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Suppression of Ischemia-induced Fos Expression and AP-1 Activity by an Antisense Oligodeoxynucleotide to c-*fos* mRNA

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Abstract

Activation of c-*fos*, an immediate early gene, and the subsequent expression of the Fos protein have been noted following focal cerebral ischemia. Fos and Jun form a heterodimer as activator protein 1 (AP-1), which transregulates the expression of several genes. To study the postischemic events related to c-*fos* expression, we suppressed the expression of c-*fos* by intraventricular infusion of an antisense Oligodeoxynucleotide (anti-rncfosr$_{115}$) of c-*fos* mRNA. The effectiveness of anti-rncfosr$_{115}$ was confirmed first by its capability to block in vitro c-*fos* mRNA translation. In vivo, after intraventricular infusion of ^{32}P-labeled anti-rncfosr$_{115}$, the oligodeoxynucleotide was internalized within 6 hours and detectable also in the nucleic acids fraction up to 41 hours. Treatment of the recovered nucleic acids with RNase H separated the labeled Oligodeoxynucleotide from the nucleic acid fraction, indicating an association of the antisense Oligodeoxynucleotide and cellular RNA after uptake. When focal cerebral ischemia was induced 16 hours after the infusion of anti-rncfosr$_{115}$, the postischemic increase in Fos expression and AP-1 binding activity were suppressed. Specificity of the effect of anti-rncfosr$_{115}$ was suggested by its failure to suppress the DNA binding activity of nuclear cyclic AMP response elements. These results support the hypothesis that increased AP-1 binding activity following focal cerebral ischemia is dependent on Fos expression and can be inhibited in vivo by antisense c-*fos* oligodeoxynucleotides.

The molecular events of brain adaptation to injury that may underlie functional recovery after stroke remain largely undefined. Recent observations of altered gene expression in ischemic brain using animal stroke models have opened new avenues for exploration of the biochemical cascades after stroke [1–11]. These postischemic events include an increase in extracellular excitatory amino acid neurotransmitters such as glutamate. Glutamate receptor–mediated activation of phospholipases and protein kinases results in the alteration of nuclear regulatory processes, including the expression of immediate early genes such as c-*fos, junB,* and c-*jun* [5,12]. The Fos, Jun, and JunB proteins have been shown to form activator protein 1 (AP-1) through a conserved dimerization domain, i.e., the leucine zipper [13]. Transcription regulator AP-1 protein binds a specific DNA motif and is believed to transactivate the expression of a number of late effector genes [14–19].

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In the ischemic brain, we have previously demonstrated an increase in AP-1 binding activity [9]. A number of genes that bear neurotrophic properties may be regulated by transcription regulator AP-1. These genes, such as heat shock protein [1,4,19–22], amyloid [23], neurotrophins [7], and protein tyrosine kinase receptor *trkB* [24], have been shown to be induced following cerebral ischemia. The causal relationship between the Fos/Jun–AP-1 cascade and the subsequent expression of the late effector genes, however, has not been studied. In an attempt to characterize the pathophysiological significance of c-*fos* expression following focal cerebral ischemia, we developed an antisense strategy that may suppress post-ischemic c-*fos* expression and consequently AP-1 binding activity.

Naturally occurring antisenseRNAs are known to regulate the plasmid copy number in bacteria. The discovery of the antisense-RNA regulatory mechanism has raised the possibility of the synthesis of antisense oligonucleotides to regulate selected genes in single cells or whole animals [25–27]. The oligonucleotides employed in the antisense strategies are usually singlestranded DNA, 15 to 30 nucleotides in length. Anti-sense oligodeoxynucleotides have been used to suppress the expression of c-*fos* gene in cell culture systems [28–34] and in an animal model [35]. The antisense oligodeoxynucleotides are designed to block selected events such as transcription, splicing, or translation of the target mRNA. In special circumstances, similar to the delivery of cloned genes in plasmid, anti-sense oligodeoxynucleotides longer than 30 nucleotides can be internalized with the aid of lipofectin [36–38]. Although the cellular uptake of antisense oligodeoxynucleotides can be mediated via carrier-mediated endocytosis [39,40], phosphorothioated oligodeoxynucleotide-lipofectin uptake by cells does not appear to involve endosomes or lysosomes [41,42]. The present study of an in vivo antisense strategy utilizes a focal cerebral ischemia model in rats, in which the expression of c-*fos* and Fos protein and the enhanced AP-1 binding activity have been established and characterized [6,8,9].

Materials and Methods

Materials

The antisense oligodeoxynucleotides tested were respectively an anti-rncfosr$_{115}$ (5′-catcatggtcgtggtttgggcaaacc-3′) and anti-rncfosr$_{143}$ (5′-cctcgtagtccgcgttgaaacccgag-3′) that are completely complementary to the rat c-*fos* mRNA sequence (at nucleotide nos. 140–115 and 168–143, respectively, locus *rncfosr*, GenBank accession no. X06769) and an anti-rncfosrA17 (mismatch antisense, 5′-gaacatcatCgtGgCgg-3′; the capital letters are variations from *rncfosr* sequence). The sense oligodeoxynucleotides (sense-rncfos$_{115}$, sense-rncfos$_{143}$, or sense-rncfosrA17) are complementary to each of the antisense sequences, respectively. Unless specified, antisense and sense oligodeoxynucleotides in this study were the rncfosr$_{115}$ sequence. In preliminary studies we compared unmodified versus phosphorothioated oligodeoxynucleotides and noted that their effects on c-*fos* suppression were similar. The modified oligodeoxynucleotide, however, appeared more stable in cellular uptake studies (see Results). In subsequent experiments, only modified oligodeoxynucleotides were used. Totally phosphorothioated oligodeoxynucleotides were synthesized by National Biosciences, Inc (Plymouth, MN). In another preliminary study we also noted that standard desalting or further purification of oligodeoxynucleotides by reverse-phase high-performance liquid chromatography (HPLC) resulted in no significant difference in Fos expression (unpublished results). Subsequently, standard desalting was used routinely in this study.

Polyclonal antibodies against Fos peptide (Ab-2) and protein A-agarose were from Oncogene Science (Manhattan, NY). Prior to application in immunohistochemistry and to avoid variabilities in different lots of Ab-2, we used the antibody generously provided by Dr T. Curran (Roche Institute of Molecular Biology, Nutley, NJ) as the standard to ensure that the Fos immunostaining based on Ab-2 was comparable with an anti-Fos antibody with specificity established in previous publication [43]. Proteinase K, T4 polynucleotide kinase, RNase H,

RNase A, double-stranded oligodeoxynucleotides with AP-1 (5′tccggctgactcatcaagcg-3′), CREB (5′-ctagctctctgacgtcaggcaatctct-3′), SP-1 (5′-gctcgccccgccccgatcgaat-3′) consensus sequences, in vitro translation kit with nuclease-treated rabbit reticulocyte lysate, recombinant RNasin (rRNasin), and human placenta RNasin (hpRNasin) were from Promega Biotech (Madison, WI). Poly(dI)-poly(dC) · poly(dI)-poly(dC) was from Pharmacia LKB (Mechanicsburg, PA). Lipofectin and Superscript II reverse transcriptase, DNA size markers, distilled phenol were from Bethesda Research Laboratories (Bethesda, MD). Phagemid pBluescript^{r}II SK(–) was from Stratagene (La Jolla, CA). Radioactive nucleotides were from New England Nuclear (Boston, MA). RNAzol B was from Biotecx (Houston, TX). Taq DNA polymerase was from Perkin-Elmer Cetus (Norwalk, CT). Chloroform, isoamyl alcohol, *n*-propanol, ethanol, paraformaldehyde, Nonidet P-40, N-laurolsarcosin, and goat anti-rabbit IgG conjugated with biotin were from Sigma Chemical Co (St Louis, MO). Ammonium persulfate, TEMED, peptide size markers, agarose, acrylamide were from Bio-Rad Laboratories (Richmond, CA). Nembutal was purchased from Abbott Laboratories (North Chicago, IL). Long–Evans male rats (body weight, 300 ± 30 gm) from Harlan (Indianapolis, IN) were used.

IN VITRO TRANSLATION OF *c-fos* mRNA TO FOS PROTEIN—The synthetic antisense and sense oligodeoxynucleotides were first tested for their effects on the in vitro translation of c-*fos* mRNA to Fos protein. A full-length c-*fos* mRNA was transcribed from a linear c-*fos* cDNA in pBluescript^{r}II SK(−) phagemid using T3 RNA polymerase. The Fos protein was translated using nuclease-treated reticulocyte lysate. Antisense and sense oligodeoxynucleotides (10 or 100 pmol each) or vehicle only were added to c-*fos* mRNA (0.6 or 2.25 μg) in 5-μl volume and heated at 65°C for 10 minutes, followed by brief centrifugation in a capsulefuge (Tomy HF-120, Peninsula Laboratory, Inc, Belmont, CA) and then cooled to room temperature. The samples were then kept on ice. A mixture of 6 μl containing RNasin (20 units) and amino acids [20 μM for each amino acid except [^{35}S]methionine at 2 μM (Expre ^{35}S^{35}S-protein labeling mix, ^{35}S at > 1,000 Ci/mmol and 5 mCi/ml)] was added to each reaction mixture and incubated for 5 minutes at room temperature, followed by an addition of the lysate (14 μl). The reaction was carried out at 30°C for 60 minutes. The RNasin used in this study was either recombinant or human placenta RNasin. The peptide was immunopurified using Ab-2 antibodies (0.5μg) and protein A-agarose for 16 hours at 4°C, followed by three washes in NET-gel buffer (50 mM, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, pH 8.0, 0.02% sodium azide) [44]. The peptide was denatured in 30 μl of 1X sodium dodecyl sulfate (SDS) gel loading buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol [DTT], 2% SDS, 0.1% bromophenol blue, 10% glycerol) at 95°C for 3 minutes, followed by brief centrifugation; 15 μl of the immunol-purified peptide in the supernatant was resolved in 7.5% stacking SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using Tris-glycine electrophoresis buffer (25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS), followed by autoradiography [44]. The autoradiograms were quantitated by using a densitometer (E-C Apparatus Corp, St Petersburg, FL).

ANIMAL STROKE MODEL—Surgery to produce focal cerebral ischemia was conducted under Nembutal anesthesia (50 mg/kg) and has been detailed in previous publications [45–48]. This model is characterized by a severe ischemia (blood flow reduction by 88–92%) confined to the cortex of the right middle cerebral artery (MCA) territory. The duration of ischemia was 30 or 90 minutes. Ischemia for 90 minutes resulted in severe ischemic brain injury leading to consistent coagulation necrosis in the right MCA cortex (infarct volume of approximately 140 mm^3, n = 6). The contralateral (left MCA) cortex and ipsilateral and contralateral hippocampi and subcortical structures sustained little or only mild ischemia (blood flow reduction of 0–20%) with no morphological evidence of ischemic injury. In this model, consistent expression of c-*fos* mRNA by Northern blot or in situ hybridization, and of

Fos antigen by immunohistochemistry and increase in AP-1 binding activity have been reported [6,8,9].

The rectal temperature was monitored and maintained at 37 ± 0.5°C via an electronic temperature controller (Versa-Therm 2156, Cole-Pharmer, Chicago, IL) connected to a heating lamp. Physiological parameters including arterial blood pressure and pulse rate were monitored and maintained within normal ranges during surgery and up to 60 minutes after ischemia. Animals were kept in air-ventilated incubators at 24 ± 0.5°C for different recirculation (reperfusion) periods and allowed free access to food and water after recovery from anesthesia. At the designated time points, animals were anesthetized and killed. For nucleic acid and protein isolation, the animals were killed by decapitation under anesthesia. The brain was quickly dissected on ice and the entire cerebral cortex and hippocampus from the right hemisphere were immediately separated, then frozen in liquid nitrogen before being transferred to a − 75°C freezer. For immunohistochemical study or in situ hybridization, the animals were anesthetized and then transcardially perfused with normal saline, followed by 4% paraformaldehyde in phosphate-buffered saline (PBS) via the left ventricle of the heart.

INTRAVENTRICULAR DELIVERY OF OLIGODEOXYNUCLEOTIDES— Intraventricular infusion of the antisense or sense oligodeoxynucleotides (10 pmol [the uptake study] or 10 nmol [Fos and AP-1 studies] in artificial cerebrospinal fluid [total volume = 40 μl]) was carried out with the aid of a stereotaxic device. In some experiments lipofectin (15 ng [the uptake study] or 15 μg [Fos and AP-1 studies]) was added to facilitate the transfer of oligodeoxynucleotide into the neurons in vivo. Lipfectin has been shown to increase transfection efficiency in vitro and in vivo [36,37,41,42]. The oligodeoxynucleotide was delivered through a custom-made intraventricular catheter directly into the left lateral ventricle. The catheters were placed using a stereotaxic device (L: −1; A–P: +2; D–V: −4.5) through a 1-mm burr hole that was drilled under constant cooling with normal saline. The location of the burr hole was also guided by the stereotaxic device. Based on the wet weight of a rat brain at an average age of 10.5 weeks, the initial intracerebroventricular concentration of the oligodeoxynucleotide was estimated to be at least 10 μM. After delivery of the oligodeoxynucleotide, the burr hole was sealed with epoxy. Approximately 17 to 18 hours (overnight) after delivery, the animals were anesthetized for surgery to produce focal cerebral ischemia as described above. Periodic infusion of 40 μ1 of 2% Evans blue in separate animals was made to verify the location of the intraventricular catheter. All experiments were carried out in a double-blinded, sense oligodeoxynucleotide-controlled fashion in random order to ensure the objectivity of the investigators who induced ischemia or conducted molecular biology and immunohistochemistry studies [47,48].

IN VIVO UPTAKE OF OLIGODEOXYNUCLEOTIDES—The single-strand anti-rncfosrA17 or anti-rncfosr$_{115}$ oligodeoxynucleotide was labeled on the 5′-end using T4 polynucleotide kinase and [γ-^{32}P]ATP (3,000 Ci/mmol), followed by gel electrophoresis purification (16% nondenaturing PAGE). The purified oligodeoxynucleotide (1–3 × 10^6 cpm in 10 pmol per animal) was mixed with 15 ng lipofectin and was delivered via a catheter into the left lateral ventricle as described above. At different intervals after oligodeoxynucleotide delivery, the animal was killed by intracardiac perfusion under anesthesia with 200 ml of PBS. The right hippocampus was then quickly dissected on ice, washed once in ice-cold PBS, and homogenized in 4 ml of ice-cold STE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The nuclear fraction was separated from the cytosol fraction by centrifugation (3,000 *g*, 15 minutes, 4°C). The nuclear fraction in the pellet was washed once in STE buffer and was centrifuged as before. The two fractions of the supernatant were combined to constitute the cytosol fraction; both nuclear and cytosol fractions were separately treated with proteinase K (500 μg/ml in 0.5% SDS) at 50°C for 16 hours, followed by extractions of nucleic acids in phenol (pH 4, RNA grade) and chloroform/isoamyl alcohol (24:1, vol/vol). An equal volume

of *n*-propanol was added to the aqueous phase and mixed. The chromosomal DNA in the nuclear fraction was removed using a glass rod. The low molecular weight nucleic acids in both cytosol and nuclear fractions were obtained by centrifugation at 4°C (30 minutes, 15,000 *g*) followed by one wash in 70% ethanol. After being air-dried in a vacuum, the nucleic acids were dissolved in 1.1 ml of Tris-EDTA (TE; pH 8.0) buffer. An aliquot each of the cytosol and nuclear fractions (100 μl) was added to 0.5 ml of counting scintillant (Amersham Corp, Arlington Heights, IL) and the radioactivity was determined in a liquid scintillation counter (Wallac 1410, Pharmacia, Gaithersburg, MD) for calculation of the recovery. The radioactivity in the nuclear acids from nuclear fractions was low and did not allow further analysis by gel electrophoresis. To assess a possible association between the oligodeoxynucleotides and the recovered nucleic acids in the cytosol fraction, 200 μl of each sample from various time points were incubated with or without RNases (2 or 7.5 units RNase H, 10 μg of heat-inactivated RNase A) at 37°C for 30 minutes. After phenol and chloroform extractions, *n*-propanol precipitation, and ethanol washes, remaining nucleic acids were dissolved in 100 μ1 of TE and a fraction (20 μl) was resolved by 16% nondenaturing PAGE [44].

FOS IMMUNOHISTOCHEMISTRY—Fos antigen expression after ischemia was studied by immunohistochemistry. Animals were transcardially perfused under anesthesia as described above, with normal saline followed by 4% paraformaldehyde in PBS, and cryoprotected in 20% sucrose/0.1 M PBS (pH 7.8) overnight at 4°C [49]. Sample slices were prepared as described previously [9,50], and frozen tissue sections were incubated in PBS containing 1% bovine serum albumin (BSA) and 0.1% NaN_3 (PBS-BSA) for 30 minutes, then in 0.03% hydrogen peroxide for 5 minutes, followed by washing in PBS-BSA five times. The sections were incubated with Ab-2 in PBS-BSA at a suitable dilution at 4°C overnight. We found that dilution of Ab-2 to 1:400 to 1:500 or 0.2–0.25 μ/ml gave the best result. After washes in PBS-BSA, the tissue sections were incubated with a secondary antibody (goat anti-rabbit IgG conjugated with biotin at 1:400 dilution) at room temperature for 45 minutes. The antigen–IgG complex was incubated with avidin-horseradish peroxidase (1:100) for 30 minutes, and then stained by diaminobenzidine (DAB) using Vectastain ABC kits (Vector Laboratories, Burlingame, CA).

GEL MOBILITY SHIFT ASSAY FOR TRANSCRIPTION FACTORS—DNA-protein interaction in nuclear extract was measured using ^{32}P-radiolabeled consensus sequences of AP-1, CREB, and SP-1 as described previously [51,52]. This assay was established as a method to detect alterations of the AP-1 binding activity in a previous publication [9]. Moreover, we found the presence of single-stranded and- or sense-rncfosr$_{115}$ did not affect the AP-1 binding activity in the gel mobility–shift assay under these conditions (data not shown). The consensus oligodeoxynucleotide was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and was purified using PAGE. The assay mixture contained, in a total volume of 20 μl, 10 μg protein equivalent of nuclear extract, 2 to 10×10^4 cpm of ^{32}P-labeled consensus oligodeoxynucleotide, 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 μg of poly(dI)-poly(dC) · poly(dI)-poly(dC). The DNA–protein complex was resolved by a nondenaturing PAGE (5%) and high ionic-strength buffer (50 mM Tris, 0.4 M glycine, 2 mM EDTA, pH 8.5).

Results

In Vitro Translation of c-*fos* mRNA to Fos Protein

The effect of antisense and sense oligodeoxynucleotides on the in vitro translation of c-*fos* mRNA was studied. In the absence of antisense c-*fos,* a single Fos peptide of 62 kd (Fig 1A, lane 1) was successfully translated under the in vitro condition employed in this study. Both anti-rncfosr$_{115}$ and anti-rncfosr$_{143}$ inhibited c-*fos* mRNA translation completely (lanes 2–4)

or almost completely (13% of control by densitometer, lane 5 versus lane 1). This Fos peptide was also translated and immunoprecipitated in the presence of sense–rncfosr$_{115}$ (Fig 1B lane 1). This assay was specific for the detection of synthetic peptide using c-*fos* mRNA (see Fig 1B, lane 3) but not its complementary mRNA (see Fig 1B, lane 4). Results from these studies confirmed that the in vitro c-*fos* mRNA translation could be inhibited by antisense c-*fos* oligodeoxynucleotides.

In Vivo Uptake of Oligodeoxynucleotides

With ^{32}P-labeled anti-rncfosr$_{115}$, we recovered approximately 1% of labeled oligodeoxynucleotide in the cytosol fractions in samples collected at 6 through 41 hours after intraventricular infusion in 16 animals. The gel electrophoresis pattern of ^{32}P-labeled antisense oligodeoxynucleotide is shown in Figure 2. The radioactivity in the cytosol fraction appeared as a low molecular weight species, approximately the size of the preinfusion oligodeoxynucleotide up to 4 hours after intraventricular infusion of the labeled oligodeoxynucleotide.

Between 6 and 41 hours, we observed an appearance of several high molecular weight species in the cytosolic fractions. One species migrated the approximate equivalent of a 75-bp double-stranded DNA, and the other stayed in the wells with little migration. Treatment of the samples with RNase H (in the presence [Fig 2A] or in the absence [Fig 2B] of heat-treated RNase A) resulted in the disappearance of the high molecular weight species and an increase in the low molecular weight species equivalent to the labeled unbound oligodeoxynucleotide. Heat-treated RNaseA in the absence of RNase H, on the other hand, did not affect the high molecular weight species (data not shown).

Inhibition of the Postischemic Fos Protein Expression by an Antisense Oligodeoxynucleotide

In 15 animals, we compared the effect of anti-rncfosr$_{115}$, anti-rncfosrA17, and their sense oligodeoxynucleotides on Fos antigen expression induced by ischemia. In animals that received no oligodeoxynucleotide, we observed an increase in the postischemic expression of Fos antigen following focal cerebral ischemia in the hippocampus (positive control, Fig 3A) compared with those without ischemia (sham control, Fig 3B). The same increase was observed in five animals that received sense c-*fos* oligodeoxynucleotide with (n = 2) or without (n = 3) lipofectin (one is shown in Fig 3C), indicating that the sense c-*fos* oligodeoxynucleotide or lipofectin did not have a deleterious effect on Fos expression. In the six animals that received antisense c-*fos* oligodeoxynudeotide with (n = 2) or without (n = 4) lipofectin, we observed a significant reduction in the Fos antigen in hippocampal neurons (one is shown in Fig 3D) to a level very similar to normal or sham-operated animals. A mismatched anti-rncfosrA17 oligodeoxynudeotide did not inhibit ischemia-induced Fos expression (n = 4; one is shown in Fig 3E), though the level of ischemia-induced Fos antigen was less intense than the positive control. This could be due to the fact that only three bases of the 17 nucleotides to the c-*fos* gene are altered. Because of coagulation necrosis in the ischemic cortex, expression of Fos antigen in the right MCA cortex was not consistently observed. The effect of anti-rncfosr$_{115}$ oligodeoxynucleotide on Fos expression in the right MCA cortex, therefore, could not be reliably determined.

Effects of Antisense Oligodeoxynudeottdes on the AP-1 Binding Activity

A total of 15 animals were used for AP-1 binding study. A postischemic increase in the hippocampal AP-1 binding activity after ischemia was noted in rats without oligodeoxynucleotide infusions (Fig 4A, lane 8, n = 3) and in rats with preischemic infusion of sense-rncfosr$_{115}$ oligodeoxynucleotide (Fig 4A, lanes 1, 3, 5; n = 4) compared with rats with sham operations (see Fig 4A, lane 7). Eight animals that received anti-rncfosr$_{115}$

oligodeoxynucleotide showed a significant reduction in the ischemia-induced hippocampal AP-1 binding activity (lanes 2, 4, and 6) compared with the positive controls and the sense-rncfosr$_{115}$ samples. The inhibitory effect was noted as early as 60 minutes after ischemia, and gradually increased to a maximal suppression at 4 (see Fig 4A, lane 4) and 16 hours (see Fig 4A, lane 6) after ischemia. Based on a semiquantitative analysis by a densitometer with sense oligodeoxynudeotide–treated rats as control (100%), anti-rncfosr$_{115}$ oligodeoxynudeotide reduced the AP-1 binding to 34% and 10% of the sense control at 1 and 4 hours, respectively, after initiation of reperfusion in the ipsilateral hippocampus. Moreover, the AP-1 binding activity at 16 hours after anti-rncfos$_{115}$ infusion was reduced to a level not distinguishable from the background (see Fig 4A, lane 6). Most important, the anti-rncfosr$_{115}$ oligodeoxynudeotide did not affect CREB (Fig 4B) or SP-1 (not shown) binding activity.

Discussion

We report here the in vivo suppression of ischemia-induced hippocampal Fos antigen expression and AP-1 binding activity using an antisense c-*fos* oligodeoxynudeotide. In this study, we first tested the ability of the antisense oligodeoxynudeotide (rncfosr$_{115}$ and rncfosr$_{143}$) to inhibit in vitro translation and confirmed that the antisense c-*fos* oligodeoxynudeotide was effective in blocking translation of c-*fos* mRNA. Anti-rncfosr$_{115}$ appears to be a better blocker than anti-rncfosr$_{143}$ (see Fig 1A). The sense-rncfosr$_{115}$, on the other hand, were without effect (see Fig 1B). Results from this experiment establish the effectiveness of the anti-rncfosr$_{115}$ for the intended objective of blocking c-*fos* mRNA translation. The lack of effects of other oligodeoxynucleotides, particularly the sense-rncfosr$_{115}$ oligodeoxynudeotide, establishes an appropriate control. These studies also confirm that inhibition of the target protein translation did not require the degradation of the target mRNA, and is consistent with the observation by Wahlestedt and colleagues [53] using antisense oligodeoxynudeotide to NMDA-1 which blocks the in vivo expression of *N*-methyl-D-aspartate (NMDA) receptor protein without the degradation of target mRNA.

In the study of the uptake of labeled antisense c-*fos* oligodeoxynucleotide, we found that when introduced into the central nervous system in vivo, the totally phosphorothioated oligodeoxynucleotide could be internalized by the brain cells, in agreement with the studies by Loke and co-workers [39]. Moreover, our recovery of the oligodeoxynucleotide in vivo is similar to that reported in cell culture system. In culture systems using cells supplemented with medium and heat-inactivated serum, about 1 to 2% of an added unmodified phosphodiester oligodeoxynucleotide can be internalized within 4 hours [37], and 25% was degraded after 24 hours [54,55]. The delay by 2 hours in our study could be due to a difference between the uptake by cells in a monolayer cell culture system and that in the in vivo multilayer cell system. On the other hand, we have not studied the cellular distribution of ^{32}P-oligodeoxynucleotide in the brain after infusion. The high background generated from ^{32}P-oligodeoxynucleotide in the extracellular space made such an in vivo study difficult.

We have shown the appearance of at least two labeled high molecular weight species between 6 and 41 hours after infusion of labeled antisense c-*fos* oligodeoxynucleotide (see Fig 2). In addition to oligodeoxynucleotide:RN A hybrids, these labeled high molecular weight species could be due to (1) contamination, (2) nonspecific binding during sample preparations, or (3) labeling of intracellular macromolecules by degraded products from the ^{32}P-nucleotide. Because the labeled high molecular weight species were not present at the zero and 4-hour time points (see Fig 2), their appearance after 6 hours suggests they did not resolve from contamination or nonspecific binding during sample preparation. While RNase A specifically digests and removes unhybridized regions of RNA from RNA: DNA hybrids, RNase H digests the hybridized RNA from RNA: DNA hybrid, whereby DNA becomes single stranded [44]. That these high molecular weight nucleic acids may be hybrids of labeled oligodeoxynucleotide

and cellular nucleic acids is suggested by the observation that an addition of RNase H facilitated the disappearance of the labeled high-molecular-weight species and led to an increase in the intensity of the labeled unbound oligodeoxynucleotide (see Fig 2, panels A and B). While an addition of RNase H and heat-treated RNase A did cause the conversion (see Fig 2A), additions of RNase A alone did not convert the high molecular weight radiolabeled nucleic acids to the preinfused oligodeoxynucleotide form (not shown). These findings reduced the possibility that the appearance of the labeled high molecular weight species was from an intracellular salvage of ^{32}P that was subsequently incorporated into nucleic acids. These results suggest that the labeled antisense c-*fos* oligodeoxynucleotide was taken up by the cells [56] and then hybridized with cellular nucleic acids, most likely RNAs, between 6 and 41 hours following intraventricular delivery.

The intraventricular infusion of the antisense c-*fos* oligodeoxynucleotide resulted in a drastic reduction in postischemic expression of nuclear Fos antigen in the ipsilateral hippocampus. The following three mechanisms have been proposed for the inhibition of translation using antisense c-*fos* oligodeoxynucleotides directed at the mRNA target: (1) inhibition of S80 ribosome formation during translation [57], (2) degradation of the target mRNA on DNA: RNA hybrid by RNase H [58], and (3) ribozyme mediated cleavage of the target mRNA [59]. Results supporting each of these mechanisms have been reported by several investigators [34,53,57–60]. We will consider only the first two mechanisms because the antisense c-*fos* oligodeoxynucleotide we used was not a ribozyme. If the first mechanism applies, no significant reduction of the target mRNA is expected [53,57]. On the other hand, a significant reduction in the target mRNA will be noted if the second mechanism prevails [34,58,60]. Despite the demonstration in this study that antisense oligonucleotides can suppress ischemia-induced Fos protein levels, we have been unable to demonstrate any reduction in c-*fos* mRNA levels using northern blot analysis or reverse transcriptase–polymerase chain reaction (unpublished data). Our results, therefore, are more consistent with the first mechanism. However, because c-*fos* mRNA transcription may be autoregulated [61], the inhibition of Fos protein synthesis may activate c-*fos* mRNA transcription, causing an increase in newly transcribed c-*fos* mRNA. Whether c-*fos* mRNA content can be maintained (and compensate for degraded c-*fos* mRNA) by such a mechanism remains to be determined. If there is a dynamic replacement of c-*fos* mRNA, a significant reduction in the c-*fos* mRNA might not be detected even if degradation of c-*fos* mRNA in DNA: RNA hybrids does occur. Our in vitro study of the inhibition of c-*fos* translation by antisense oligodeoxynucleotide does not distinguish the two mechanisms. More studies are needed to further delineate the mechanism of antisense suppression of Fos expression in vivo.

In the present study, the inhibition of ischemia-induced Fos expression by antisense c-*fos* oligodeoxynucleotide is supported by the following two lines of evidence: (1) a reduced Fos antigen expression in an immunohistochemical assay, and (2) a reduced AP-1 binding activity by using the gel mobility–shift assay. These findings are consistent with the physiological role of Fos as a major component of the AP-1 heterodimer in the postischemic increase in AP-1 binding activity [9]. Moreover, in the gel mobility–shift assay we noted that the activity of CREB, another major transcription factor, was also increased after ischemia. In contrast to AP-1, CREB activity was not reduced by this antisense c-*fos* oligodeoxynucleotide. The binding activity of another transcription regulator SP-1 activity was also not affected by these antisense c-*fos* oligodeoxynucleotides. This finding supports the specific action of the antisense c-*fos* oligodeoxynucleotide and suggests that the reduced AP-1 binding activity by the antisense c-*fos* oligodeoxynucleotide is unlikely to be a nonspecific toxic effect. The successful suppression of ischemia-induced Fos expression by this antisense strategy will allow us to explore further the pathophysiological roles of c-*fos* expression after cerebral ischemia. These include neuronal viability and neurotrophic gene expression in the hippocampus.

We have observed a delay in the suppression of AP-1 activity compared with that of Fos antigen. The expression of Fos antigen was completely suppressed within 1.5 hours (see Fig 3), but the AP-1 activity in the hippocampus was only reduced by 66% (see Fig 4A, lane 2) at 1 hour. The complete inhibition of AP-1 activities occurs at a much later time (at least 4 hours) than the inhibition of Fos expression. Because the turn over time for Fos protein is approximately 30 minutes [62], and the half-life of AP-1 activity is estimated to be 6 hours [63], the residual AP-1 activity could be from a preexisting Fos/Jun heterodimer or Jun/Jun homodimer. Moreover, AP-1 activity regulates Jun expression at both the transcription and posttranslation. The delayed inhibition in AP-1 activity could be a result of preexisting but diminishing c-*jun* expression to form Jun/Jun homodimer as a consequence of autoregulation [63].

In the ipsilateral hippocampus, the reduction in AP-1 binding activity correlated well with the decrease of Fos antigen expression induced by antisense c-*fos* oligodeoxynucleotide. In the ischemic cortex, we did not demonstrate a consistent expression of Fos antigen by immunohistochemical technique. The discrepancy in immunohistochemical findings between the hippocampus and the ischemic cortex is not clear. It should be noted that in this stroke model, the ipsilateral hippocampus sustains little or no ischemia but does show an intense regional expression of c-*fos* [8,9], presumably due to a probable secondary neurotransmitter mechanism [5,12]. A consistent coagulation necrosis develops in the right MCA cortex. It is possible that the capability to translate c-*fos* mRNA into Fos may be compromised in the severely ischemic cortex destined for degeneration. Alternatively, a limited diffusion of the antisense oligodeoxynucleotide to regions remote from the lateral ventricles might be partially responsible for the inconsistent effects on cortical Fos expression.

Impaired expression of other genes such as heat shock protein in the infarcted area has also been noted by others [64]. Fos/Jun heterodimer functions as a transcription activator by a sequence-specific interaction with the consensus AP-1 sequence and stimulates the transcription of nearby promoters by a yet undefined mechanism. AP-1 has been proposed as a master switch for the expression of several genes. Postischemic increase in AP-1 binding activity may underline one of the molecular mechanisms allowing the surviving neurons to adapt to the degenerative processes after ischemic brain injury [65–67]. In summary, our studies demonstrate the feasibility of an in vivo antisense strategy to suppress the postischemic expression of an immediate early gene. The subsequent reduction of the DNA binding activity of a transcription factor relevant to Fos supports the contention that Fos is a major component of AP-1 binding activity in the ischemic brain. These results also raise the possibility that the antisense c-*fos* oligodeoxynucleotide thus designed is capable of modulating the functional consequences related to c-*fos* expression and offers an alternative strategy to regulate c-*fos* expression. This mode of gene suppression may be useful in dissecting the molecular mechanism and in exploring the pathophysiological significance of postischemic c-*fos* expression [59].

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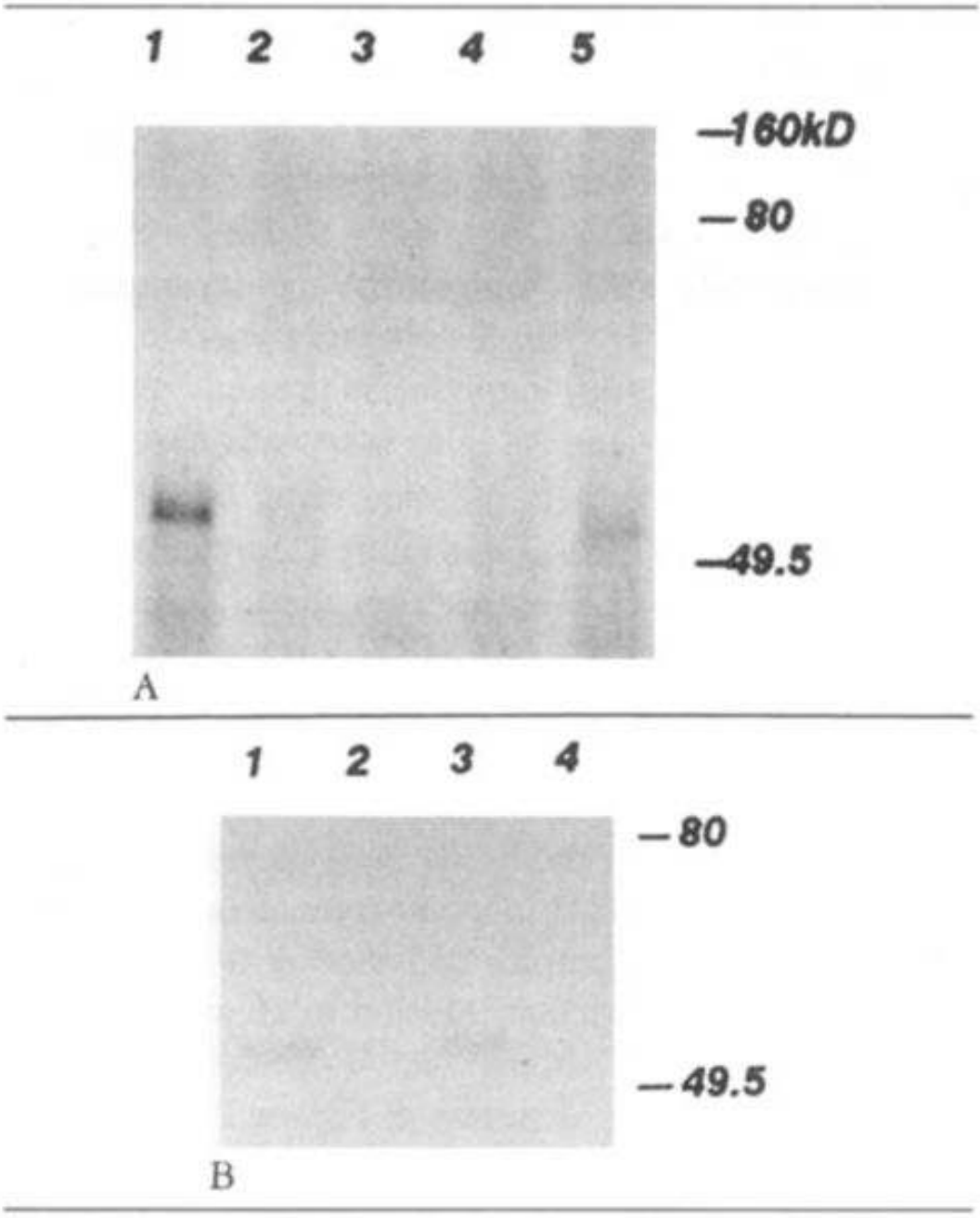

Fig 1.

Inhibition of c-*fos* translation in the presence of anti-sense c-*fos* oligodeoxynudeotide. (A) A full length c-*fos* mRNA (0.6 μg) was translated using rabbit reticulocyte lysate and {^{35}S} methionine with RNasin in the absence (lane 1 or in the presence of antisense oligodeoxynudeotide (10 pmol of anti-rncfosr$_{115}$ in lanes 2 and 3 or anti-rncfosr$_{143}$ in lanes 4 and 5). The peptide was immunoprecipitated by Ab-2 and protein A-agarose and then resolved in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7.5%). Three molecular mass markers were depicted, and a single peptide of 62 kd was shown. The intensity of the 62-kd protein as measured using a laser densitometer was 100% (lane 1), 13% (lane 5), and at a level not distinguishable from the background (lanes 2–4). (B) A full-length c-*fos* mRNA (lanes 1–

3) or a complementary c-*fos* mRNA (lane 4), each at 2.25 μg, was translated with the addition of hpRNasin and then the peptide was immunoprecipitated by Ab-2 and protein A-agarose as described in the text, except in the presence of 100 pmol unmodified sense-rncfosr_{115} (lane 1), anti-rncfosr_{115} (lane 2), or no oligodeoxynudeotide (lanes 3 and 4). Molecular mass markers were 80 and 49.5 kd.

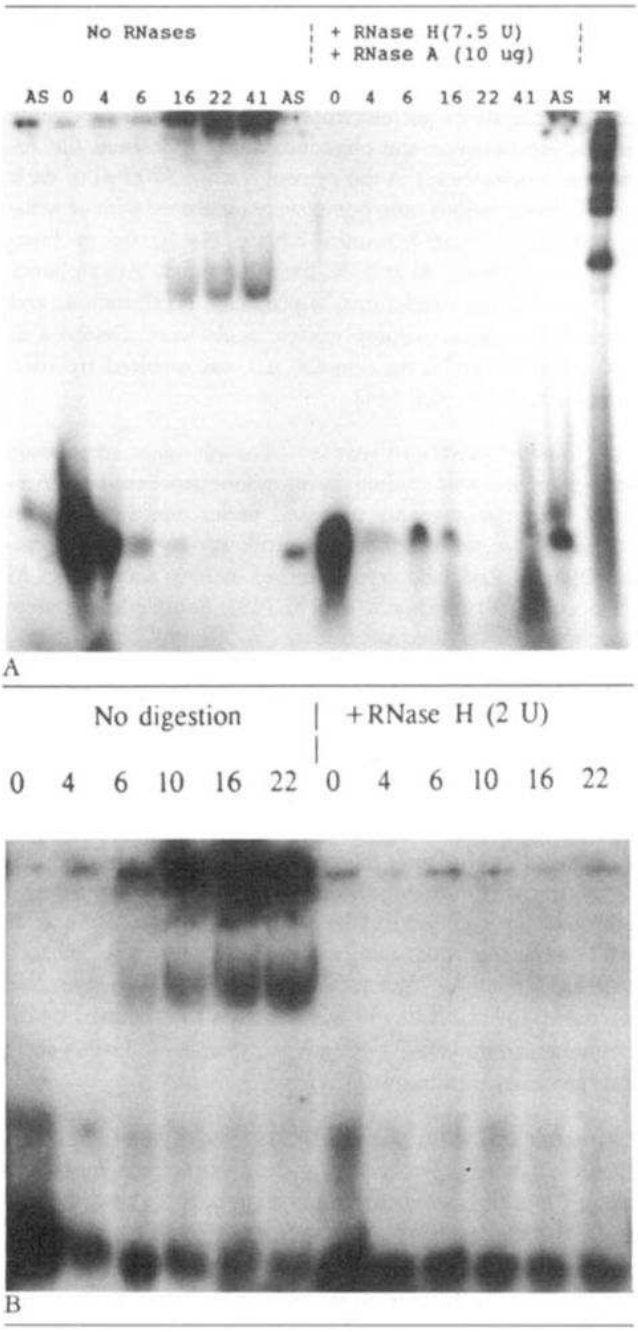

Fig 2.

The uptake and stability of antisense c-*fos* oligodeoxynudeotide after intraventricular infusion. A mixture of ^{32}P-antisense oligodeoxynudeotide (10 pmol) to c-*fos* and lipofectin (15 ng) was delivered into the left lateral ventricle as described in the text. At a specific time (hours) after delivery as indicated on the top of each lane, the cytosolic nucleic acids were extracted after proteinase K digestion. The nucleic acids were treated with or without RNase before being resolved in nondenaturing polyacrylamide gel electrophoresis. In one study (A), a double-stranded radiolabeled DNA size marker (1 kb ladder from BRL) was added to indicate a relative molecular weight of the oligodeoxynudeotide. AS = antisense oligodeoxynudeotide without

lipofectin before transfection. (B) Results from another uptake study in which the recovered nucleic acids were treated with RNase H compared with those without RNase.

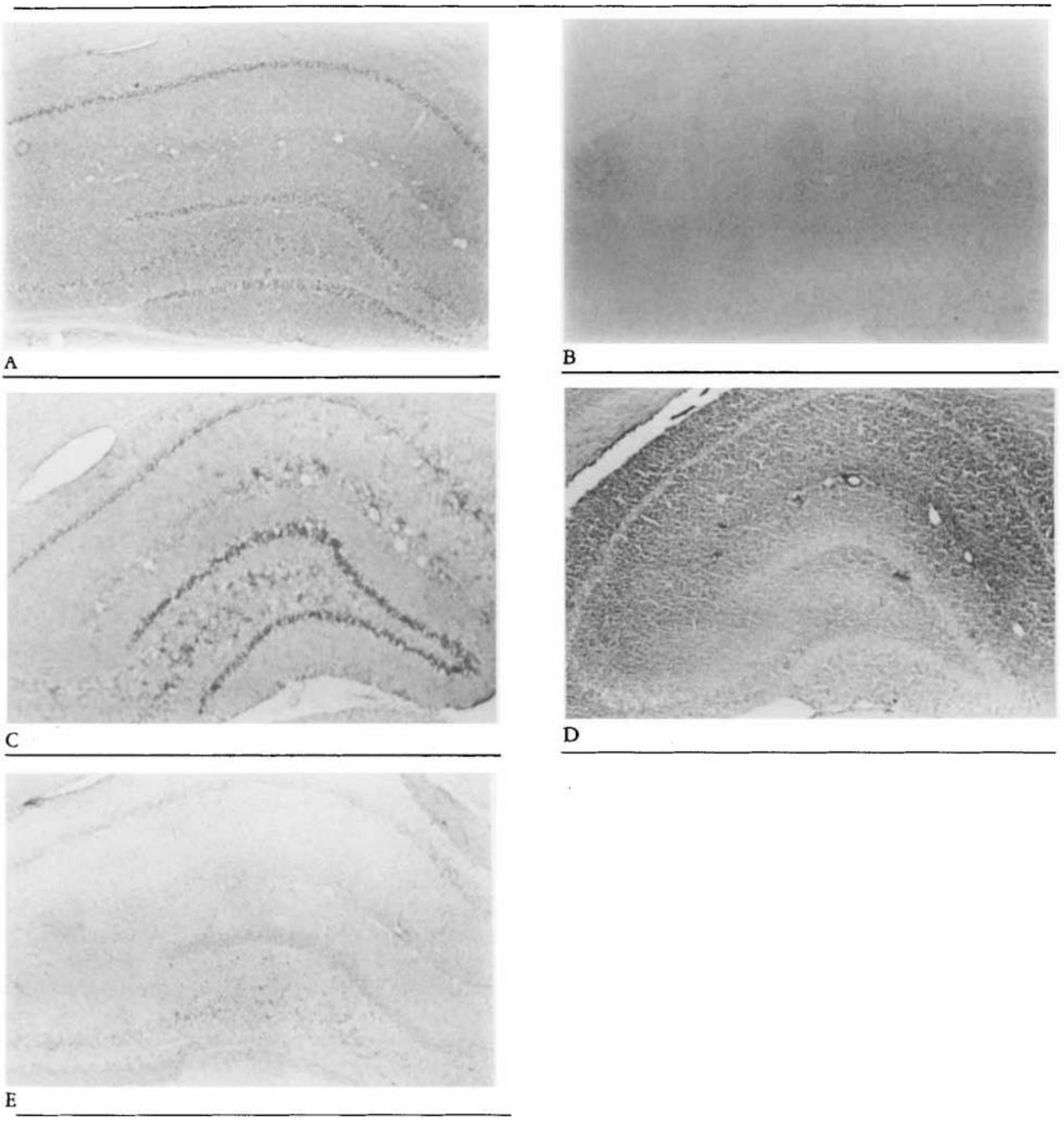

Fig 3.

Immunohistochemical demonstration of hippocampal Fos protein using polydonal antibodies. Representative specimens were shown for the positive control (A), sham operated (B), and seme c-*fos* oligodeoxynucleotide (C), antisense c-*fos* oligodeoxynucleotide (D), or mismatched anti-rncfosrA17 oligodeoxynudeotide-treated groups (E).

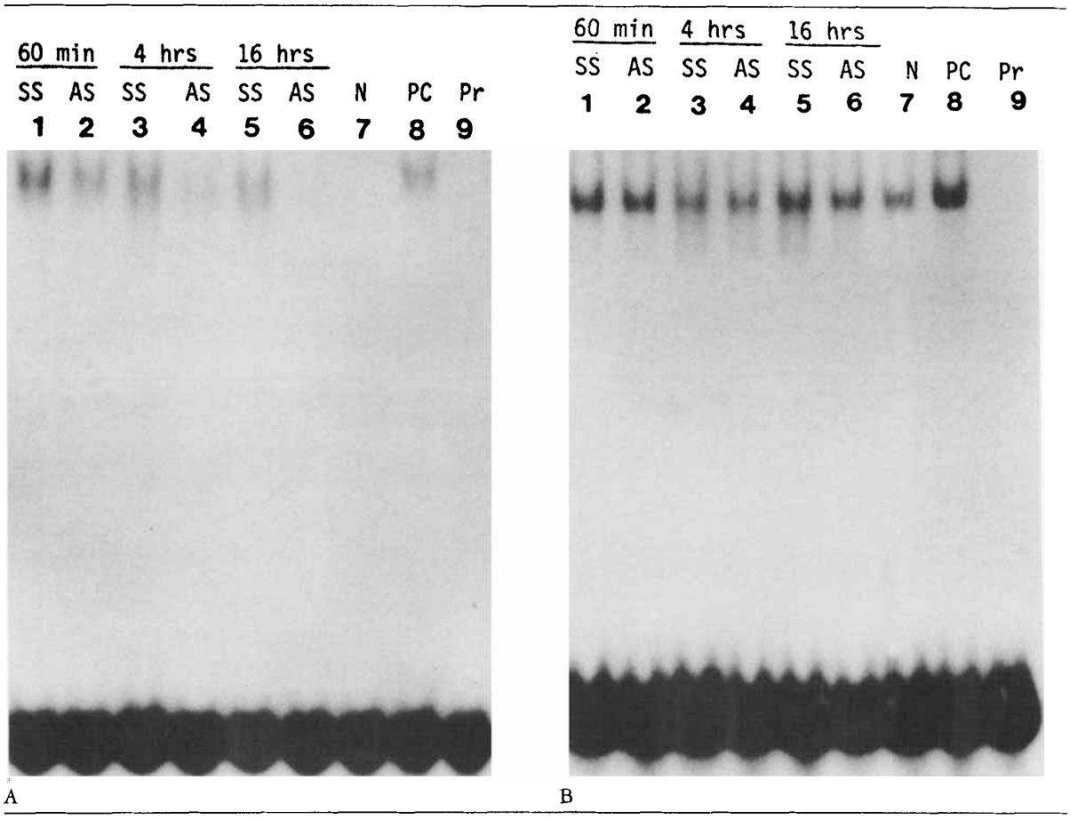

Fig 4.

Analysis ofAP-1 and CREB activities using nuclear extract from ipsilateral hippocampus in gel mobility–shift assay. Ischemia was induced 6 hours after infusion of sense (*SS*) or antisense (*AS*) oligodeoxynudeotide. Nuclear extract from brain samples were obtained 60 minutes to 16 hours after ischemia as described in the text and indicated on the top of each lane. Positive control (*PC*; ischemia plus 4 hours' reperfusion) and normal (*N*; no ischemia.) were animals that received no oligodeoxynudeotide. The radiolabeled consensus DNA probe (*Pr*) without extract was also shown. (A) AP-1 binding activity. (B) CREB activity.