Cloning and Characterization of an ATBF1 Isoform That Expresses in a Neuronal Differentiation-dependent Manner*

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The human ATBF1 cDNA reported previously, now termed ATBF1-B, encodes a 306-kDa protein containing 4 homeodomains and 18 zinc fingers including one pseudo zinc finger motif. Here, we report the isolation of a second ATBF1 cDNA, 12 kilobase pairs long, termed ATBF1-A. The deduced ATBF1-A protein is 404 kDa in size and differs from ATBF1-B by a 920-amino acid extension at the N terminus. Analysis of 5'-genomic sequences showed that the 5'-noncoding sequences specific to ATBF1-A and ATBF1-B transcripts were contained in distinct exons that could splice to a downstream exon common to the ATBF1-A and ATBF1-B mRNAs. The expression of ATBF1-A transcripts increased to high levels when P19 and NT2/D1 cells were treated with retinoic acid to induce neuronal differentiation. Preferential expression of ATBF1-A transcripts was also observed in developing mouse brain. Transient transfection assays showed that the 5.5-kilobase pair sequence upstream of the ATBF1-A-specific exon (exon 2) supported expression of the linked chloramphenicol acetyltransferase gene in neuronal cells derived from P19 cells but not in undifferentiated P19 or in F9 cells, which do not differentiate into neurons. These results showed that ATBF1-A and ATBF1-B transcripts are generated by alternative promoter usage combined with alternative splicing and that the ATBF1-A-specific promoter is activated during neuronal differentiation.

The ATBF1 (AT motif binding factor 1) cDNA was first isolated from HuH-7 human hepatoma cells based on the ability of its product to bind to an AT-rich enhancer element of the human α-fetoprotein gene (AFP) (1). This protein is characterized by a large size (306 kDa) and the presence of four homeodomains (I–IV) and 18 zinc fingers including 1 pseudo zinc finger motif. Transient transfection assays showed that ATBF1 suppressed the activity of the AT-rich element of the enhancer and promoter of the AFP gene but not those of the albumin gene (2). This effect is thought to be mediated by specific interaction between homeodomain IV of the ATBF1 molecule and the AT-rich element of the AFP gene (1, 2).

Besides ATBF1, there are three transcription factors that contain both homeodomain and zinc finger motifs. Chicken δEF1 has one homeodomain and nine zinc finger motifs. This protein has been shown to repress activities of the DC segment of the α-crystallin enhancer (3, 4) and the E2 element of the immunoglobulin κ chain and muscle creatine kinase enhancer (5). Its expression pattern in chicken embryos suggests that δEF1 plays a role in mesoderm development and embryonic myogenesis (3–5). Drosophila ZFH-1 contains one homeodomain and nine zinc finger motifs (6). It is expressed in the embryonic mesoderm and nervous system (7), and phenotypic analysis of loss-of-function mutant embryos has shown that ZFH-1 determines cell fate or positioning (8). Drosophila ZFH-2 contains 3 homeodomains and 16 zinc finger motifs (6) and is expressed almost exclusively in the central nervous system of Drosophila embryos (7). It binds to the RCS element of the opsin gene through homeodomain III (6, 9) and activates the SERK element of the DOPA decarboxylase gene through homeodomain II (6, 9).

Sequence comparison shows that homeodomains I, II, and III of ATBF1 are 77, 69, and 61% identical with the corresponding homeodomains of ZFH-2 (9, 10). Homeodomain IV of ATBF1 is 46% identical with homeodomain III of ZFH-2. 13 zinc fingers of ATBF1 and ZFH-2 show identities ranging from 22 to 89%. All of these homologous domains are colinearly arranged in the ATBF1 and ZFH-2 molecules. These observations suggest that ATBF1 and ZFH-2 may have similar functions, raising the possibility that ATBF1 plays a role in mammalian central nervous system development. In fact, the level of ATBF1 transcripts is highly elevated in embryonic and neonatal mouse brains (11). In addition, ATBF1 expression is activated during retinoic acid-induced neuronal differentiation of P19 embryonal carcinoma cells (11).

In this paper, we report the isolation of a second ATBF1 cDNA, termed ATBF1-A, which is 3.3 kb longer than the previously reported clone, now termed ATBF1-B. We show that ATBF1-A transcripts are generated by alternative splicing and alternative usage of a promoter region that is activated in neurally differentiating cells.

MATERIALS AND METHODS

Cells—M426 human fibroblasts were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. P19 mouse embryonal carcinoma cells, kindly provided by Dr. M. W. McBurney, were grown in 1 The abbreviations used are kb, kilobase pairs; bp, base pairs; CAT, chloramphenicol acetyltransferase; RT-PCR, reverse transcriptase-linked polymerase chain reaction; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); UMS, upstream mouse sequence.
RESULTS

Isolation of ATBF1-A cDNA—Screening of λCEV15 cDNA library with the λ488 human ATBF1 cDNA (λ) as a probe resulted in the isolation of λE and λME clones (Fig. 1). These clones were then used as probes to screen ATBF1-A and ATBF1-B cDNA libraries for Northern blot analysis, RNase protection assays, and screening genomic libraries.

Total RNA (5 μg) was hybridized with 5 × 10^6 cpm of probe in 40 μl
of the 32P-labeled probes were obtained by hybridizing with λME and λE probes. These probes were labeled with 32P-dCTP to produce a radioactive probe for use in Northern blot analysis. Northern blots were hybridized with λME and λE probes to detect ATBF1-A and ATBF1-B transcripts. The probe was detected as a 1.5-kb band in both human and mouse samples. Glyceraldehyde-3-phosphate dehydrogenase mRNA was used as an internal control.

The day of adding retinoic acid to the medium was assigned day 0. F9 mouse embryonal carcinoma cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. To induce neuronal differentiation, the cells were incubated with 10 nM retinoic acid for 4 days in the presence of 0.5 μg/ml retinoic acid.

To prepare MD14 probe for RNase protection assays, oligonucleotide probes were used for Northern blot analysis. RNase protection assays, and screening genomic libraries. Oligonucleotide probes were used for Northern blot analysis. RNase protection assays, and screening genomic libraries.

Neuronal Cell-associated Expression of ATBF1 Gene

ATBF1-A cDNA. Open bar, full-length ATBF1-A cDNA; solid bars, partial ATBF1-A and ATBF1-B cDNA clones, and an RT-PCR product. Short bars with vertical lines at both ends indicate probes used for Northern blot analysis, RNase protection assays, and screening genomic libraries.
**Fig. 2. Nucleotide sequence of ATBF1-A cDNA and deduced amino acid sequence.** Dotted lines, zinc finger motifs; solid lines, homeodomains; (a), 5'-end of ME cDNA; (b), exon 2-exon 3 junction; (c), DEAH box-like sequence; (d), vitamin K-dependent carboxylase recognition motif; (e), casein kinase II phosphorylation motif; (f), DEAD box-like sequence; (g), SAT box-like sequence; (h), RNA-binding motif; (j), exon 3-exon 4 junction (alternative splicing site); (k), translation initiation codon of ATBF1-B; (l), ATP-binding site; (m), nuclear targeting sequence.
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24 nucleotides deleted in HuH-7 ATBF1 cDNA (1);
variable number of GGC triplet. The termination codon and two potential polyadenylation signals are underlined.

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(m), 24 nucleotides deleted in HuH-7 ATBF1 cDNA (1); (n), variable number of GGC triplet. The termination codon and two potential polyadenylation signals are underlined.
represented a new cDNA sequence with a longer coding sequence. To complete sequence analysis of the new ATBF1 cDNA, we isolated a short region between λME and λI by RT-PCR. The sequence of the RT-PCR product was identical with the corresponding region of ATBF1 previously reported. Combining λME, λI, and the RT-PCR product, the sequence of the new ATBF1 cDNA was constructed (Fig. 2). This cDNA is referred to as ATBF1-A and the ATBF1 cDNA reported previously as ATBF1-B.

The ATBF1-A cDNA encodes a protein of 3703 amino acids with a molecular mass of 404 kDa. This protein contains 4 homeodomains and 23 zinc finger motifs including 1 pseudo zinc finger motif. ATBF1-A is longer than ATBF1-B by 920 amino acids added at the N terminus. This extended region contains five zinc fingers, two acidic domains (amino acids 110–145 and 432–510), and one region rich in both serine and threonine (39%, amino acids 396–431) (Fig. 3). In addition, computer analysis (22) revealed several sequences that are similar to consensus motifs of RNA and DNA helicases. They include a DEAH box-like sequence, SfrVFDvrHk (amino acids...
transcripts was very weak but visible after long exposure. In retinoic acid-treated neuroblastoma cells (Fig. 4A), small amounts of ATBF1-A and ATBF1-B transcripts were detected. The absolute levels of these transcripts were low. Similarly, the expression of ATBF1 transcripts was much less, giving rise to a 50:1 ratio of ATBF1-A and ATBF1-B transcripts in differentiated cell lines described above. The results showed high levels of ATBF1 expression, predominantly in the form of ATBF1-A, in brains of 15-day-old embryos and 1-day-old neonates (Fig. 5). Expression of ATBF1 transcripts was decreased in brains of 5- and 7-day-old neonates.

Identification of Alternatively Spliced Exons—To explore the mechanism by which ATBF1-A and ATBF1-B mRNAs are generated, we analyzed 5′-genomic sequences encompassing the 5′-ends of these transcripts. Two genomic clones were isolated. One clone, \( \lambda \)FIX-17 (Fig. 6), could hybridize to ME and E probes representing the 5′-noncoding sequences of ATBF1-A and ATBF1-B mRNAs, respectively (Fig. 1). The other clone, \( \lambda \)FIX-13 (Fig. 6), could hybridize to ME probe and also to MEM probe, which represents a coding region common to ATBF1-A and ATBF1-B (Fig. 1).

To analyze more precisely the relative amount of ATBF1-A and ATBF1-B transcripts, we conducted RNase protection assays using the \( \lambda \) clone as a probe detected faint bands in the region of 10–13 kb in M426, HuH-7, and huH-1 human cell lines (Fig. 4A, lanes 1–3). These bands corresponded to ATBF1-A and ATBF1-B transcripts, but the levels of their expression were very low in all these cell lines. Similarly, P19 embryonal carcinoma cells contained small amounts of ATBF1-A and ATBF1-B transcripts (Fig. 4A, lane 4). However, when P19 cells were treated with retinoic acid to induce neuronal differentiation, there were large increases in the levels of these transcripts (Fig. 4A, lane 5) (11).

The results showed that exon 1 carried most of the 5′-noncoding sequence of ATBF1-A mRNA and exon 2 that of ATBF1-B mRNA (Fig. 2). Exon 3 in \( \lambda \)FIX-13 contained the remaining ATBF1-A noncoding sequence (49 bp) followed by the ATBF1-A protein coding sequence. Exon 4 contained the remaining ATBF1-B noncoding sequence (23 bp) followed by the ATBF1-B protein coding sequence. Analysis of exon-intron junctions confirmed the presence of the consensus splice donor (GT) and acceptor (AG) sites (results not shown). In addition, it was found that exon 4 could splice to either exon 1 or exon 3 (Fig. 6). In the former case, the ATBF1-B mRNA sequence is generated, and in the latter case, the ATBF1-A mRNA sequence is generated with the ATBF1-B noncoding sequence in exon 4 becoming a part of the ATBF1-A coding sequence (Fig. 2). These results are consistent with the mechanism that ATBF1-A and ATBF1-B mRNAs are generated by alternative splicing.

Determination of Promoter Activity—The presence of distinct 5′ exons specific to ATBF1-A and ATBF1-B sequences suggested that two different promoters may be used to initiate transcription of the two types of ATBF1 mRNAs. To examine whether the 5′-flanking sequences of exons 1 and 2 have transcriptional activity, they were linked to the CAT reporter gene (Fig. 8A) and transfected into undifferentiated and neurally differentiated P19 cells. The 5.5-kb 5′-flanking sequence of ATBF1-A-specific exon 2 (pA5.5-CAT) supported CAT expression in differentiated P19 cells (Fig. 8B, lane 7) but not in undifferentiated P19 cells (Fig. 8B, lane 3). No CAT activity was expressed in F9 embryonal carcinoma cells with or without retinoic acid treatment (Fig. 8C). Treatment of F9 cells with retinoic acid resulted in induction of endodermal cells but not...
neuronal cells. pA5.5-CAT expressed CAT activity in other neuronal cells, such as Neuro-2a and retinoic acid-treated NT2/D1 cells, but not in non-neuronal cells, such as HepG2 (results not shown). These results showed that the 5' flanking sequence of exon 2 has neuronal cell-specific promoter activity.
The 3.5-kb 5' flanking sequence of ATBF1-B-specific exon 1 (pB3.5-CAT), on the other hand, did not support CAT expression in undifferentiated or differentiated P19 cells or any other cell lines described above.

**DISCUSSION**

We report here the isolation of a second human ATBF1 cDNA, termed ATBF1-A. This cDNA differs from the previously reported ATBF1 cDNA, termed ATBF1-B, by an extra 3.3-kb sequence at the 5'-end. Since the extended region can

![FIG. 4. Analysis of ATBF1-A and ATBF1-B transcripts in various cell lines.](image)

![FIG. 5. Analysis of ATBF1-A and ATBF1-B transcripts in developing mouse brain.](image)

![FIG. 6. Exon-intron organization of 5'-region of the ATBF1 gene.](image)

![FIG. 7. RNase protection mapping of the 5'-ends of exons 1 and 2.](image)
encode five additional zinc fingers, helix-loop-helix-related sequences, and domains rich in acidic amino acids and serine and threonine, it is possible that ATBF1-A may have functions not associated with the ATBF1-B isoform.

RNase protection assays detected two sizes of mRNAs corresponding to ATBF1-A and ATBF1-B in various cell lines and mouse brain. In all cases, ATBF1-A transcripts were present in larger amounts than ATBF1-B transcripts, but the absolute levels of these transcripts were low in various cell lines and adult mouse brain. Similarly, the amounts of these ATBF1 mRNAs were low in undifferentiated P19 and NT2/D1 embryonal carcinoma cells. However, neuronal cells derived from these cells by treatment with retinoic acid contained much higher levels of these transcripts. The increased expression was particularly pronounced with ATBF1-A transcripts, relative to ATBF1-B transcripts. Preferential expression of the ATBF1-A form was also observed in developing mouse brain.

The isolation and analysis of 5'-genomic sequences defined the basis for the generation of the two species of ATBF1 mRNAs. We found that 5'-noncoding sequences specific to ATBF1-A and ATBF1-B mRNAs were contained in distinct exons. We also found a downstream exon that can splice to either the ATBF1-A- or ATBF1-B-specific exon. These results showed that alternative splicing is involved in the generation of the two ATBF1 isoforms. Transient transfection experiments showed that the 5'-flanking region of exon 2, the first exon specific to ATBF1-A, exhibited promoter activity in neuronal cells derived from P19 cells but not in undifferentiated P19 cells. No promoter activity was expressed in F9 embryonal carcinoma cells or other non-neuronal cells. These results indicate that the 5'-flanking sequence of the ATBF1-A-specific exon functions as a neuronal cell-specific promoter. It is likely that this promoter is responsible for the observed increase in ATBF1-A transcripts in association with neuronal differentiation of P19 or NT2/D1 cells. Recent CAT assays showed that the 5'-flanking sequence of exon 2 could be shortened to 300 bp without losing promoter activity in neuronal cells. This indicates that the relatively short promoter region is sufficient to confer neuronal cell specificity. Computer search has revealed that the 300-bp region contains putative binding sites for several transcription factors, including c-fos, AP-2, SP-1, and zif268 (also known as egr-1, krax24, or NGF-A). Whether these factors are in fact involved in promoter activity in neuronally differentiating P19 cells is being investigated in our laboratory. Although it is possible that ATBF1-B transcripts are produced by a differentially regulated promoter, we have not yet detected transcriptional activity associated with the 5'-flanking region of exon 1 (ATBF1-B-specific exon). Our failure to detect promoter activity in this region is likely due to very low levels of ATBF1-B expression in the cell lines used for CAT transfection assays. Obviously, these cells are deficient of certain transcription factors important for the synthesis of the ATBF1-B isoform. We are currently searching for cell lines expressing higher levels of ATBF1-B transcripts to be used for determination of ATBF1-B-specific promoter activity.

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Neuronal Cell-associated Expression of ATBF1 Gene

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