Isolation and Characterization of MRF-1, a Brain-derived DNA-binding Protein with a Capacity to Regulate Expression of Myelin Basic Protein Gene*

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The 5-flanking region of the myelin basic protein (MBP) contains several regulatory elements that differentially contribute to the cell type-specific transcription of MBP in cells derived from the central nervous system. The distal regulatory element, termed MB3, had previously been shown to have characteristics of a cell type-specific enhancer element and bind to multiple brain-derived nuclear proteins in vitro. We now report the isolation of a recombinant cDNA clone, named myelin regulatory factor-1 (MRF-1) from a mouse brain expression library that encodes a novel protein which interacts with the MB3 domain. Computer-assisted analysis of MRF-1 revealed substantial sequence homology in the central and the COOH-terminal regions of this protein with the previously identified splicing factor SC35. Co-transfection studies indicated that MRF-1 increases transcription of the MBP promoter in glial cells and that this activation requires an intact MRF-1-binding site within the MB3 region. MRF-1 cDNA hybridized to three RNA species 1.8, 2.5, and 3.0 kilobases which are expressed in all tissues analyzed. The gene encoding MRF-1 is located on the distal half of mouse chromosome 11 in a region where the human homolog would be predicted to reside on human chromosome 17.

In the murine brain, myelination of central nervous system and concomitant synthesis of myelin proteins such as myelin basic protein (MBP) occurs during postnatal development (for review, see Ref. 1). The most rapid period of myelin accumulation in mouse brain occurs between 10–25 days after birth, which overlaps with peak accumulation of MBP synthesis at 15–20 days postnatal (2–4). The temporal regulation of myelinoogenesis and expression of the MBP gene in the brain occurs in oligodendrocytes, which are believed to be independent of axonal contact (5, 6). Several studies have led to the conclusion that the cell type- and stage-specific expression of MBP gene is regulated primarily at the level of transcription (7–11). However, the details of the regulatory pathways that govern the programmed transcription of MBP are not fully understood. Studies on other eukaryotic promoters have led to the assumption that the basal regulatory sequences have little role in cell type-specific or developmentally regulated transcriptional initiation of the inducible promoters. It is postulated that the protein factors which bind these basal sequences are constitutively active and that specific transcriptional regulation is mediated by upstream regulatory elements which bind proteins that, in turn, interact with the basal transcriptional complex in order to exert their effects (12). Analysis of MBP upstream regulatory region by transient transfection and in vitro transcription assays revealed that the MBP regulatory region is composed of multiple cis-acting motifs positioned within 400 bp upstream of the transcription start site, some of which stimulate transcription preferentially in glial cells or cell-free extracts derived from brain tissues (13–16). The proximal element, termed MB1, spans the sequences from −14 to −50 and confers glial specificity to a heterologous promoter in transient transfection assay (13, 14). Because of its location directly upstream of the transcriptional start site, this element was initially thought to be involved in a direct interaction with basal transcription factor(s) including TFIIID (17). Sequence analysis of this region, however, indicates that the MB1 element lacks a classical TFIIID-binding site and has the capacity to interact with multiple proteins derived from brain tissues (13, 14, 18, 19). The distal regulatory element between −93 to −130 bp, named MB3 has the ability to stimulate transcription of a heterologous viral promoter in astroglial cells (14) and modulate expression of its own promoter in neuronal cells (15, 20). A binding site for the CTF/NF1 family of transcriptional activators has been identified within MB3, and a novel CTF/NF1 family member has been identified which can stimulate transcription of the MBP gene through this element in transient transfection assays (20). Binding sites for the CTF/NF1-like proteins are also found in the glial fibrillary acidic protein gene, a gene which is expressed exclusively in non-myelinating glial cells and astrocytes. In addition, CTF/NF1-like binding sites are found in the human neurotropic JC virus which is expressed in both astrocytes and oligodendrocytes (21). These results, along with the notion that CTF/NF1 sites are absent in the other myelin-specific gene promoters such as proteolipid protein (22), suggest that the presence of CTF/NF1-binding sites may not be sufficient for appropriate expression of oligodendrocyte-specific promoters. Previously, we utilized a series of DNA-binding techniques and demonstrated that the sequences between −94 to −130 interact with nuclear proteins from mouse brain at various stages of development (19). Methylation interference experiments have allowed the identification of the binding motif apart from the CTF/NF1 of MB3 that forms
MBP, Transcription Factor, DNA-binding Protein

complexes with the brain nuclear factors. We now report the isolation of a complete cDNA clone derived from mouse brain RNAs that encodes a protein with the ability to bind to the MB3 regulatory region and to increase transcription of the MBP promoter in cells derived from the central nervous system.

EXPERIMENTAL PROCEDURES

Screening of a Mouse cDNA Library—A mouse brain (15-day postnatal) cDNA library in agt11 was screened according to the method described previously (23). Briefly, bacterial strain Y1990 was incubated with recombinant phages, plated, and incubated at 42°C for 3-5 h. The nitrocellulose filters of colony plaques in 10 ml IPTG were overlaid in the plates and incubated at 37°C for 24 h. The filters containing phages (approximately 60,000) were incubated in binding buffer containing 25 mM HEPES, pH 8.9, 25 mM NaCl, 5 mM MgCl2, and 0.1% diethiothreitol supplemented with 6 x guanidine hydrochloride. After 30 min, filters were washed in binding buffer with no guanidine hydrochloride, after which they were incubated in binding buffer supplemented with 5% non-fat dry milk. Binding was carried out in the buffer containing 0.25% non-fat dry milk and 32P-labeled double-stranded MB3 oligonucleotide representing rat MBP sequence with G → T alteration at nucleotide -123. Filters were then washed in binding buffer with 0.25% milk three times. The MBP oligonucleotide is as follows: 5'-TGC-CTTGCAGATTGCCCCGACCAGCGGG-3'; 3'-AGCAGATACTTACGGTTGGTGGTACGTGCCC-5'. Two positive clones were plaque purified and the inserts were subcloned in Bluescript II for DNA sequencing. The sequences of the clones were carried out using the dyeoxy chain termination method (24) which proceeded from both ends. The sequences were verified by sequencing of the opposite strand. Sequence analysis was performed on GCG sequence analysis software according to the manufacturer's instructions (New England Biolabs).

For expression of MRF-1 in eukaryotic cells, the MRF-1 fragment was placed in a pcMV vector which contains an initiator methionine codon and the translation consensus sequence downstream of the eukaryotic promoter derived from a cytomegalovirus (CMV) early gene. The pcMV-MRF-1 cDNA fragment from the recombinant phage was cloned into the EcoRI site of pMAL-CRI vector (New England Biolabs). The plasmid was introduced into HB101, and the transformed cells were cultured in L-broth to OD600 = 0.5 after which IPTG (0.3 mM) was added, and cultures were incubated for 4-5 h at 37°C. The recombinant phage was cloned into the EcoRI site of MRF-1 in a bacterial system, the EcoRI MRF-1 cDNA fragment from MRF-1 in eukaryotic cells, the MRF-1 fragment was isolated of which two, named Myelin Regulatory Factor 1 and MRF-2, revealed sequence length polymorphism markers were detected by amplifying DNA oligonucleotides used for detecting the simple sequence length polymorphism marker were made using an Applied Biosystems model 384 DNA synthesizer. Simple sequence length polymorphism markers were detected by amplifying genomic DNA from N, animals using the specified DNA oligonucleotide probes (31) and Taq DNA polymerase as described (30). At least one restriction fragment length polymorphism was identified for each of the probes tested. The probes used and the sizes of the genomic restriction fragments detected by each probe are listed in Table I.

RESULTS AND DISCUSSION

isolation of a Complementary DNA That Encodes an MB3-binding Protein—A 15-day postnatal mouse brain cDNA library cloned in agt11 was screened using a double-stranded oligonucleotide probe spanning the distal regulatory element of MBP that resides between -93 to -130 with respect to the RNA initiation site. In the initial screening, three positive clones were isolated which two, named Myelin Regulatory Factor 1 and 2 (MRF-1 and MRF-2), remained positive during the subsequent screenings. Fig. 1A illustrates the cDNA sequence and deduced amino acid sequence of MRF-1. Sequencing of the cDNA fragments from MRF-1 and MRF-2 revealed sequence identity between these two clones and the presence of 1771 nucleotides with an open reading frame for a protein of 146 amino acids in length. A GenBank homology search of the deduced amino acid sequence indicated that the MRF-1 cDNA encodes a novel proline-glycine and serine-arginine rich protein with the highest degree of sequence homology to the mammalian splicing factor SC35 (33). The SC35 protein is believed to
be critical for assembly of the ATP-dependent splicing complex A and interaction with the other participant proteins bound to the 5' and 3' splice sites during spliceosome assembly (34, 35). The SC35 consisting 221 amino acid residues is characterized by the presence of a ribonucleoprotein (RNP) RNA binding motif at the NH₂ terminus, proline-glycine-rich sequence in the middle, and multiple serine-arginine-rich domains at the COOH terminus (35). Similar structural organization was ob-

**Fig. 1. Sequence analysis of cloned MRF-1 cDNA.** A, nucleotide sequence of MRF-1 and the deduced primary structure of the protein. The region homologous to SC35 is underlined. B, comparison of the structural features of MRF-1 and SC35. The positions of the proline-glycine-rich and the serine-arginine dipeptide repeat regions are shown in the central and COOH-terminal regions of the MRF-1 and SC35 proteins. The RNP1 and RNP2 boxes indicate the positions of the RNA binding consensus sequence at the NH₂-terminus of SC35 which are absent in MRF-1.
MBP, Transcription Factor, DNA-binding Protein

A. Band-shift analysis of bacterially produced maltose-binding protein-MRF-1 fusion protein probed with the MB3 double-stranded oligonucleotide. Binding reactions were carried out in the absence (lane 1) or presence of 50 and 100 ng of the competitor oligonucleotides from MB3 (lanes 2 and 3); unrelated DNA fragment of similar size (lanes 4 and 5); coding strand of MB3 (lanes 6 and 7); and non-coding strand of MB3 (lanes 8 and 9). B, methylation interference assay of MRF-1 binding to MB3 sequences. The coding and non-coding strands of MB3 were 5'-end-labeled and after annealing to the complementary unlabeled strand treated with DMS, and used in protein-binding reactions using purified MRF-1. Free (F) and bound (B) DNA were isolated and chemically cleaved and analyzed on a denaturing urea gel. Modification of the G residues with the most significant effects on binding are shown by the arrows on the gels and marked by an asterisk on the MB3 sequence. C, sequence specificity of MRF-1 for the MB3 sequence. The bacterially produced MRF-1 was prepared and used in the band-shift assay in the absence (lane 1) and presence of wild type (MB3a) competitor derived from MB3 sequence (lanes 2 and 3); or mutant competitor DNA (Mut.2) with altered nucleotides (G → T) at the critical binding sites (shown in panel B).

FIG. 2. DNA binding specificity of bacterially produced MRF-1. A, band-shift analysis of bacterially produced maltose-binding protein-MRF-1 fusion protein probed with the MB3 double-stranded oligonucleotide. Binding reactions were carried out in the absence (lane 1) or presence of 50 and 100 ng of the competitor oligonucleotides from MB3 (lanes 2 and 3); unrelated DNA fragment of similar size (lanes 4 and 5); coding strand of MB3 (lanes 6 and 7); and non-coding strand of MB3 (lanes 8 and 9). B, methylation interference assay of MRF-1 binding to MB3 sequences. The coding and non-coding strands of MB3 were 5'-end-labeled and after annealing to the complementary unlabeled strand treated with DMS, and used in protein-binding reactions using purified MRF-1. Free (F) and bound (B) DNA were isolated and chemically cleaved and analyzed on a denaturing urea gel. Modification of the G residues with the most significant effects on binding are shown by the arrows on the gels and marked by an asterisk on the MB3 sequence. C, sequence specificity of MRF-1 for the MB3 sequence. The bacterially produced MRF-1 was prepared and used in the band-shift assay in the absence (lane 1) and presence of wild type (MB3a) competitor derived from MB3 sequence (lanes 2 and 3); or mutant competitor DNA (Mut.2) with altered nucleotides (G → T) at the critical binding sites (shown in panel B).

served in other splicing factors including SF2/ASF and tra 2 (36–38). As schematized in Fig. 1B, MRF-1 lacks the RNP domains, and in the middle it contains 15 amino acid residues entirely different from those in SC35. The MRF-1 contains no other commonly recognized regulatory domains such as leucine zipper, zinc finger, helix-turn-helix, etc. Results from our RNA binding studies indicated that MRF-1 in addition to binding DNA has the capacity to interact with RNA molecules.2

Interaction of MRF-1 with MB3 Sequences—To study the DNA binding activity of MRF-1, a 1,771 nucleotide DNA fragment of the cDNA isolate was placed into bacterial expression vector pMAL-CR1 which led to the generation of a maltose-binding protein (MBP)-MRF-1 fusion protein. To examine the specificity of DNA-protein interaction, band-shift assays were performed using crude extract obtained from the bacterial cells treated with IPTG which allows for overexpression of the fusion protein. As shown in Fig. 2A, the (MBP)-MRF-1 fusion protein binds to the MB3 oligonucleotide probe and forms the shifted band (lane 1). The intensity of the complex was decreased in the presence of an excess amount of the unlabeled double-stranded MB3 (lanes 2 and 3) and remained virtually unchanged when excess amounts of a heterologous unlabeled DNA fragment (see “Experimental Procedures”) were included in the binding reaction (lanes 4 and 5), demonstrating that the interaction of MRF-1 with MB3 is sequence-specific. Moreover, inclusion of the single-stranded DNAs representing the coding and non-coding strand of MB3 in the reaction mixture effectively abrogated assembly of the MRF-1-MB3 complex, suggesting that MRF-1 has the capacity to interact with the double- and single-stranded DNA fragments from the MB3 region.

To determine which purine bases in the MB3 motif are in close contact with the MRF-1 we performed methylation interference assays. A 32P end-labeled MB3 DNA was first modified with DMS and then incubated with bacterially produced MRF-1. The DNA-protein complexes were resolved by electrophoresis on a native 9% polyacrylamide gel. DNA present in the bound and free fractions was eluted from the gel and treated with piperidine to cleave the DNA backbone adjacent to N2-methylated guanosyls and N6-methylated adenosyls (25). Comparison of the bound and free DNA showed that the DNA binding capability of the MRF-1 complex was reduced by the presence of methyl groups on the 2 and 3 guanosine residues of the coding and non-coding strands, respectively, (Fig. 2B). Site-directed mutagenesis of the affected guanosine residues within the MB3 probe followed by competition band-shift assay using wild type (MB3a) or the mutant (mut.2) competitors revealed
that the protected guanosine residues are critical for formation of a stable MRF-1-MB3 complex (Fig. 2C).

Transcriptional Activity of MRF-1 in Glial Cells—To assess transcriptional activity of the MRF-1 protein, a complete 1,771-bp DNA fragment containing the MRF-1 open reading frame was cloned in a eukaryotic expression vector (pCMV) which contains the eukaryotic promoter from cytomegalovirus early genome. For efficient translation of the MRF-1, an initiator methionine codon (AUG) and the consensus flanking sequences for translation were engineered in frame with the MRF-1 cDNA between the CMV promoter and the MRF-1 cDNA insert. Indicator plasmids contained the bacterial CAT gene under the control of the myelin basic protein promoter sequence spanning from −402 bp upstream of the transcription start site to +21 bp (pMBP402) or its mutant (pMBP402 mut.3) which has base substitutions in the critical G residues (G → T) within the MB3 domain for binding of MRF-1. Examination of the transcriptional activity of the wild type and mutant constructs by transient transfection of glial cells revealed the importance of the MRF-1-binding site in the overall transcriptional activity of the MBP promoter (Fig. 3A). Co-transfection of the glial cells with pMBP402 plus pMRF-1 consistently enhanced the basal CAT activity of the MBP promoter approximately 3-fold relative to the basal level obtained in the control cells (Fig. 3B, left panel, compare lanes 1 and 2). Increasing concentration of pMRF-1 in transfection mixture had no substantial effect on the activity of the promoter (Fig. 3B, left panel, compare lane 2 with lanes 3–5). Under identical conditions, transfection of non-glial/Hela cells with similar plasmids showed no activation of MBP promoter by MRF-1.1 Co-transfection of glial cells with the pMBP402 mut.3 and pMRF-1 showed no significant effect on the basal CAT activity (Fig. 3B, right panel, compare lane 2 with lanes 3–5). These observations strongly suggest that the MRF-1-binding site located within the MB3 regulatory region is important for the basal transcriptional activity of the MBP promoter and that MRF-1 is a sequence-specific DNA-binding protein with the capacity to stimulate transcription of the MBP promoter in glial cells.

Tissue Distribution and Developmental Accumulation of MRF-1 mRNA—To determine the profile of expression of MRF-1 mRNA in various tissues, as well as its temporal expression in brain, Northern blot analysis was performed. Results from analysis of 20 μg of total RNAs from brain, heart, kidney, lung, and spleen of 60-day-old mice revealed the presence of three RNA species of 1.8, 2.5, and 3.0 kb in all tissues (Fig. 4A, left). Whether or not these three transcripts represent alternatively processed forms of the single primary RNAs remains to be investigated. As anticipated, MBP mRNA was expressed exclusively in brain tissue (Fig. 4B, left). Ethidium bromide staining demonstrates that similar amounts of RNA were loaded on the gel (Fig. 4C, left). Developmental expression of these transcripts in mouse brain at various stages of myelination revealed a slight increase in the abundance of the 3.0-kb transcript at 30 and 60 days after birth (Fig. 4A, right, lanes 4 and 5). We also noticed that the levels of the 1.8-kb RNA species decreased at 18 and 30 days in comparison to 3 and 7 days after birth (Fig. 4A, right, compare lanes 1 and 2 to lanes 3 and 4). Northern blot analysis of RNAs from brain of mice at various stages showed a developmental increase in the levels of the MBP transcript in 18-, 30-, and 60-day-old mice (Fig. 4B, right, lanes 3–5) as previously reported (9, 18).

Identification of Restriction Fragment Length Polymorphisms—To further characterize the Mrfl locus, its chromosomal location in the mouse was determined using interspecific backcross analysis. Genomic DNAs from AEJ/Gn and Mus spretus parental control animals were digested with several restriction endonucleases and analyzed by Southern blot hybridization using the MRF-1 probe. These results indicated that digestion with TaqI identified...
an informative polymorphism between AEJ/Gn and M. spretus. (Table I). The segregation pattern of the M. spretus allele in 144 backcross animals was then determined for Mrfl. Comparison of the allele segregation pattern of Mrfl with the loci that are typed in our backcross panel revealed close linkage with markers located at the distal end of mouse chromosome 11 (Table I). The order of loci mapping to distal chromosome 11 was determined by minimizing the number of recombinants between loci; there were no double recombinants observed for the interval analyzed. The haplotype information derived from the subset of animals in which all the loci were typed is shown (Fig. 5A). The order to the loci and the ratio of the number of recombinants to the total number of N<sub>0</sub> offspring examined are as follows: Wnt3, 2:187; D11Mit10, 12:144; Mrfl, 2:145; D11Jkn1e, 2:195; Thbp. The genetic distances between loci in centiMorgans (± S.E.) are: Wnt3, 1.1 centiMorgans (± 0.8); D11Mit10, 8.3 centiMorgans (± 2.3); Mrfl, 1.4 centiMorgans (± 1); D11Jkn1e, 1.0 centiMorgans (± 0.7); Thbp. Fig. 5B shows the placement of these genes on the map of mouse chromosome 11. MRF-1 maps in the vicinity of js (jackson shaker), tn (teetering), cod (cerebellar outflow degeneration), and Ts (tail-short) (39). The js, tn, and cod all result in mice exhibiting neurological defects (40-42). The fact that MRF-1 regulates myelin gene expression and that MRF-1 maps in the vicinity of these mutations makes MRF-1 a candidate gene for any of these neurological mutations. Moreover, since MRF-1 expression is not limited to the central nervous system, it is conceivable that a mutation in MRF-1 could be responsible for the Ts mutation (43).

Determining the binding activity of MRF-1 through gel shift assays in the various mouse mutants should resolve whether these mutations are a result of a defect in the gene encoding MRF-1.

The region of chromosome 11 where Mrfl is localized shows exclusive linkage synteny with human chromosome 17 (39). Therefore, the human homolog of MRF-1 most likely resides in the distal region of human chromosome 17q. Based on mouse human linkage relationships the most likely location for the human homolog of MRF-1 is human chromosome 17q23-25 (44).

It is well-established that programmed expression of the MBP gene, encoding the major constituent of the myelin sheath in brain is determined by a series of cis-acting transcriptional elements positioned upstream of the MBP RNA start site. Studies in our (14) and other (45) laboratories have demonstrated that sequences between -93 to -130 play an important role in transcriptional activation of the MBP promoter. This regulatory motif, named MB3, which contains a target sequence for binding to NF-1 transcription/replication factor interacts with several proteins present in brain nuclear extract. Use of in vitro transcription and footprinting assay led to the identification of a ubiquitous nuclear protein that by binding to the region spanning -130 to -106 activates transcription of MBP in cell-free extract from Hela cells (45). In the present study, we have isolated a cDNA from a mouse brain expression library which encodes a protein (MRF-1) with binding affinity to the MB3 sequences spanning -128 to -102 including the NF-1 site. Expression of MRF-1 augments the transcriptional activity of the MBP promoter, and this activation requires intact binding site for MRF-1 within the MB3 region. Sequence analysis of the MRF-1 revealed striking homology with splicing factor SC35 (33); while the NH<sub>2</sub>-terminal of MRF-1 is distinct from SC35, the central and COOH-terminal regions of these proteins exhibit a substantial degree of homology with each other. Although the two RNP-type RNA binding motifs found in the NH<sub>2</sub>-terminal region of the SC35 are absent in the NH<sub>2</sub> terminus of MRF-1, our preliminary studies indicated that MRF-1 binds to RNA molecule similar to SC35. Thus, MRF-1 may belong to an emerging family of proteins which bind to DNA in a sequence-specific manner and have the capacity to form a complex with RNA molecules (46). Of interest is the observa-

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**FIG. 4. Expression of MRF-1 RNAs in mouse tissues.** Total RNAs were extracted from various tissues of 18-day-old mice (left panel) or from brain tissue of mice aged 3 to 60 days (right panel) and analyzed by Northern blot hybridization using MRF-1 and MBP probes. Panel C represents ethidium bromide staining of the gels corresponding to the blots.

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**TABLE 1**

<table>
<thead>
<tr>
<th>Locus</th>
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<th>Restriction enzyme</th>
<th>Restriction fragment sizes</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>AEJ/Gn fragment</td>
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<tr>
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* Underlined restriction fragment size indicates the segregated allele(s) that was typed in the backcross.

* Locus typed by polymerase chain reaction amplification of microsatellite sequences.

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MBP, Transcription Factor, DNA-binding Protein
mouse mutants which exhibit neurological deficits are mapped in these animals. Human homologs reside on human chromosome 12 in the region defined by MRF-1, raising an intriguing possibility currently in progress in our laboratory to determine whether these mice carry a mutation in their MRF-1. The Northern blot studies also indicated that expression of the most abundant 1.8 kb species was developmentally down-regulated during myelination while the higher molecular weight species of 2.6 kb were developmentally up-regulated suggesting that the product of this transcript may participate in transcription of the responsive promoter. Results from our and others' laboratories strongly suggest that MRF-1 by binding to its cognate site in DNA regulates their expression in different cell contexts. MRF-1 may be a transcription factor which, through interaction with other regulatory proteins, determines which of its several target promoters are activated. For example, we have found that MRF-1 is strongly expressed in the lens of both normal and cataractous mice. The lens is a highly specific tissue which expresses only a limited number of genes, and our data indicate that MRF-1 plays a role in the regulation of these genes.

Fig. 5. A, the interspecific backcross analysis. Genes mapped in the analysis are listed on the left. Each column represents chromosome 12 of the mouse genome.

REFERENCES

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