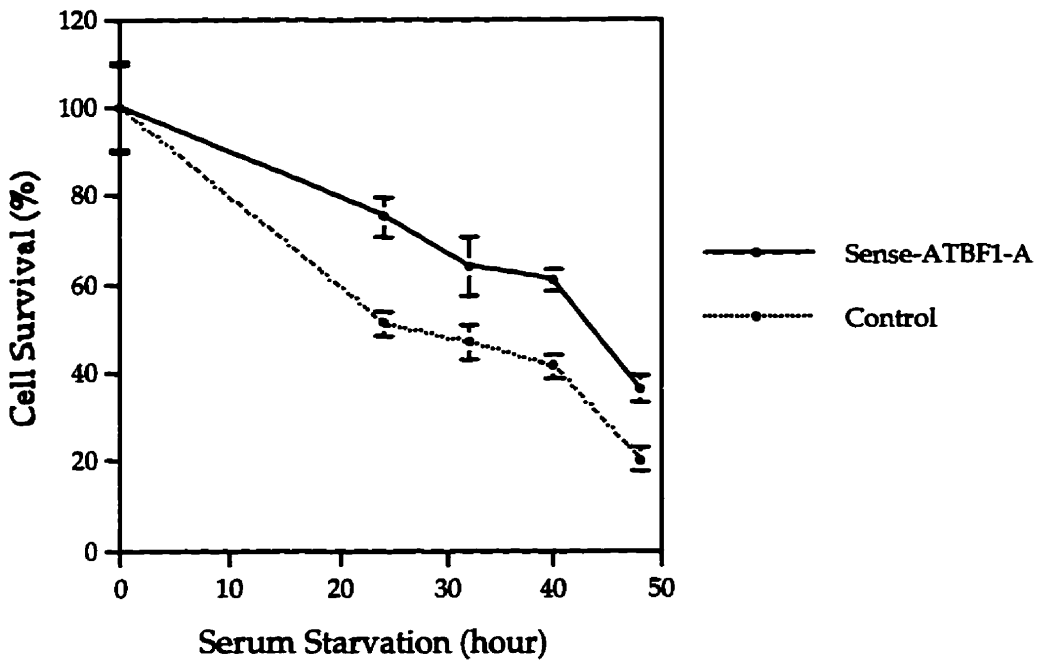

Figure 32. Serum Starvation Resistance of P19 Cells Transfected with Sense-*ATBF1-A* Expression Vector.

Figure 33. *LacZ* Expression in P19 Cells Transfected with pCH110 Expression Vector

P19 cells were transfected with pCH110, a *LacZ* gene expression vector (panel B) or the empty expression vector (panel A) and stained with X-gal as described in Section 4.19. In panel B, about 15% of cells showed blue staining.

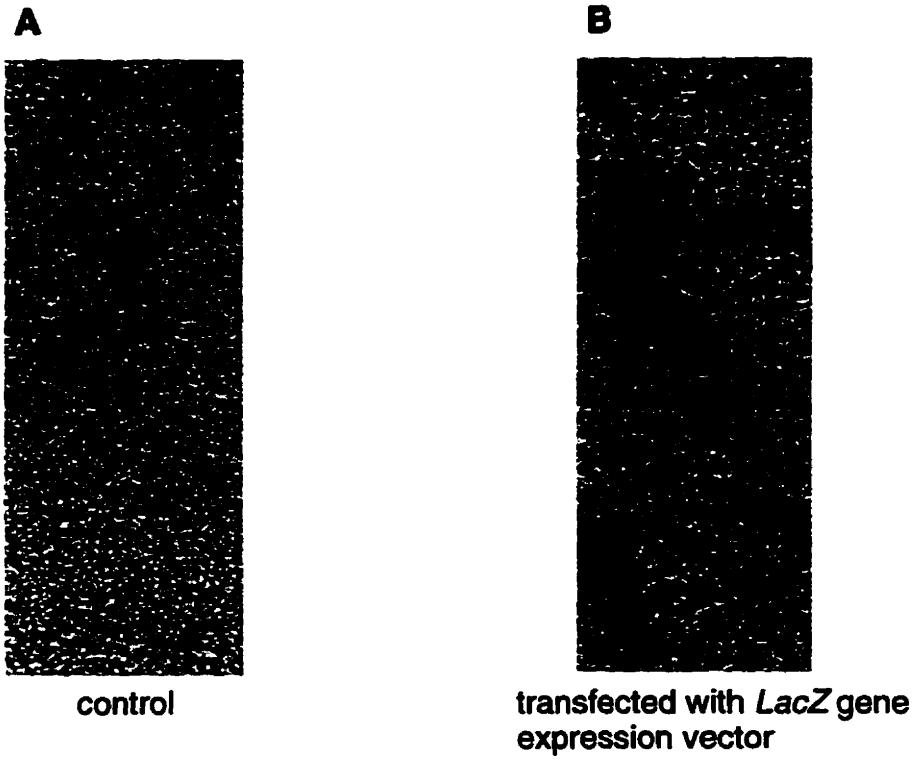

Figure 33. *LacZ* Expression in P19 Cells Transfected with pCH110 Expression Vector.

CHAPTER VI: DISCUSSION

ATBF1 protein is a transcription factor containing multiple homeodomains and zinc fingers. *ATBF1* transcripts have been identified in human and mouse so far studied, and two isoforms of *ATBF1* transcripts are identified and cloned (Morinaga *et al.*, 1991; Ido *et al.*, 1994; Miura *et al.*, 1995; Ido *et al.*, 1996). They are transcribed from the same *ATBF1* gene by the mechanism of alternative promoters/alternative 5′ splice sites (Miura *et al.*, 1995). That ATBF1 protein acts as a transcription factor was first established by its ability to suppress human α-fetoprotein gene expression (Yasuda *et al.*, 1994). Later, our laboratory showed that the *ATBF1* gene was expressed in the neuronally differentiated P19 embryonal carcinoma cells (Ido *et al.*, 1994). Analyses of the gene expression in mouse tissues using Northern hybridizations, ribonuclease protection assays, and *in situ* hybridizations indicate that the *ATBF1* gene is expressed mainly in the central nervous system during embryogenesis (Miura *et al.*, 1995; Ido *et al.*, 1996; Watanabe *et al.*, 1996).

The mouse *ATBF1* gene has been cloned and contains 10 exons (Figure 16). The size of the gene is over 170 kb including 1.5 kb of 5′-flanking and 3.6 kb of 3′-flanking regions. Functional studies of the *ATBF1* in P19 cells revealed that the enforced expression of antisense-*ATBF1* in P19 cells blocked the neuronal differentiation induced by retinoic acid (described in Section 5.2.1). This was demonstrated by the morphological alternations as well as the

significantly decreased expression of marker genes corresponding to the neuronal differentiation. As well, it was also shown that the expression of sense-*ATBF1* in P19 cells may induce a certain degree of differentiation (described in Section 5.2.2).

6.1 Molecular Cloning of the Mouse *ATBF1* Gene

The cloned and characterized mouse *ATBF1* gene makes it possible to study the function of the *ATBF1* gene product during embryonic development and differentiation in mouse, a well-established animal model.

The human *ATBF1* gene is mapped on human chromosome 16q22.3-23.1 (Yamada *et al.*, 1995). A subclone with a 4.8-kb insert containing the region of intron 2 at the 5'-end of clone λ27 (Figure 16) was used as probe for the mouse *ATBF1* gene chromosome mapping and, the mouse gene mapped to chromosome 8E1 (Yamada *et al.*, 1996). The mouse chromosome 8E1 is syntenic to the human chromosome 16q (O'Brien and Graves, 1991). In the human *ATBF1* gene, several 5′ exons have been identified (Miura *et al.*, 1995), and the characterized exon-intron junction sequences between exon 2 and exon 3, and between exon 3 and exon 4 are shown to be the same as that of the mouse counterpart. These data further indicate that the mouse and the human *ATBF1* genes share high similarities.

The mouse *ATBF1* gene is expressed in the neuronally differentiated P19 mouse embryonal carcinoma cells (Ido *et al.*, 1994). Likewise the human

ATBF1 gene is expressed in the neuronally differentiated NT2/D1 human embryonal carcinoma cells (Miura *et al.*, 1995). Furthermore, the 5'-flanking region of human *ATBF1-A* transcript showed promoter activity in neuronally differentiated P19 cells (Miura *et al.*, 1995). Based on these observations, it is very likely that the 5'-flanking regions of the mouse and the human *ATBF1-A* may contain similar transcription factor binding sites. However, sequence analyses show (Figure 21) that there are different potential transcription factor binding sites in the mouse sequence as are identified in the human sequence. Since some sequence similarity exists at several locations in the 5'-flanking regions in mouse and in human(Figure 21), it is possible that other as yet uncharacterized transcription factor(s) regulate(s) both the mouse and the human *ATBF1* gene expression. On the other hand, the 5'-boundary of human exon 2 (the transcription initiation site of *ATBF1-A*) was obtained by ribonuclease protection assays, by means of which the transcription initiation site can not be conclusively determined. In addition, without the genomic DNA sequence of the corresponding region, the transcription initiation site of the human *ATBF1-A* can not be precisely identified. In the promoter analysis of the human *ATBF1-A* transcript, a 5.5-kb fragment of the 5'-flanking region was used and it showed promoter activity in neuronally differentiated P19 cells (Miura *et al.*, 1995). That 5.5-kb fragment probably includes all the regulatory regions required for the transcription of human *ATBF1-A*. The human *ATBF1-A* promoter must be within that 5.5-kb fragment but the precise

location needs to be identified. For the putative promoter regions (about 200 base pairs in length) in mouse and in human, it is important that the promoter activities of these regions should be analyzed before further sequence comparisons are carried out.

Sequence analysis showed (Figure 21) that there were three potential MZF-1 binding sites within 230-nucleotides upstream of exon 2 (the first exon of the *ATBF1-A* transcript) of the mouse *ATBF1* gene. MZF-1 protein contains 13 zinc fingers and, the *MZF-1* gene is preferentially expressed in myeloid leukemia cell lines with the highest mRNA levels observed in HL-60 cells after being induced into differentiation with retinoic acid (Hromas *et al.*, 1991). Our laboratory previously showed that the *ATBF1* gene was expressed in differentiated HL-60 cells which were treated with retinoic acid (Tamura and Tamaoki, personal communications). These observations implicate MZF-1 in regulating *ATBF1* gene expression.

The mouse *ATBF1* gene promoter needs to be identified and characterized so that the regulation of *ATBF1* gene expression can be studied in the future. The *in vivo* study of *ATBF1* gene expression during embryogenesis can be carried out by generating transgenic mice with the *ATBF1* promoter fused to a reporter gene.

6.2 The Expression of Sense- and Antisense-*ATBF1* in P19 Cells

Our laboratory previously showed that *ATBF1* mRNA was expressed in the neuronally differentiated P19 cells treated with retinoic acid (Ido *et al.*, 1994). The expression of sense- and antisense-*ATBF1* in undifferentiated P19 cells were used to analyze the function of the *ATBF1* gene expression during neuronal differentiation of P19 cells.

The results showed that overexpression of antisense-*ATBF1* in P19 cells blocked the neuronal differentiation induced by retinoic acid. In all seven clones of antisense-*ATBF1* transfectants, the endogenous *ATBF1* mRNA was expressed after retinoic acid treatment. The clones of antisense-*ATBF1* transfectant showed relatively low levels of *ATBF1* mRNA expression compared to that of the control, although the expression levels varied from clone to clone. The possible mechanism of the blockage of neuronal differentiation by antisense-*ATBF1* in P19 cells is that normal processing and/or transport of the sense-*ATBF1* RNA is blocked by the formation of sense-antisense *ATBF1* RNA duplexes in nucleus, thus, leading to the prevention of ATBF1 protein from being translated. This can be elucidated by analyzing the ATBF1 protein levels in those clones of antisense-*ATBF1* transfectants treated with retinoic acid.

In all seven clones of antisense-*ATBF1* transfectants, however, very few neuron-like cells were visualized upon retinoic acid treatment. The gene expression analyses showed that there were still some levels of *MASH1* and

GAD67 mRNA, although very low, present in the antisense-*ATBF1* transfectants. These may be due to the property of P19 cells as shown by McBurney *et al.* (1994b). In general, it is difficult to isolate clones of P19 cells stably expressing transfected genes and, even in an isolated colony which contains the transgene, the introduced gene is expressed only in some but not all cells. Therefore, the clones of the antisense-*ATBF1* transfectants may be a mixed population of P19 cells with and without antisense-*ATBF1* expression, although all those cells carry the antisense-*ATBF1* transgene.

Inhibition of retinoic acid-induced neuronal differentiation by blocking the *ATBF1* expression suggests that the *ATBF1* gene expression is required for neuronal differentiation of P19 cells induced by retinoic acid. The results shown in Section 5.2.1 indicate that *ATBF1* gene expression is required during neuronal differentiation of P19 cells treated with retinoic acid. On the other hand, retinoic acid treatment of P19 cells can give rise to the expression of many genes and some of them may be required for the neuronal differentiation other than *ATBF1*. Therefore, overexpression of *ATBF1* alone may not be sufficient for P19 cells to commit to fully neuronal differentiation. The results shown in Section 5.2.2 indicate that expression of sense-*ATBF1* in P19 cells prevents cells from proliferation. These cells showed a certain degree of differentiation.

The effect of *ATBF1* on P19 cells overexpressed with sense-*ATBF1* may not entirely reflect the physiological function of *ATBF1* in the mouse. The P19

cells transfected with sense-*ATBF1-A* expression vector probably produced abnormal levels of *ATBF1-A* mRNA, which might lead to abnormal P19 cell behavior. In P19 cells, enforced expression of antisense-*ATBF1* can block the retinoic acid-induced neuronal differentiation. However, it is possible that other proteins having redundant functions of ATBF1 exist in mouse. In this case, blocking *ATBF1* may have no effect on the differentiation and development. This possibility may be investigated by analyzing *ATBF1* knockout mice.

ATBF1 protein contains many potential functional domains and, therefore, could have multiple functions. The target gene(s) which is/are regulated by ATBF1 during neural development remain(s) to be identified. In P19 cells, *ATBF1* mRNA reaches its highest level as early as 3 hours following retinoic acid treatment (Miura and Tamaoki, unpublished). The established P19 cell line with antisense-*ATBF1* expression provides us with an approach to further characterize the ATBF1 function as a transcription factor to regulate its target gene(s) along the neuronal differentiation pathways in P19 cells.

CHAPTER VII: CONCLUSIONS

The mouse *ATBF1* gene was cloned and characterized. This gene contains 10 exons over 170 kb of gene sequence. The P19 cell line expressing antisense-*ATBF1* has been established, in which the neuronal differentiation induced by retinoic acid is blocked. The enforced expression of sense-*ATBF1-A* in P19 cells results in preventing cells from proliferation, and these cells show a certain degree of differentiation. This is the first time that *ATBF1* gene expression has shown to be required during the retinoic acid-induced neuronal differentiation of P19 cells. These results are consistent with the *ATBF1* gene expression patterns in mouse and in P19 cells.

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