Chronic Fos-Related Antigens: Stable Variants of ΔFosB Induced in Brain by Chronic Treatments

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Fos family transcription factors are believed to play an important role in the transcriptional responses of the brain to a variety of stimuli. Previous studies have described 35 and 37 kDa Fos-like proteins, termed chronic Fos-related antigens (FRAs), that are induced in brain in a region-specific manner in response to several chronic perturbations, including chronic electroconvulsive seizures, psychotropic drug treatments, and lesions. We show in this study that the chronic FRAs are isoforms of ΔFosB, a truncated splice variant of FosB that accumulate in brain after chronic treatments because of their stability. ΔFosB cDNA encodes the expression of 33, 35, and 37 kDa proteins that arise from a single AUG translation start site. The 35 and 37 kDa proteins correspond to the chronic FRAs that are induced in brain by chronic treatments, whereas the 33 kDa protein corresponds to a Fos-like protein that is induced in brain by acute treatments, findings based on migration on one- and two-dimensional Western blots with anti-FRA and anti-FosB antibodies. Using cells in which ΔFosB or FosB expression is under the control of a tetracycline-regulated gene expression system, we show that the 37 kDa ΔFosB protein exhibits a remarkably long half-life, the 35 kDa ΔFosB protein exhibits an intermediate half-life, and the 33 kDa ΔFosB protein and all FosB-derived proteins exhibit relatively short half-lives. Moreover, we show that the 33 kDa ΔFosB protein is the first to appear after activation of ΔFosB expression. Finally, ΔFosB proteins are shown to possess DNA-binding activity and to exert potent transactivating effects in reporter gene assays. Together, these findings support a scheme wherein ΔFosB, expressed as a 33 kDa protein, is modified to form highly stable isoforms of 35 and 37 kDa. As a result, these stable isoforms gradually accumulate in the brain with repeated treatments to mediate forms of long-lasting neural and behavioral plasticity.

Key words: seizure; cocaine; FosB; chronic Fos-related antigens; gene expression; neural plasticity

Regulation of gene transcription is proposed to be an important mediator of long-term responses of the brain to chronic perturbations (Hyman and Nestler, 1996). One transcription factor that has received a great deal of attention for its potential role in these responses is activator protein-1 (AP-1) (Morgan and Curran, 1991, 1995). AP-1 is a dimer composed of various combinations of Fos- and Jun-like proteins. AP-1 complexes interact with AP-1 sites, with a consensus sequence of TGA(G/C)TCA, present in the promoter regions of target genes to increase or decrease the rate of the transcription of these genes.

There is now a large literature reporting that Fos- and Jun-like proteins are induced in brain, in a region-specific manner, in response to a wide variety of stimuli. Induction of these proteins, and the resulting AP-1 complex they form, is both rapid and transient. Consequently, these proteins could mediate some of the short-lived changes in gene expression elicited by these stimuli (Morgan and Curran, 1991). In contrast, work in several laboratories over the last few years has identified novel Fos-like proteins, termed chronic Fos-related antigens (FRAs), that are induced in brain in response to chronic perturbations and are much more long-lived than other Fos-like proteins induced acutely. These features make the chronic FRAs attractive candidates to mediate some of the longer-lasting transcriptional changes involved in the regulation of brain function (Hope et al., 1992, 1994a).

The chronic FRAs, identified as 35 and 37 kDa proteins, are induced in specific brain regions in response to chronic, but not acute, administration of cocaine (Hope et al., 1994b; Nye et al., 1995; Moratalla et al., 1996), electroconvulsive seizures (ECSs) (Hope et al., 1994a), morphine (Nye and Nestler, 1996), nicotine (Pich et al., 1997), antipsychotic drugs (Nye et al., 1995), and antidepressant drugs (Hope et al., 1994b). The chronic FRAs also are induced after kainate lesions of the hippocampus and cortex (Pennypacker et al., 1994; Kasof et al., 1995) or 6-hydoxydopamine lesions of the striatum (Jian et al., 1993; Doucet et al., 1996). In each case, the chronic FRAs, once induced, are relatively stable proteins that persist in brain for long periods. For example, after a course of chronic cocaine or ECS administration, chronic FRAs remain detectable in striatum and frontal cortex, respectively, for at least 2 weeks. Similarly, in lesion paradigms the chronic FRAs remain detectable for several months.

The identity of the chronic FRAs has remained elusive despite considerable effort. The chronic FRAs are immunochemically related to ΔFosB, a truncated splice variant of FosB, but show different M_r values compared with ΔFosB-induced acutely in brain or cultured cells (Hope et al., 1994b; Chen et al., 1995, Doucet et al., 1996). These findings, coupled with the observation that levels of ΔFosB mRNA are not elevated at times when the chronic

FRAs are induced (Chen et al., 1995; Pennypacker et al., 1995), have raised doubt as to whether the chronic FRAs are indeed ΔFosB-like proteins.

Despite this controversy, recent work has provided definitive evidence that the chronic FRAs are products of the *fosB* gene. Induction of the chronic FRAs by repeated cocaine or ECS treatment is completely abolished in *fosB* knock-out mice (Hiroi et al., 1996, 1997). However, this finding leaves unanswered the question of whether the chronic FRAs represent ΔFosB, short-ened forms of FosB, or novel products of the *fosB* gene. We show here, using an inducible expression system *in vitro*, that the chronic FRAs are, in fact, modified forms of ΔFosB that are highly stable proteins, which could account for their accumulation in brain in response to chronic perturbations.

MATERIALS AND METHODS

Construction of plasmids. FosB and ΔFosB cDNAs in pcDEBdelta vectors, under the control of a constitutive SR-alpha promoter (Nakabeppu et al., 1993), were placed under the control of an inducible promoter as follows. The FosB and ΔFosB cDNAs were subcloned into the pTet splice (Gibco/BRL, Gaithersburg, MD) by insertion of the *Sal*I-*Bam*HI fragment into the blunted *Sal*I site of the pTet splice. The new plasmids were designated as pTetop-FosB and pTetop-ΔFosB. To place expression of the tetracycline transactivator (tTA) under the control of the neuron-specific enolase NSE promoter, we cloned the NSE promoter (~1.8 kb) in pNSE-*Lac*Z (a gift from Dr. J. Gregor Sutcliffe, Scripps) into pTet-tTAk (Gibco/BRL; the k denotes an added Kozak sequence to enhance translation) in place of the Tetop-minimal cytomegalovirus (CMV) promoter. The new plasmid was designated pNSE-tTAk, in which the *Hin*dII-*Xho*I fragment of pTet-tTAk was replaced by the *Hin*dII-*Sac*I fragment of pNSE-*Lac*Z.

There are four AUG codons in ΔFosB cDNA (Zerial et al., 1989; Nakabeppu and Nathans, 1991). To delete the first AUG, we removed the *Hin*dII fragment of pcDEB-ΔFosB, and the plasmid was religated. The new plasmid was designated p-1AUG. To delete the first two AUGs, we removed the *Bst*EII-*Eco*RV fragment of pcDEBdelta-ΔFosB, and the plasmid was religated and designated p-1,2AUGs.

The 4xAP-1/RSV-Luc construct (a gift from Dr. Steven Hyman, National Institute of Mental Health) consists of a promoter region of four consensus AP-1 sites, in tandem with a minimal RSV promoter, and a luciferase reporter gene under the control of this promoter.

Transfections. C6 glioma, SH-SY5Y (Biedler et al., 1978), and CATH.a (Suri et al., 1993) cells were cultured in DMEM with 5% fetal bovine serum (FBS), DMEM with 10% FBS, and RPMI 1640 with 8% horse serum plus 4% FBS, respectively. Transient transfections of the SH-SY5Y and CATH.a cells were performed by the calcium phosphate method. Approximately 75% confluent cultures in six-well plates were transfected with 10 μg of plasmid DNA overnight and then washed with PBS (10 mM sodium phosphate, pH 7.4, 150 mM NaCl) three times. The transfected cells were incubated in fresh medium for 24 hr, after which the cells were harvested for Western blotting or gel shift assays as described below. For luciferase reporter gene assays, the transfected cells were lysed by 1× reporter lysis buffer (Promega, Madison, WI). Relative luciferase activity, assayed as described in the luciferase assay protocol of Promega and measured in a luminometer, was calculated as enzyme activity per microgram of total protein (determined by Bradford assays).

For stable transfection, C6 glioma cells were transfected overnight, washed with PBS three times, and reincubated in fresh medium for 24 hr. The transfected cells were then split and incubated for another 24 hr. Stable C6 cell lines transfected with constitutive expression plasmids pcDEB-ΔFosB and pcDEB-FosB using the gene for hygromycin-B phosphotransferase (the hygromycin-B resistance gene) were selected by hygromycin (100 μg/ml). Stable C6 cell lines transfected with inducible expression plasmids pNSE-tTAk plus pTetop-FosB or pTetop-ΔFosB were selected by the neomycin resistance marker G418 (100 μg/ml) using cotransfection with a plasmid containing the gene for aminoglycoside phosphotransferase (the neomycin resistance gene).

In vivo *ECS treatment.* Male Sprague Dawley rats (initial weight, 140–260 g; Camm Research Institute, Wayne, NJ) were used for all experiments. An ECS was administered, as described previously (Hope et al., 1994a), via ear clip electrodes (45 mA, 0.3 sec). Chronic animals received a single ECS daily for 10 d. Control and acute animals received

daily sham treatments, in which electrodes were clipped onto the ears of the rats, but no current was applied. On day 11, acute animals were given an acute ECS, and control and chronic animals were given sham treatment. Animals were killed 2 hr later. Previous sham treatments were used in the control and acute animals to reduce the effects of stress (see Campeau et al., 1991; Sharp et al., 1991). Cerebral cortex was obtained by gross dissection.

Gel shift assays. Gel shift assays were performed as described previously (Hope et al., 1992, 1994a). The transfected cells (~5 × 10⁷) were lysed in 300 μl of electrophoretic mobility shift assay (EMSA) buffer of Korner et al. (1989): 20 mM HEPES, pH 7.9, 0.4 M NaCl, 20% glycerol, 5 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 10% μg/ml leupeptin, 0.1 mM *p*-aminobenzamidine, 1 μg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, and 5 mM dithiothreitol. Cerebral cortex was homogenized with Dounce homogenizers in 20 vol of the EMSA buffer. Crude homogenates were incubated on ice for 30 min before centrifugation at 15,000 × *g* for 20 min at 4°C. Aliquots of supernatants (containing 20 μg of protein) were incubated at room temperature for 20 min with 1 μg of poly(dI-dC)·poly(dI-dC), 40 μg of bovine serum albumin, 10 mM Tris-HCl, pH 7.9, 10 mM KCl, 1 mM EDTA, 4% glycerol, and 1 ng of the radioactively labeled AP-1 probe derived from the AP-1 site of the human metallothionein II gene (Hope et al., 1992). Then the samples were electrophoresed at 150 V for 2 hr in a nondenaturing 6% acrylamide/ 0.16% *N,N*′-methylenebisacrylamide gel containing 25 mM Tris-borate buffer, pH 8.3, 1 mM EDTA, and 1.6% glycerol. The gels were dried and exposed to x-ray film. Levels of AP-1 binding were quantified by measuring the optical density of specific bands using an image analysis system with National Institutes of Health (NIH) image software.

Western blotting. One-dimensional Western blotting was performed as described previously (Hope et al., 1994a). Transfected cells and cerebral cortex were homogenized in EMSA buffer as described above for gel shift assays. Aliquots (containing 50 μg of protein) were then applied to a 10% acrylamide/0.27% *N,N*′-methylenebisacrylamide resolving gel for SDS-PAGE overnight at 75 V and then electrotransferred to nitrocellulose at 200 mA for 3 hr. The blots were blocked with four 15 min changes of 2% (for anti-FRA antibody; kindly provided by Dr. Michael Iadarola, National Institute of Dental Research, NIH) or 0.5% (for anti-FosB antibodies; see Chen et al., 1995) nonfat dry milk in PBS-Tween (PBS containing 0.1% Tween 20). The blots were then incubated overnight on a shaker at 4°C in a 1:4000 dilution of anti-FRA antibody or a 1:1000 dilution of anti-FosB antibodies in blocking buffer with 0.05% sodium azide. The blots were washed four times for 15 min each in blocking buffer and incubated for 2 hr in a 1:4000 dilution of goat anti-rabbit antibody conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA) in blocking buffer. The blots were washed eight times for 15 min each with PBS-Tween alone, developed with the enhanced chemiluminescence (ECL) system of Amersham (Arlington Heights, IL), and exposed to Hyperfilm-ECL (Amersham) for 5–60 sec. Levels of FRA immunoreactivity were quantified either by measuring the optical density of specific bands using an image analysis system or by measuring light intensity using the Bio-Rad (Hercules, CA) GS-363 phosphor-imager.

For two-dimensional Western blotting, samples (containing 225 μg of protein) were separated by isoelectric focusing in tube gels for the first dimension according to published procedures (Hope et al., 1994b). The resulting tube gels were then layered across SDS-polyacrylamide slab gels (10% acrylamide/0.4% bisacrylamide) and electrophoresed in the second dimension. The proteins in the resulting gels were transferred onto nitrocellulose membranes, and Western blotting was performed as described above.

RESULTS

The 35 and 37 kDa chronic FRAs are products of ΔFosB mRNA

As stated in the introductory remarks, the 35 and 37 kDa chronic FRAs can be recognized by anti-FosB antibodies but can be distinguished from FosB (~46 kDa) and ΔFosB (~33 kDa) on one-dimensional gels (Hope et al., 1994b; Chen et al., 1995). One possible explanation is that the chronic FRAs are proteolytic products of FosB. To test this possibility, we established stable C6 glioma cell lines constitutively expressing FosB or ΔFosB and analyzed proteins generated from the cell lines by Western blotting using a pan-FRA antibody (Fig. 1). Extracts of cerebral cortex
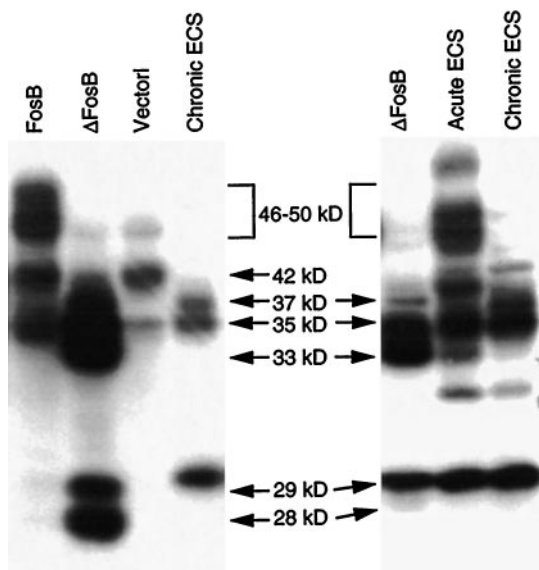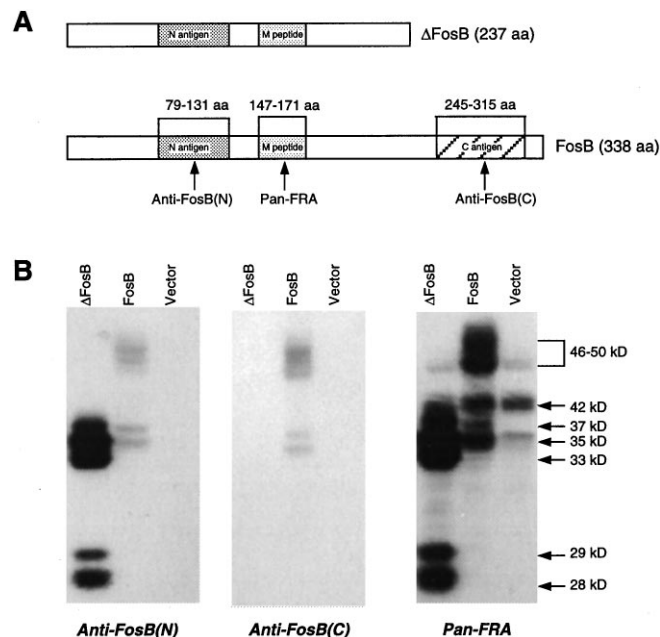
Figure 1. Western blots showing expression of FosB- and ∆FosB-derived proteins in stably transfected C6 glioma cells (*left*) and in transiently transfected Cath.a cells (*right*). Cells constitutively expressing FosB or ∆FosB cDNA were prepared. Cell extracts of these lines and of nontransfected cells (vector) were analyzed by Western blotting using the pan-FRA antibody (see Materials and Methods), which recognizes a domain conserved in all Fos-like proteins. Aliquots of cerebral cortex from rats treated acutely or chronically with ECS were analyzed for comparison. In both types of cells, FosB cDNA encodes the expression of proteins at 46–50 and 35 kDa. ∆FosB cDNA encodes the expression of proteins at 37, 35, 33, 29, and 28 kDa. The 35 and 37 kDa ∆FosB-encoded proteins comigrate with the 35 and 37 kDa chronic FRAs induced by chronic ECS treatment. The 33 kDa ∆FosB-encoded protein comigrates with the 33 kDa acute FRA induced by acute ECS treatment. The 29 and 28 kDa ∆FosB-encoded proteins do not correspond to FRAs detected in the brain; they are distinct from an ~30 kDa protein seen in the chronic ECS sample that is constitutive and not regulated by ECS treatment. Note that a prominent ~42 kDa protein (possibly FRA-1 or -2) is present in control C6 cells (*vector1*) and in the FosB transfectants but is not in the ∆FosB transfectants. Note also that an ~40 kDa protein (possibly FRA-1 or -2) is induced in cortex by acute ECS treatment only. The results shown are representative of at least four separate determinations. All of the bands recognized by the anti-FRA antibody represent specific labeling, because detection was abolished by preincubation of the antibody with the immunizing peptide antigen (see Hope et al., 1994a).

from rats treated chronically with ECSs were analyzed for comparison. FosB-transfected cells expressed FRA-immunoreactive proteins in the M_r range of 46–50 kDa, which is consistent with the reported M_r of FosB (Zerial et al., 1989; Nakabeppu and Nathans, 1991). The cells also expressed lower levels of FRAs in the M_r range of 35 kDa. Although the 35 kDa band comigrated with the 35 kDa chronic FRA, no 37 kDa chronic FRA-like band was generated by FosB cDNA.

∆FosB-transfected cells expressed high levels of five FRAs with M_r values of 37, 35, 33, 29, and 28 kDa (Fig. 1). The 35 and 37 kDa ∆FosB-derived proteins migrated at the same position as the 35 and 37 kDa chronic FRAs. The 33 kDa ∆FosB-derived protein migrated at the same position as a FRA induced by acute ECS (Fig. 1) or acute cocaine treatment (see Chen et al., 1995). In contrast, the 29 and 28 kDa ∆FosB-derived proteins did not correspond to FRAs detected in the brain.

Whereas a 37 kDa FRA was generated by only ∆FosB cDNA, a 35 kDa FRA was generated by both ∆FosB and FosB cDNA. To characterize the protein products from FosB- and ∆FosB-transfected cells further, we used antibodies selective for the N
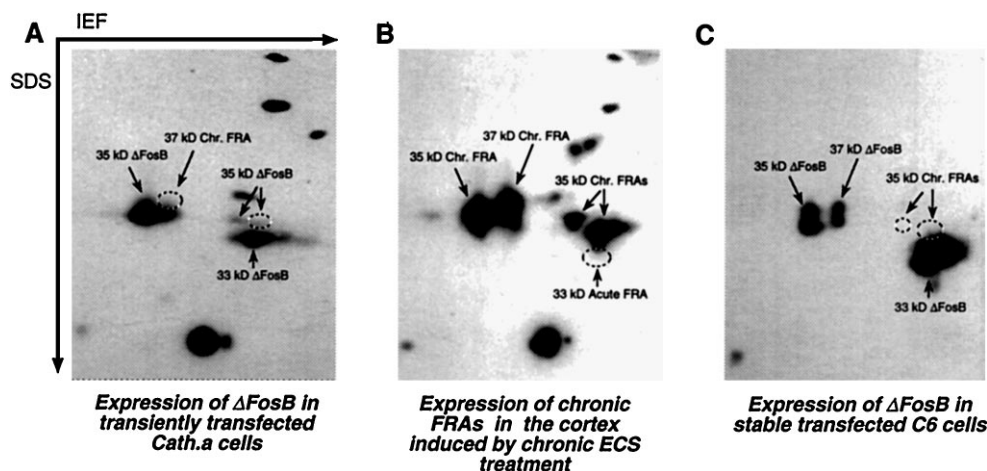

Figure 2. Western blots showing the expression of FosB- and ∆FosB-derived proteins in stably transfected C6 glioma cells. *A*, Regions of the ∆FosB and FosB proteins against which the anti-FosB(N), anti-FosB(C), and pan-FRA antibodies are directed. *B*, Extracts of stable cell lines (see Fig. 1) that allow constitutive expression of FosB or ∆FosB cDNA or extracts of nontransfected cells (*Vector*), analyzed by Western blotting using an antibody directed against the N terminus of FosB [anti-FosB(N)], an antibody directed against the C terminus of FosB [anti-FosB(C)], or the pan-FRA antibody. Note that all of the ∆FosB-derived proteins were recognized by the anti-FosB(N) antibody but not by the anti-FosB(C) antibody, whereas the FosB-derived proteins were recognized by both antibodies. Note also that none of the several proteins expressed in control cells and recognized by the pan-FRA antibody were recognized by the anti-FosB antibodies. The results shown are representative of three separate determinations.

terminus [anti-FosB(N)] or the C terminus [anti-FosB(C)] of FosB to distinguish ∆FosB- from FosB-derived proteins. This is based on the fact that ∆FosB lacks the C terminus of FosB and is therefore recognized by only the anti-FosB(N) antibody, whereas FosB is recognized by both antibodies. As shown in Figure 2, all five of the ∆FosB-derived proteins were recognized by anti-FosB(N) but not by anti-FosB(C), which confirms that these proteins were generated from ∆FosB cDNA. In contrast, all of the FosB-derived proteins were recognized by both anti-FosB(N) and anti-FosB(C) antibodies. Because the 35 and 37 kDa chronic FRAs are recognized only by the anti-FosB(N) antibody (Hope et al., 1994b; Chen et al., 1995), the results suggest that the ~35 kDa proteins in the FosB-transfected cells are not alternatively spliced ∆FosB products and are distinct from the chronic FRAs. Rather, these proteins would seem to be the same as the 35 kDa FRAs induced in the brain by acute ECS or cocaine treatment (Chen et al., 1995).

To confirm that the chronic FRAs are ∆FosB-derived proteins, two-dimensional Western blotting was used to compare the chronic FRAs induced in the brain by chronic ECS treatment with the ∆FosB proteins expressed in cell culture (Fig. 3). Two cell culture systems were analyzed: transiently transfected CATH.a cells, in which the 35 and 33 kDa ∆FosB proteins predominate, and stably transfected C6 glioma cells, in which the 37 kDa ∆FosB protein is expressed as well. The results of these experiments show

Figure 3. Two-dimensional Western blots showing the expression of chronic FRAs in the brain and of ΔFosB-derived proteins in cultured cells. *A,* CATH.a cells transiently transfected with ΔFosB cDNA. *B,* Aliquots of cerebral cortex from rats treated chronically with ECSs, showing the migration of the 35 and 37 kDa chronic FRAs as demonstrated previously (Hope et al., 1994a; Chen et al., 1995). *C,* C6 glioma cells stably transfected with ΔFosB cDNA. Cell and brain extracts were analyzed by two-dimensional Western blotting using the pan-FRA antibody. The figure shows the comigration of the 37 kDa ΔFosB with the 37 kDa chronic FRA and of the 35 kDa ΔFosB with the 35 kDa chronic FRA. The 33 kDa ΔFosB comigrates with a 33 kDa FRA induced in the brain by an acute ECS treatment (see Chen et al., 1995). The results shown are representative of two to three separate determinations.


Expression of ΔFosB in transiently transfected Cath.a cells

Expression of chronic FRAs in the cortex induced by chronic ECS treatment

Expression of ΔFosB in stable transfected C6 cells

that the chronic FRAs correspond precisely with the 35 and 37 kDa ΔFosB proteins. The 35 kDa chronic FRA migrated on two-dimensional gels as two to three widely spaced bands that correspond to the positions of 35 kDa ΔFosB protein bands, whereas the 37 kDa chronic FRA corresponds to the position of the 37 kDa ΔFosB protein. In contrast, the 33 kDa ΔFosB protein migrated at the same position as a 33 kDa FRA induced in the brain by acute ECS or cocaine treatment (Chen et al., 1995). Another observation from these studies, as mentioned above, is that levels of the 37 kDa ΔFosB protein are barely detectable in the transiently transfected cells and that, even in the stably transfected cells, only a relatively small amount of the 37 kDa ΔFosB was detected (see Figs. 1, 2). These results suggest that the accumulation of the 37 kDa protein *in vitro* may be slower than the more robust accumulation of the 37 kDa chronic FRA in the brain after chronic perturbation.

It should be noted that there was one major protein of ~42 kDa, detected by the anti-FRA antibody, in the untransfected and FosB-transfected cells (Figs. 1, 2). This band was not recognized by either the anti-FosB(N) or anti-FosB(C) antibody. Interestingly, expression of this endogenous 42 kDa FRA (which could be FRA-2) was repressed in the ΔFosB-transfected cells. This suggests the possibility that the ΔFosB proteins are functional and serve as negative regulators of the expression of this protein.

The 33, 35, and 37 kDa ΔFosB proteins are isoforms of the same gene product

Analysis of the ΔFosB cDNA sequence reveals that there are four AUG start codons in the ΔFosB mRNA, of which the first AUG locates in the first exon and the other three AUGs locate in the second exon (Fig. 4*A*). Based on this sequence information, we hypothesized that the multiple ΔFosB proteins expressed from ΔFosB cDNA (i.e., the 37, 35, 33, 29, and 28 kDa bands in Figs. 1, 2) are alternative translation products from different translation start sites. To test the hypothesis, we deleted the first AUG, or both the first and second AUGs, from the ΔFosB cDNA and analyzed the expression patterns of the deletion mutants in transiently transfected CATH.a cells (Fig. 4*B*). Contrary to our hypothesis, we found that the 33, 35, and 37 kDa protein bands disappeared when the first AUG was deleted and that further deletion of the second AUG did not eliminate more bands, namely, the 28 and 29 kDa proteins.
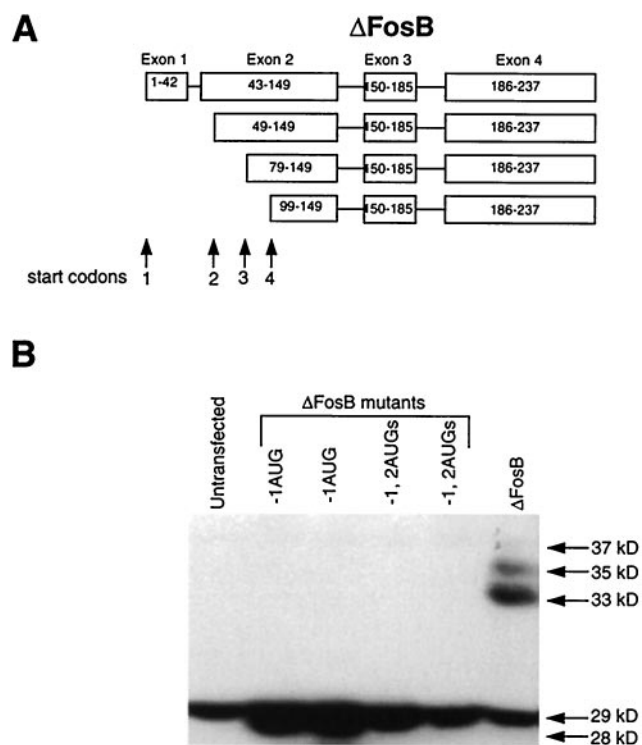
Figure 4. Analysis of translation start sites for ΔFosB-derived proteins. *A,* Genomic map for ΔFosB and the location of four alternative start (AUG) codons present in the resulting mRNA. *B,* Western blot of extracts of CATH.a cells transiently transfected with full-length ΔFosB cDNA or with mutants lacking the first (−*1AUG*) or the first and second (−*1,2AUGs*) start codons. Note that deletion of the first AUG completely obliterated the 37, 35, and 33 kDa proteins, whereas deletion of the first and second AUGs did not abolish the 28–29 kDa ΔFosB proteins, as indicated by the still higher levels of immunoreactivity present in this region of the gel compared with that in untransfected cells. The results shown are representative of three separate determinations.

These results indicate that the 33, 35, and 37 kDa ΔFosB proteins are encoded by the same open reading frame of the ΔFosB mRNA and that they are isoforms of one another. In contrast, the 28 and 29 kDa ΔFosB proteins seem to be translated from start codon(s) downstream of the second AUG, although we

cannot rule out the possibility that deletion of the first AUGs altered the use of the downstream ones. Because FosB and ΔFosB mRNAs contain identical exons 1 and 2, it would be expected that FosB mRNA would encode a similar pattern of protein products, namely products from the first AUG and smaller products from the downstream AUG. Indeed, deletion studies confirm that the 35 kDa FosB protein (see Figs. 1, 2) is translated from a downstream start codon (data not shown).

The 35 and 37 kDa ΔFosB proteins are highly stable

Previous work has shown that the stability of FosB and ΔFosB mRNAs are similar, with both returning to control levels within a few hours of acute ECS or cocaine treatment (Chen et al., 1995). Moreover, the two mRNAs show a reduced ability to be induced after repeated treatment. This is in contrast to the gradual accumulation of the chronic FRAs during a course of chronic treatment. One possible explanation for these findings is that the chronic FRAs, once induced, are relatively stable proteins. To test this possibility directly, we compared the stability of ΔFosB and FosB proteins using transfected cells in which ΔFosB or FosB expression is under the control of the tetracycline-regulated gene expression system. It was necessary to use such an inducible expression system, in which the expression of proteins can be turned on and off, to assess protein stability, in contrast to a constitutive system in which the expression of proteins is always on and would therefore interfere with measures of protein turnover.

In the tetracycline system (Shockett et al., 1995), ΔFosB and FosB cDNAs were placed under the control of a promoter consisting of the tetracycline operator (Tetop) and minimal CMV promoter, as shown in Figure 5*A*. In the absence of tetracycline, tTA binds to the promoter and activates the transcription of the ΔFosB and FosB cDNAs. In the presence of tetracycline, tTA undergoes a conformational change and cannot bind to the Tetop promoter, so that transcription of FosB and ΔFosB is inhibited. In this manner, the expression of FosB and ΔFosB proteins can be activated or repressed, respectively, by the absence or presence of tetracycline.

The tetracycline system was first used to encode FosB and ΔFosB transcription in transiently transfected CATH.a cells. The transfected cells were cultured in the absence of tetracycline for 24 hr, after which time tetracycline was added to the culture medium to turn off transcription of ΔFosB and FosB cDNAs (Fig. 5*B*). As shown in Figure 5*B*, levels of ΔFosB and FosB proteins decreased in the cells as the proteins were degraded. It was found that the ΔFosB proteins were more stable than the FosB proteins. Note, for example, in Figure 5*B* that levels of all FosB products were at barely detectable levels 2 d after addition of tetracycline, whereas the ΔFosB proteins remained at much higher levels. This impression is verified by quantitative analysis of the data. For example, Figure 5*C* shows a comparison of the rate of disappearance of the 35 kDa ΔFosB protein and the 46 kDa FosB protein. Note that because only ~10% of the cells were transfected in these transient transfection experiments, and expression of ΔFosB was permitted for a relatively short time only (24 hr), no 37 kDa ΔFosB protein was detected.

To overcome this limitation of transient transfections, and thereby to study the stability of the 37 kDa ΔFosB protein, we made stable C6 glioma cell lines transfected with the FosB and ΔFosB cDNAs under the control of the tetracycline-regulated promoter. The use of these cell lines, in which expression of ΔFosB can be turned on and off in repeated cycles of tetracycline exposure and withdrawal, is shown in Figure 6. These initial
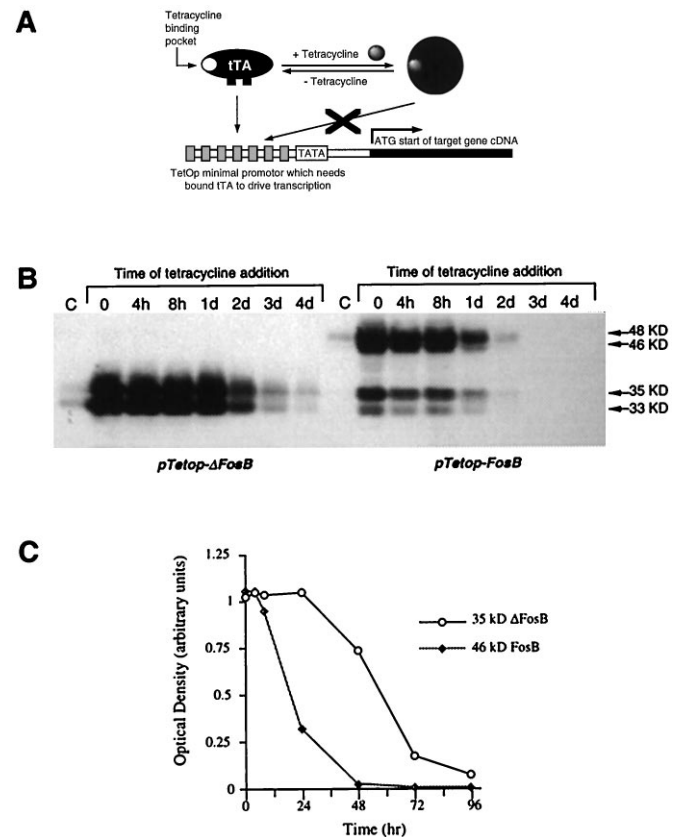
Figure 5. Analysis of the stability of ΔFosB and FosB proteins in CATH.a cells transiently transfected with ΔFosB or FosB cDNA under the control of the tetracycline expression system. *A*, Schematic illustration of the tetracycline-regulated gene expression system, as adapted from Dr. Rene Hen (Columbia University, Neuroscience Short Course, Society for Neuroscience, 1996). *B*, Western blot of ΔFosB and FosB proteins in CATH.a cells 24 hr after transfection (time 0) and at varying times after the addition of tetracycline (4 hr–4 d) to turn off expression. *C*, Quantitative representation of the time-dependent reduction in the 35 kDa ΔFosB protein and the 46 kDa FosB protein after the addition of tetracycline. The results shown are representative of two separate determinations.

experiments indicated that the 37 kDa ΔFosB protein appears at significant levels only in cells in which the expression of ΔFosB cDNA is turned on for relatively long periods.

To compare the stability of the 33, 35, and 37 kDa ΔFosB proteins, we cultured the ΔFosB stable cell lines in the absence or presence of tetracycline for varying periods. The results of this experiment are shown in Figure 7. When the cells were cultured in the absence of tetracycline for 11 d, the 37 kDa protein accumulated to a significant level, approximately comparable to that of the 33 and 35 kDa ΔFosB proteins (Fig. 7, *lane 1*). In contrast, when the cells were cultured first in the absence of tetracycline for 5 d and then in the presence of tetracycline for 6 d, the 35 and 37 kDa proteins were detectable at low levels, whereas the 33 kDa protein was not detectable (Fig. 7, *lane 2*). This suggests that the 35 and 37 kDa proteins are more stable than the 33 kDa protein. When ΔFosB expression was on for 5 d, then switched off for 3 d and back on for 3 d, the first new ΔFosB protein detected was the 33 kDa protein (Fig. 7, *lane 3*). This suggests that the 33 kDa protein is the native form of ΔFosB, that is, the first expressed. When ΔFosB expression was on for 8 d and then turned off for 3 d, the 33 kDa protein was not detectable, whereas the 35 and 37 kDa proteins remained (Fig. 7, *lane 4*). This suggests, again, that the 35
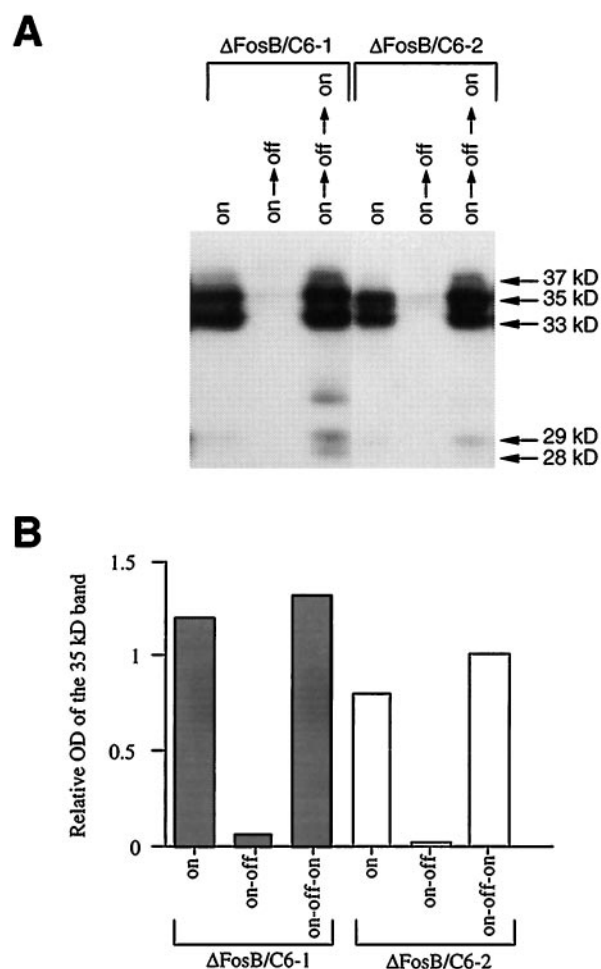
Figure 6. Western blots showing the expression of ΔFosB proteins in C6 glioma cells stably transfected with ΔFosB cDNA under the control of the tetracycline expression system. *A*, Western blot analysis, using the pan-FRA antibody, of extracts of stable C6 glioma cell lines. *B*, Quantitative representation of the Western blots. Results from two independent stable cell lines are shown. One set of cells was harvested after being cultured for 3 d in the absence of tetracycline to turn ΔFosB expression on (*on*); a second set of cells was harvested after being cultured for 3 d in the absence of tetracycline, followed by 1 wk in the presence of tetracycline to turn ΔFosB expression off (*on → off*); a third set of cells was harvested after being cultured for 3 d in the absence of tetracycline, followed by 1 wk in the presence of tetracycline and then 1 wk in the absence of tetracycline to turn ΔFosB expression back on (*on → off → on*). Note the ability to turn ΔFosB expression repeatedly on and off by use of the tetracycline expression system. Note also that the 37 kDa ΔFosB protein appears appreciably only after more prolonged periods of expression (i.e., in the *on → off → on* condition). The results shown are representative of two separate determinations of six independent cell lines examined.

and 37 kDa proteins are more stable than the 33 kDa protein and are presumably derived from the 33 kDa protein via some form of covalent modification.

A quantitative analysis of the stability of ΔFosB and FosB proteins was performed by culturing cells in the absence of tetracycline for 11 d and harvesting them at varying times after the addition of tetracycline (Fig. 8). In this analysis the 33 kDa ΔFosB protein and the FosB-derived proteins showed relatively short half-lives of 9–10 hr. This short half-life is consistent with the highly transient appearance of these proteins in the brain *in vivo* after acute ECS or cocaine treatment (Hope et al., 1994a,b; Chen et al., 1995). In contrast, the 35 and 37 kDa ΔFosB proteins
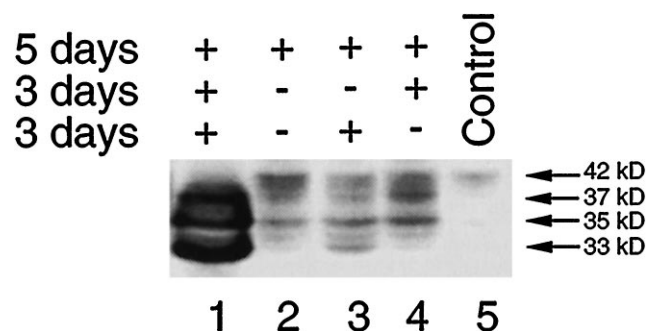
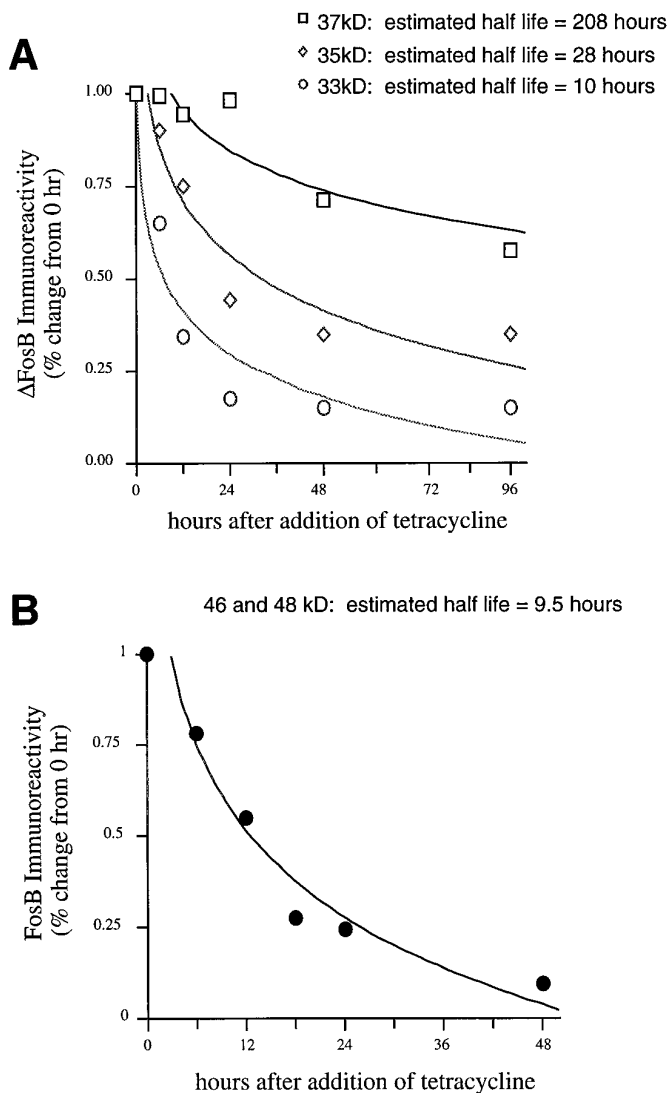*Figure 7.* Western blot showing the relative stability of the 37, 35, and 33 kDa ΔFosB proteins in stable C6 glioma cells transfected with ΔFosB cDNA under the control of the tetracycline expression system. Cells were grown for varying times in the absence or presence of tetracycline to turn ΔFosB expression on (+) or off (−), respectively, and cell extracts were then analyzed by Western blotting using the pan-FRA antibody. In *lane 1*, cells were harvested after being grown in the absence of tetracycline for 11 d. In *lane 2*, cells were harvested after being grown in the absence of tetracycline for 5 d, followed by an additional 6 d in the presence of tetracycline. In *lane 3*, cells were harvested after being grown in the absence of tetracycline for 5 d, followed by 3 d in the presence of tetracycline and another 3 d in the absence of tetracycline. In *lane 4*, cells were harvested after being grown in the absence of tetracycline for 8 d, followed by an additional 3 d in the presence of tetracycline. *Control* (untransfected) cells are shown for comparison and illustrate the presence of an endogenous FRA, possibly FRA-2, of ~42 kDa (see also Fig. 1). The results demonstrate that the 33 kDa ΔFosB protein is the first to appear after activation of ΔFosB expression, but this protein is less stable compared with the 35 and 37 kDa ΔFosB proteins. The results shown are representative of three separate determinations using two independent, stable cell lines.

showed longer half-lives. The 35 kDa protein decayed with an estimated half-life of 28 hr, and the 37 kDa protein decayed with an estimated half-life of 208 hr. This dramatic stability of the 37 kDa protein of >8 d *in vitro* is comparable to the half-life of 7 d calculated for the chronic FRAs *in vivo* after chronic administration of ECSs or cocaine (Hope et al., 1994b).

Transcriptional activity of ΔFosB and FosB proteins

To study the function of ΔFosB and FosB proteins, we first analyzed their DNA-binding activity. As shown in Figure 9*A*, expression of ΔFosB proteins was associated with the appearance of high levels of AP-1 binding. The AP-1 complex so formed migrated at the same position as the chronic AP-1 complex induced in the brain by chronic ECS treatment. Expression of FosB proteins was also associated with high levels of AP-1 binding, but the AP-1 complex comigrated in this case with the upper, acute AP-1 complex induced in brain by acute ECS treatment (Fig. 9*B*). These results demonstrate that the ΔFosB proteins exhibit DNA-binding activity. Moreover, the ΔFosB–AP-1 complex seems to be identical to the chronic AP-1 complex induced in brain by chronic ECS or cocaine treatment (Hope et al., 1994a,b).

To test whether ΔFosB proteins can regulate the activity of promoters containing AP-1 sites, we transiently transfected SH-SY5Y cells with ΔFosB (in the tetracycline expression system) and with the 4×AP-1/RSV-Luc plasmid (a construct that contains four tandem consensus AP-1 sites driving expression of a luciferase reporter gene). Analysis of luciferase activity (Fig. 10*A*) showed that AP-1 promoter activity was upregulated to a small extent by ΔFosB. This transactivation activity of ΔFosB on the AP-1 promoter was confirmed in stable ΔFosB-transfected C6 cells, which showed a dramatic induction of luciferase activity in response to ΔFosB expression (Fig. 10*B*). In contrast to ΔFosB,

*Figure 8.* Analysis of the stability of ΔFosB and FosB proteins in stable C6 glioma cells transfected with ΔFosB or FosB cDNAs under the control of the tetracycline expression system. Cells were grown in the absence of tetracycline for 11 d to turn ΔFosB (*A*) or FosB (*B*) expression on, after which time the cells were cultured in the presence of tetracycline for varying periods to turn expression off. The results demonstrate the dramatic stability of the 37 kDa ΔFosB protein compared with the other ΔFosB and FosB proteins. The results shown are representative of two separate determinations using two independent, stable cell lines.

FosB downregulated the activity of the promoter; this is illustrated in Figure 10*C*, which shows a decrease in luciferase activity in stable FosB-transfected C6 cells after FosB expression. Tetracycline itself exerted no effect on AP-1 promoter activity in SH-SY5Y or C6 cells that lack FosB and ΔFosB plasmids, which excludes the possibility of a tetracycline artifact (data not shown).

DISCUSSION

The major finding of this study is that the 35 and 37 kDa chronic FRAs, induced in the brain by a variety of chronic treatments (see introductory remarks), are the stable products of ΔFosB. We detected five proteins generated from ΔFosB cDNA, of which the 33, 35, and 37 kDa proteins are isoforms. The 33 kDa protein is the first to appear after induction of ΔFosB expression, with the other forms presumably generated by covalent modification. The 35 and partic-
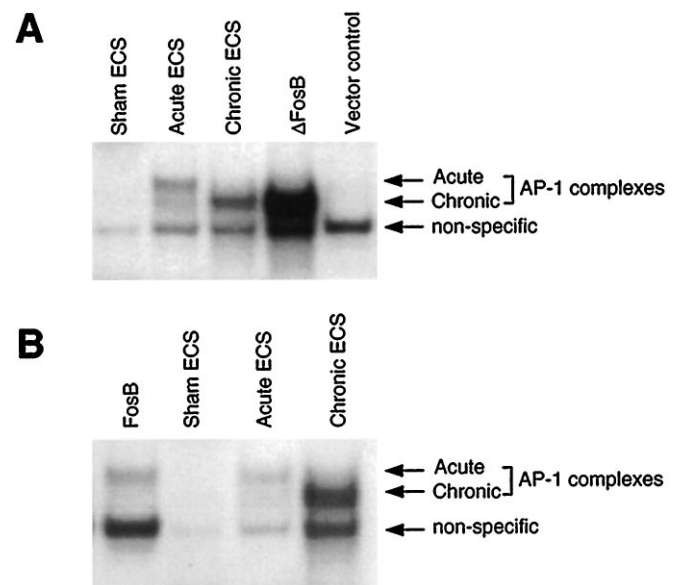
*Figure 9.* Gel shift assays showing the induction of AP-1 DNA-binding activity after the expression of ΔFosB or FosB in stable C6 glioma cells. Extracts of a stable cell line that allows constitutive expression of ΔFosB (*A*) or FosB (*B*) cDNA and of cells transfected with vector DNA only (*Vector control*) were analyzed for AP-1 DNA-binding activity using gel shift assays. Extracts of cerebral cortex from rats that were treated acutely or chronically with ECSs, or with sham treatments, were analyzed for comparison. As shown in *A*, the expression of ΔFosB results in the dramatic induction of an AP-1 complex that comigrates with the AP-1 complex induced by chronic, but not acute, ECSs. In contrast, as shown in *B*, the expression of FosB results in the induction of an AP-1 complex that comigrates with the AP-1 complex induced in brain by acute ECS. The results shown are representative of three separate determinations.

ularly the 37 kDa isoforms are more stable than the 33 kDa isoform, which provides the probable mechanism for the accumulation of the 35 and 37 kDa chronic FRAs in the brain after repeated perturbations. Accumulation of the 37 kDa ΔFosB occurs very slowly *in vitro*, but the stability of this protein is highest among the ΔFosB isoforms, which could explain how both the 35 and 37 kDa proteins reach similar levels *in vivo* after chronic treatments. Together, these findings provide direct support for our earlier working hypothesis (Hope et al. 1994b) that proposed the slow accumulation of stable chronic FRAs by repeated treatments.

Recently, *fosB* knock-out mice were developed (Brown et al., 1996). Induction of the 35 and 37 kDa chronic FRAs was completely absent after repeated ECS or cocaine treatment in the −/− mutant mice, as opposed to the robust induction seen in +/+ wild-type mice (Hiroi et al., 1996, 1997). Although the knock-out disrupts both FosB and ΔFosB mRNAs, information from the knock-out mice provides strong confirmation of the conclusions of the current study that the chronic FRAs are ΔFosB proteins.

Previous work has shown that FosB and ΔFosB mRNAs have similar stability, as revealed by RNase protection analysis (Chen et al., 1995). Both mRNAs are induced rapidly and transiently by acute cocaine or ECS treatment, which is consistent with the properties of *fosB* as an immediate early gene. However, in addition to the unstable protein products of the gene (i.e., the 33 kDa ΔFosB and all FosB proteins), ΔFosB mRNA gives rise to products (i.e., the 35 and 37 kDa proteins) that are relatively stable. Indeed, the 37 kDa protein exhibits an estimated half-life that is far longer than that of other immediate early gene products. This explains how the 35 and 37 kDa proteins accumulate in
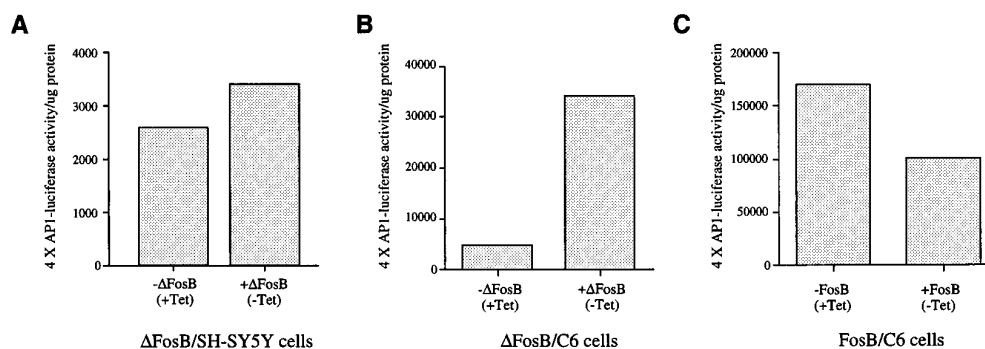
Figure 10. Analysis of the transactivating properties of ΔFosB and FosB proteins in SH-SY5Y and C6 glioma cells. *A*, Results from SH-SY5Y cells transiently transfected with ΔFosB cDNA under the control of the tetracycline expression system. *B, C,* Results from C6 glioma cells stably transfected with ΔFosB and FosB, respectively, under the control of the tetracycline expression system. Both types of cells were also transiently transfected with 4×AP-1/RSV-Luc, which contains a promoter of four tandem consensus AP-1 sites driving the expression of a luciferase reporter gene. The results demonstrate the ability of ΔFosB to upregulate AP-1 promoter activity in both cell lines. In contrast, FosB downregulates AP-1 promoter activity in the C6 cells. The results shown are representative of three separate determinations.

Table 1. Potential PEST sequences in the full-length FosB and ΔFosB proteins

Protein	PEST sequence	Amino acid location of PEST sequence	PEST-FIND score
FosB/ΔFosB	1. RCSSSPSAESQYLSSVDSFGSPPTAAASQECAGLGEMPGSFVPT VTAITTSQDLQWLVQPTLISSMAQSQGQPLASQPPAVDPYAMPGTS STPGLSAYSTGGASGSGGPSTSTTTSGPVSAR	14–136	2.832
	2. REETLTPEEEEK	146–157	23.004
FosB	3. KEDGFGWLLPPPPPPPLPFQSSR	248–270	0.565
	4. RTSGSEQPSDPLNSPSLLAL	319–338	1.365

Amino acid sequences in FosB and ΔFosB proteins that contain consensus PEST sequences, as well as the location of the amino acid sequences in the two proteins. The PEST-FIND score provides a measure of the predicted activity of the consensus sites; the larger the score, the greater the predicted activity.

the brain after chronic treatments when levels of ΔFosB mRNA are low. Moreover, these results suggest that the *fosB* gene is an atypical immediate early gene, in that it generates unstable protein products in response to a single acute stimulation and stable products in response to repeated stimulations. The stable products can be viewed in two ways from a functional point of view. They could exert the same transcriptional effect as the unstable products and thereby play a role in saving "energy" for the cell as it responds in the same way to repeated stimulations. Alternatively, they could exert the opposite transcriptional effect as the unstable products and thereby play a negative feedback role in opposing further responses to the stimulations. Of course, these possibilities are not mutually exclusive, in that the chronic FRAs could play both roles, depending on the various target genes in question and the Jun family member with which they form functional AP-1 complexes.

The mechanism responsible for the stability of the 35 and 37 kDa ΔFosB proteins remains unknown. For some immediate early genes, such as c-*fos* and *myc*, the encoded proteins contain several potential PEST (P, proline; E, glutamatic acid; S, serine; and T, threonine) sequences, which are correlated with fast protein degradation (Rogers et al., 1986). Computer analysis of FosB and ΔFosB amino acid sequences by the PEST-FIND program (Rechsteiner and Rogers, 1996) revealed four potential PEST sequences in FosB and two potential PEST sequences in ΔFosB (Table 1). The fact that the 35 and 37 kDa ΔFosB proteins are more stable than FosB proteins may be attributable to the smaller number of PEST sequences in their

amino acid sequences. However, the PEST sequences alone cannot explain the stability of these proteins, because the 33 kDa ΔFosB protein also contains only two PEST sequences. Rather, it would seem that the 33 kDa protein is modified in some way to result in the 35 and 37 kDa proteins and that this modification is important for stabilization of these proteins. A potential role for phosphorylation in generating the 35 and 37 kDa isoforms is currently under investigation.

Accumulation of ΔFosB isoforms would be expected to have an important impact on brain function, because we show that these proteins exhibit AP-1 DNA-binding activity and can regulate the activity of a promoter containing AP-1 sites in a reporter gene assay. Such an important role for ΔFosB proteins is supported by studies of *fosB* knock-out mice, which exhibit impaired behavioral plasticity to chronic ECS and cocaine treatments (Hiroi et al., 1996; Hiroi, Brown, Haile, Hong, Greenberg, and Nestler, unpublished observations). Numerous neural genes are known to contain AP-1 sites in their promoter regions (see Morgan and Curran, 1991; Chen et al., 1995). Work is now needed to identify which of these putative target genes are regulated by the stable ΔFosB isoforms that are induced in specific brain regions by chronic treatments and that are ultimately responsible for the resulting behavioral plasticity. Although further research is needed to delineate the precise mechanisms involved, the long-lasting nature of the stable ΔFosB proteins make them attractive candidate molecules to mediate some of the long-term adaptive changes in the brain associated with a variety of chronic perturbations.

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