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Kainic Acid–Induced Neuronal Death Is Associated with DNA Damage and a Unique Immediate-Early Gene Response in c-*fos*–*lacZ* Transgenic Rats

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Previously, we established that persistent upregulation of c-*fos* expression preceded kainic acid (KA)-induced neuronal death in mice. To discriminate between events that are products of the seizures elicited by KA and those that are specifically associated with its neurotoxic actions, we have examined the expression of cellular immediate-early genes (cIEGs) following KA or pentylenetetrazol (PTZ) treatment in c-*fos*–*lacZ* transgenic rats. While both chemoconvulsants elicit seizures, only KA causes selective neuronal death. Following treatment of transgenic rats with KA there was a protracted expression of Fos-lacZ that lasted for 2–3 d. In contrast, PTZ elicited a transient increase in the transgene product that lasted about 6 hr. Normally, Fos and Fos-lacZ were detected only in neuronal nuclei. However, 6 hr following kainic acid (but not PTZ) administration, β-galactosidase activity appeared in the cytoplasm of neurons within vulnerable regions (as determined by the terminal transferase biotinylated-UTP nick end labeling (TUNEL) procedure). Like c-*fos*, transcripts for other cIEGs were elevated for longer periods in the KA-treated rat hippocampus. In addition, *fra*-1 and *fra*-2 were only induced in the KA-treated rat. These changes in mRNA levels were paralleled by a sustained increase in AP-1 DNA binding activity. Thus, quantitative and qualitative changes in AP-1 DNA binding complexes accompany neurotoxic cell death that are not observed following seizures.

[Key words: excitotoxicity, pentylenetetrazol, kainic acid, TUNEL, oxidative stress, fos, jun]

Excitotoxicity produced by overstimulation of glutamate receptors has been proposed to play a key role in a number of significant neuropathological disorders including stroke, epilepsy, and Huntington's disease (Rothman and Olney, 1987). Despite the fact that this subject has attracted intense interest, the molecular mechanisms involved in coupling glutamate receptor activation to cell death are incompletely understood. However, there is evidence to indicate that changes in gene expression contribute to this process. For example, protein synthesis inhibitors can attenuate damage in stroke models and they can block glutamate toxicity *in vitro* and *in vivo* (Goto et al., 1990; Shigeno et al., 1990; Kure et al., 1991; Schreiber et al., 1993). The issue, therefore, is to identify which gene products are associated with neuronal death and to establish their role in this process.

A number of studies have shown that cellular immediate-early genes (cIEGs), such as c-*fos* and c-*jun*, are induced by conditions that result in the death of neurons (Sonnenberg et al., 1989a; Herdegen et al., 1992; An et al., 1993; Dragunow et al., 1993; Smeyne et al., 1993a,b). Furthermore, cell culture experiments indicate that several cIEGs, notably c-*myc*, c-*fos*, c-*jun* and NGFI-B, may actually contribute to the death process (Askew et al., 1991, Colotta et al., 1992; Evan et al., 1992; Shi et al., 1992; Smeyne et al., 1993a; Liu et al., 1994; Woronicz et al., 1994). The major difficulty in demonstrating an involvement of cIEGs in excitotoxicity is that they are induced by many types of stimuli, the vast majority of which do not lead to cell death. Therefore, we have attempted to assess whether the cIEG response accompanying neuronal death has any unique properties.

Rather than monitor the expression of the entire spectrum of cIEGs we have focused upon members of the Fos and Jun gene families. These proteins contribute, along with other members of the basic-zipper superfamily, to homo- and heterodimeric complexes that can interact with defined DNA elements (reviewed in Morgan and Curran, 1991). Thus, they can contribute to AP-1 (activator protein 1) and cAMP response element binding complexes. It was our interest to determine whether particular, and perhaps unique, AP-1 complexes are associated with excitotoxicity.

Previously we demonstrated the utility of a transgenic mouse line carrying a c-*fos*–*lacZ* marker gene in the analysis of programmed cell death (Smeyne et al., 1993a). Here we have extended this approach to a transgenic rat carrying c-*fos*–*lacZ*. These rats offer a rapid and unambiguous method to follow Fos expression with single cell resolution, while providing sufficient material to permit a rigorous biochemical and molecular genetic analysis of additional AP-1 components.

Kainic acid (KA) is a glutamate mimetic that is an agonist for the AMPA/kainate class of glutamate receptor and that induces a cIEG response *in vivo* (London and Coyle, 1979; Popovici et al., 1988; Sonnenberg et al., 1989a). When administered to rodents, KA initially elicits a period of generalized seizures that is followed, hours or days later, by the degeneration of specific subpopulations of neurons (Nadler et al., 1978; Repressa et al., 1987). To determine the alterations in cIEG ex-

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pression that might be unique to neuronal death, we administered another chemoconvulsant, pentylenetetrazol (PTZ). PTZ, like KA, is known to elicit seizures and induce c-*fos* expression, but it does not lead to neuronal death (Squires et al., 1984; Morgan et al., 1987; Dragunow and Robertson, 1988; Saffen et al., 1988; Sonnenberg et al., 1989a,b). While one must add the caveat that any differences in the cIEG response elicited by KA or PTZ may reflect the fact that they have distinct mechanisms of action, at a first approximation this permits us to begin the identification of candidate genes that might contribute, either positively or negatively, to neuronal death. Here we establish that there are several features of the cIEG response that are specifically associated with KA treatment.

Materials and Methods

Production of c-fos-lacZ transgenic rats. The murine c-*fos–lac*Z fusion gene used to generate transgenic mice has been described previously (Schilling et al., 1991; Smeyne et al., 1992). The linearized DNA was microinjected into fertilized rat oocytes according to published procedures (Hammer et al., 1990). Transgenic rats were identified by Southern analysis of DNA isolated from tail clips using a *lac*Z probe as described for mice (Smeyne et al., 1992).

Handling of rats. Seizures were induced by intraperitoneal (i.p.) injection of PTZ (55 mg/kg, Sigma) or KA (10 mg/kg, Sigma). Control animals received ip. saline injections (0.9% NaCl). At specified time points, animals were anesthetized with sodium pentobarbital (100 mg/kg, i.p.; Abbott Laboratories) and euthanized.

Preparation of tissue and β-galactosidase histochemistry. Rats were transcardially perfused with 100 ml of saline followed by 500 ml of 3% paraformaldehyde in 0.1 M PIPES buffer (pH 6.9). Following perfusion, brains were dissected, and postfixed overnight in 3% paraformaldehyde in 0.1 M PIPES containing 10% sucrose at 4°C. Tissues were then cryoprotected in 20% sucrose at 4°C. For sectioning, tissues were mounted in tissue freezing medium (Triangle Biomedical Sciences) at −54°C and then warmed to −23°C. Frozen sections were cut at 20 μm and thaw mounted onto Fisher brand premium microscope slides. Sections were then incubated overnight with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranosidase; Boehringer Mannheim) to visualize β-galactosidase activity and counterstained with neutral red (Oberdick et al., 1990).

Terminal transferase biotin-UTP nick end labeling (TUNEL) procedure. For the TUNEL procedure, frozen sections were prepared as for β-galactosidase histochemistry except that brains were fixed with 4% paraformaldehyde and slices were 6 μm in thickness. Sections were hydrated in H_2O then rinsed for 5 min. in 10 mM Tris-HCl, pH 8 before incubating for 15 min with 10 mg/ml proteinase K in 10 mM Tris-HCl, pH 8. Nonspecific peroxidase staining was eliminated with 3% H_2O_2 for 5 min. Sections were preincubated with terminal transferase (TdT) buffer (30 mM Tris base, 140 mM sodium cacodilate, pH 7.2, 1 mM $CoCl_2$) for 15 min at 37°C. Positive control sections were treated with 2.5 μl of RQ1 DNase. Subsequently, the sections were incubated with 40 μM bio-16-dUTP (Boehringer Mannheim), 0.3 U/μl TdT (IBI/Kodak), in TdT buffer for 2 hr at 37°C. The reaction was terminated with 2 × SSC and then stained with peroxidase using a Vectastain elite kit (Vector Labs; cat. no. PK-6100 and SK-4100). A qualitative assessment was made by at least two observers.

Hoechst 33258 staining. Morphological evaluations were made using Hoechst 33258 (Molecular Probes). Frozen sections were prepared as for β-galactosidase histochemistry. Sections 10 μm in thickness were stained for 5 min in Hoechst dye (1 μg/ml in distilled water), washed in water, and mounted with 50% glycerol/water. Sections were photographed, and then incubated overnight in X-gal to visualize β-galactosidase activity.

Isolation of RNA and Northern blotting. Nontransgenic sibling rats were treated with either PTZ or KA. At specified time points, animals were anesthetized with pentobarbital, decapitated, and the hippocampus dissected on ice and frozen in liquid nitrogen. Total RNA was isolated according to the RNAzol method (Biotecx Laboratories Inc.).

Aliquots of total RNA were analyzed by Northern blots according to described procedures (Molinar-Rode et al., 1993). cDNA probes were labeled with ^{32}P-dCTP using either the megaprime labeling system (Amersham RPN.1601Y) or nick translation (Amersham N.5000). The probes used have been described previously: c-*fos*, c-*jun*, and *jun*B (Sonnenberg et al., 1989c); *fra*-1 (Cohen and Curran, 1988); *fos*B (Zerial et al., 1989); *jun*D (Ryder et al., 1989); and *fra*-2 (GenBank accession number U18982).

Preparation of hippocampal nuclear extracts and gel shifts. Hippocampi were dissected and nuclear extracts prepared as described previously (Sonnenberg et al., 1989b).

Gel shift assays were performed using a ^{32}P-end-labeled, 20 base double-stranded oligonucleotide corresponding to the AP-1 site of the human collagenase gene (5′-AAGCATGAGTCAGACACCTC-3′; Angel et al., 1987). Binding reactions involved incubating 2 μg of hippocampal nuclear extract with 1.4 μg of poly (dI-dC) for 10 min at room temperature. Approximately 10 nM of the radiolabeled oligonucleotide was then added and the reaction was allowed to incubate for an additional 10 min. The DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel in buffer containing 25 mM Tris base and 195 mM glycine buffer (pH 8.5). The gels were dried and the results were visualized by autoradiography. For the competition assays, either a 20- or 50-fold molar excess of the designated cold oligonucleotide (mutated collagenase AP-1, 5′-AAGCAGGAGGCAGACACCTC-3′; hMT-II_A, GCCGCAAGTGACTCAGCGCGG-3′; Enk-1, 5′-GATC-GCGGGGCTGGCGTAG GG-3′) was added to the nuclear extract prior to the addition of the poly (dI-dC) and was allowed to incubate at 4°C for 30 min.

Results

Transgenic Fos-lacZ rats

Transgenic rats were generated using the c-*fos–lac*Z fusion gene described previously (Schilling et al., 1991; Smeyne et al., 1992). Since variability can exist between different lines of transgenic animals, offspring from four independent founder rats were analyzed. One line (denoted 1-8) was selected since the localization of the transgene product was similar to that reported using Fos immunohistochemistry in the rat following acute administration of PTZ and KA (Dragunow and Robertson, 1988; Popovici et al., 1988; Figs. 1–4 and data not shown). In addition, the basal and stimulated distribution of Fos-lacZ in this transgenic rat closely paralleled that seen in Fos-lacZ transgenic mice (Smeyne et al., 1992; data not shown).

Basal expression of Fos-lacZ was generally low in the brains of untreated rats of the 1-8 line, although sporadic staining was observed in the anterior olfactory nucleus and the cerebral cortex. In animals receiving intraperitoneal injections of saline, a small, transient increase of Fos-lacZ staining was observed (Fig. 1). This staining was most evident in the hippocampus two hours after injection where it was mostly confined to neurons within the CA1 field. This transient induction of Fos-lacZ may be attributed to the stress associated with handling and injecting the animals. Indeed Fos expression has been reported in the CNS, including the hippocampus, following stress (Deutch et al., 1991; Honkaniemi et al., 1992; Kononen et al., 1992; Imaki et al., 1993; Senba et al., 1993).

Influence of KA and PTZ on Fos-lacZ expression

Within 5 min of an intraperitoneal injection of PTZ (55 mg/kg) into c-*fos–lac*Z rats, clonic–tonic seizures were observed that lasted approximately 10 min. Expression of Fos-lacZ was not detectable in the hippocampus at 30 min following PTZ treatment, but it was highly induced by 2 hr (Fig. 2). With the exception of CA2, expression of β-galactosidase was seen in most neurons of the hippocampus where it was confined to the nucleus. Fos-lacZ staining decreased markedly by 6 hr and was absent by 24 hr postinjection. Besides the hippocampus, PTZ induced expression of the transgene in the anterior olfactory nucleus, caudate putamen, and frontal, pyriform and cingulate cortices (data not shown).

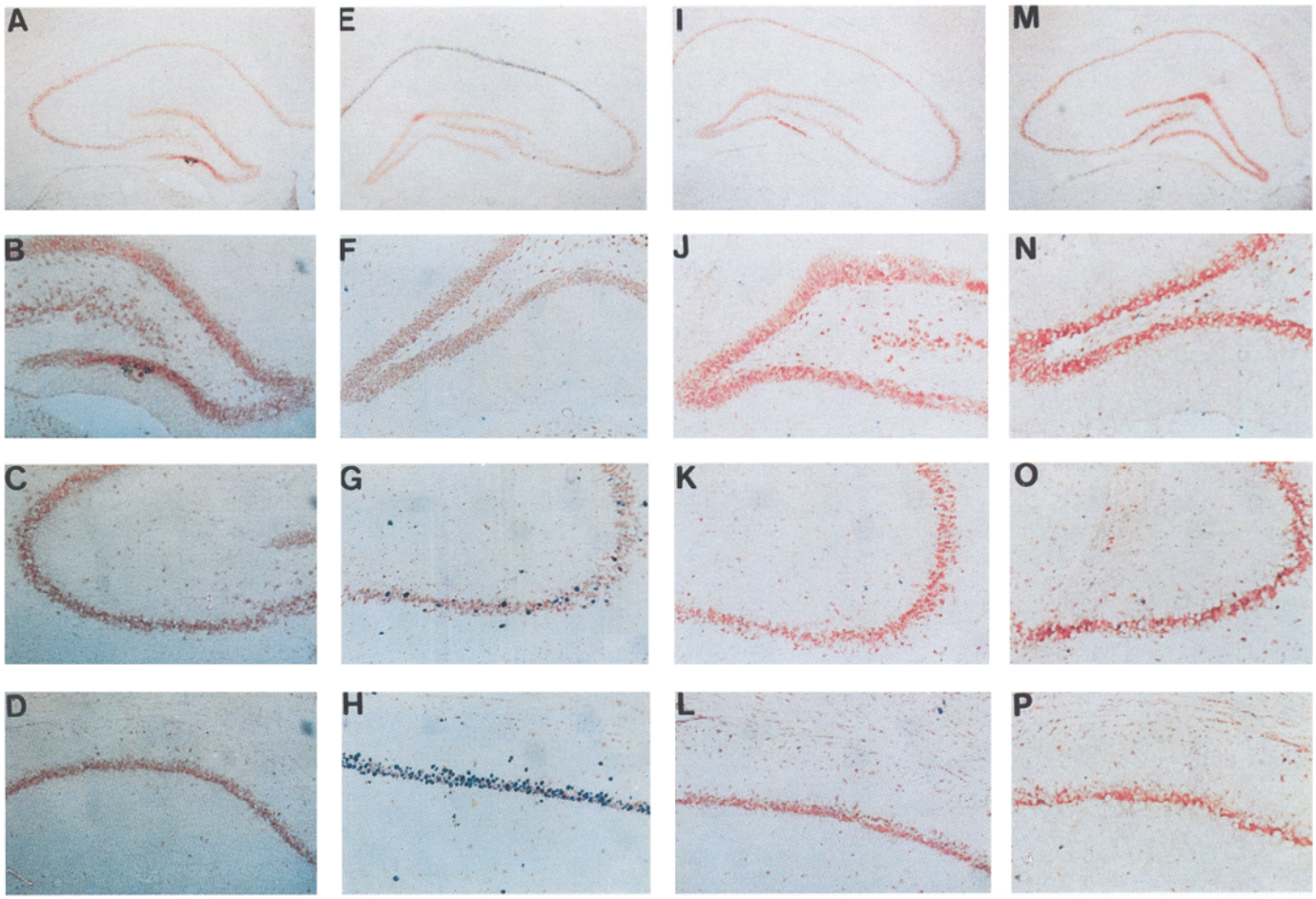

Figure 1. Time course of Fos-lacZ expression in the hippocampus of transgenic rats following saline injection. c-*fos*–*lac*Z transgenic rats were given an intraperitoneal injection of saline (1 ml/kg) and sacrificed at 0 (*A–D*), 2 (*E–H*), 6 (*I-L*), and 24 hr (*M–P*) following treatment. A transient increase in β-galactosidase activity was observed in CA1 pyramidal neurons while a few neurons within CA3 were also induced. Shown here are the hippocampus (*A, E, I, M*), dentate gyrus (*B, F, J, N*), CA3 (*C, G, K, O*), and CA1 (*D, H, L, P*). Magnification: 4X for *A, E, I,* and *M*; 10X for *B–D, F–H, J–L, N,* and *O*.

Compared to PTZ, intraperitoneal injection of KA (10 mg/kg) elicited a delayed but protracted behavioral response. Overt evidence of seizures first became apparent approximately 1 hr after injection of KA and increased in severity over the next 2–3 hr as the animals entered into status epilepticus. After 3 hr the intensity of the seizures waned and the animals appeared exhausted. Occasionally, seizures recurred upon subsequent handling of the rats.

Two hours following administration of KA, a large induction of Fos-lacZ was observed throughout the hippocampus in a pattern similar to that seen with PTZ (Fig. 3). However, in contrast to PTZ-treated rats, Fos-lacZ expression increased further by 6 hr post KA treatment. Furthermore, by 6 hr, β-galactosidase activity began to appear in the cytoplasm of neurons located in CA1 and CA3, a finding never encountered following treatment with PTZ. After 24 hr, extensive cytoplasmic β-galactosidase activity was observed in CA1 and CA3, a time when there was no detectable Fos-lacZ expression in PTZ-treated rats. In addition to the hippocampus, cytoplasmic Fos-lacZ was also detected in the pyriform cortex (Fig. 4), the anterior olfactory nucleus and cingulate cortex (data not shown). We have previously reported the presence of cytoplasmic Fos-lacZ in the hippocampus of kainic acid-treated mice (Smeyne et al., 1993). Also consistent with these observations is the finding by Popovici et al. (1988) of faint cytoplasmic Fos-like immunoreactivity in rat hippocampal pyramidal neurons following administration of KA.

Previously, it was demonstrated that barbiturates block seizure activity and cIEG induction in the mouse (Morgan et al., 1987; Sonnenberg et al., 1989a). Similarly, the cIEG response in the rat elicited by KA was blocked by the barbiturate, sodium pentobarbital (data not shown). Furthermore, a nonconvulsant dose of KA (7.5 mg/kg) did not cause a cIEG response (data not shown). This indicates that the mere presence of KA is not sufficient to elicit this response but that it requires seizure activity.

Expression of cIEGs following KA and PTZ treatment

To confirm the protracted expression of c-*fos* and to identify other gene products that might contribute to gene regulatory events following seizures, mRNA levels of other cIEGs were analyzed in PTZ- and KA-treated rat hippocampus. Of particular focus was the expression of genes that encode proteins related to Fos or Jun; namely *fra*-1, *fra*-2, *fos*B, *jun*B and *jun*D.

Both PTZ and KA induced a cIEG response in rat hippocampus as determined by Northern blot analysis (Fig. 5). However, there were a number of differences between the responses elicited by the two chemoconvulsants. Some of these differences

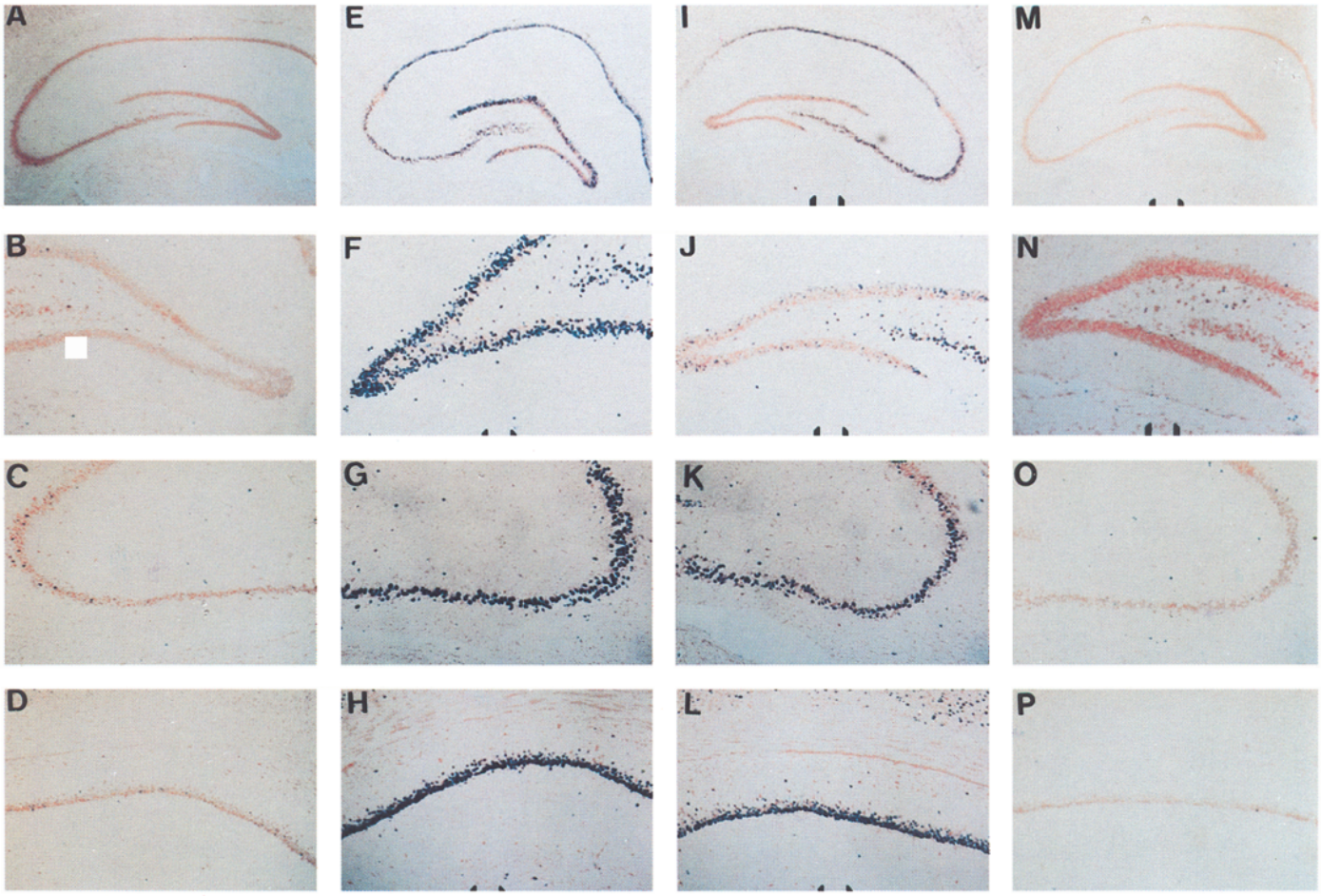

Figure 2. Time course of Fos-lacZ expression in the hippocampus of transgenic rats following PTZ treatment. Seizures were induced in c-*fos*–*lac*Z transgenic rats by intraperitoneal injection of PTZ (55 mg/kg). Rats were sacrificed at 0.5 hr (*A–D*), 2 hr (*E–H*), 6 hr (*I–L*), and 24 hr (*M–P*) following treatment. A transient increase in β-galactosidase activity was observed throughout the hippocampus. Shown here are the hippocampus (*A, E, I, M*), dentate gyrus (*B, F, J, N*), CA3 (*C, G, K, O*), and CA1 (*D, H, L, P*). Magnification: 4X for *A, E, I,* and *M*; 10X for *B–D, F–H, J–K, L,* and *N–P*.

were anticipated based upon the time course of the behavioral responses to the two agents. For example, the delayed onset of seizures in KA-treated, compared to PTZ-treated, rats is associated with a delayed rise in cIEG mRNA levels in the former. Furthermore, the more intense and extended period of seizures in KA-treated animals was paralleled by alterations in cIEG mRNA levels that were in general of greater magnitude and duration than in PTZ-treated rats. For example, following PTZ treatment, cIEG mRNA levels were highest at 30 min, declined markedly by 2 hr and were absent at 6 hr. In contrast, following KA administration cIEG mRNA levels were not elevated at 30 min, rose dramatically at 2 hr and 6 hr and were still well above baseline at 48 hr posttreatment. These results provide an independent confirmation of the data obtained in the c-*fos*–*lac*Z rats.

From a qualitative perspective, there were differences between the responses of individual cIEGs to KA and PTZ. Thus, the expression of several cIEGs was only detected in KA-treated rats. For example, c-*jun*, *fra*-1, and *fra*-2 transcripts were only robustly elevated by KA. Interestingly, in the case of *fra*-2, only the larger transcript was persistently elevated. Although *fra*-1 is a cIEG, its induction in fibroblasts is delayed in respect to c-*fos* and c-*jun* (Cohen and Curran, 1988). Here a similar temporal relationship is observed for *fra*-1. Several cIEGs showed sustained elevation, such as c-*fos* and *jun*B, although others increased and declined to baseline within 24 hr (e.g., *fos*B). Finally, *jun*D mRNA levels were not altered by either agent, indicating that it might be a constitutive component of AP-1 complexes in the hippocampus. In some cases a biphasic induction of cIEGs was observed. For example, c-*fos* and *jun*B levels declined between 6 and 24 hr and then increased again. On the other hand, c-*jun*, *fra*-1, and *fra*-2 exhibited a single phase of induction.

Characterization of AP-1 activity in KA-treated rat brain

The Northern blot data suggested that mRNA levels for many cIEG products that can contribute to AP-1 (and related) DNA binding complexes were elevated prior to neuronal death in the hippocampus. However, with the exception of Fos-lacZ expression, there was no independent proof that these mRNAs were translated into proteins. This is not a trivial point since inhibition of protein synthesis leads to an upregulation of cIEG mRNA levels (Muller et al., 1984). Furthermore, some neuropathological states, such as cerebral ischemia, are associated with compromised protein synthesis, which has led to confusing results regarding cIEG expression (Thilman et al., 1986; Kogure et al., 1987; Dragunow et al., 1993). Therefore, to demonstrate the

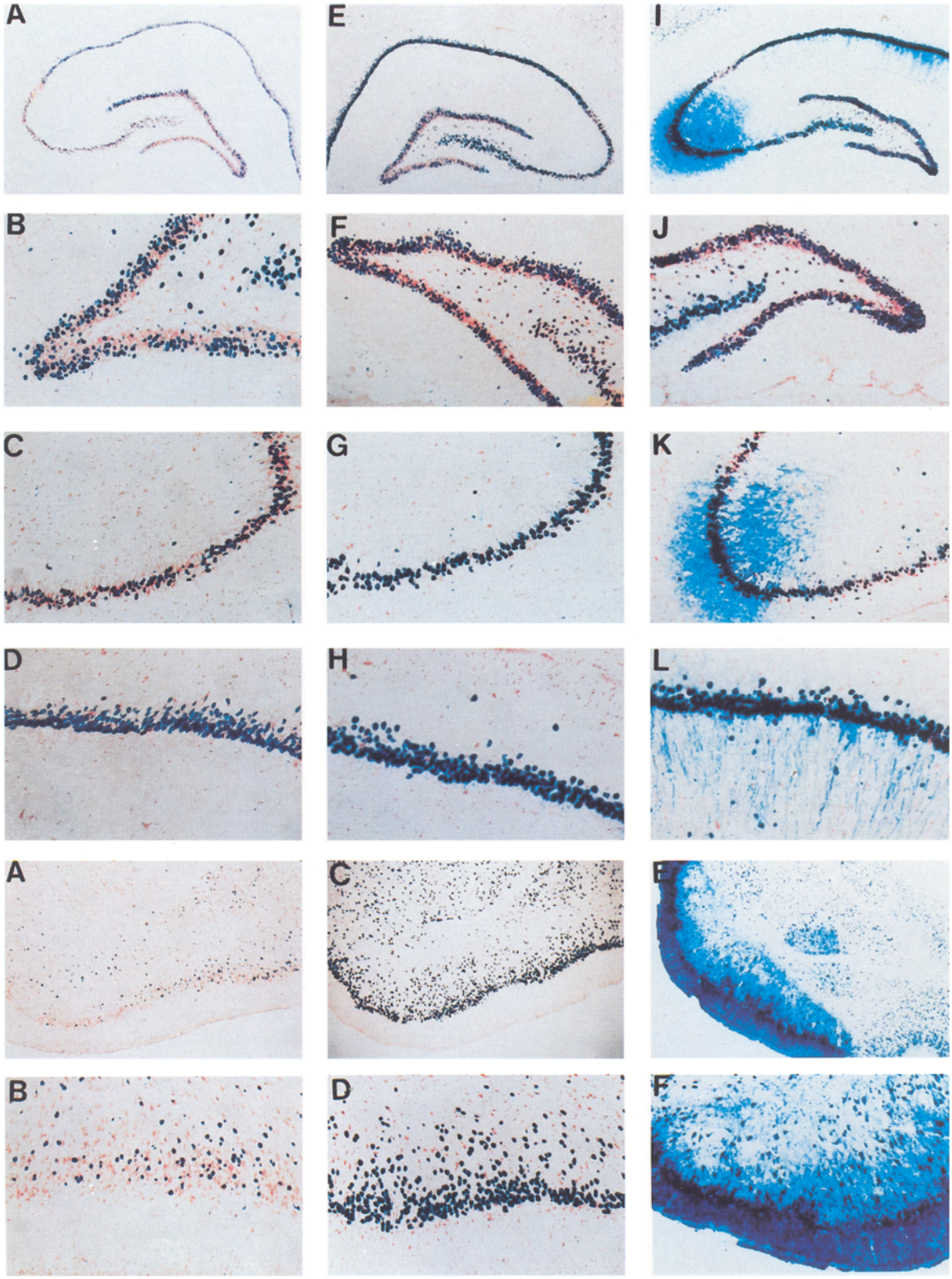

Figure 3 (*top*). Time course of Fos-lacZ expression in the hippocampus of transgenic rats following KA treatment. Seizures were induced in c-*fos*–*lac*Z transgenic rats by ip. injection of KA (10 mg/kg). Rats were sacrificed at 2 (*A–D*), 6 (*E–H*), and 24 hr (*I–L*) following treatment. A sustained increase in β-galactosidase activity was observed throughout the hippocampus. Cytoplasmic staining began to appear 6 hr following KA treatment in pyramidal neurons within CA3 (*G*) and CA1 (*H*) and was more abundant at 24 hr (*K, L*). Shown here are the hippocampus (*A, E, I*), dentate gyrus (*B, F, J*), CA3 (*C, G, K*), and CA1 (*D, H, L*). Magnification: 4X in *A, E,* and *I*; 10X in *B–D, F–H,* and *J–L*.

Figure 4 (*bottom*). Time course of Fos-lacZ expression in the pyriform cortex of transgenic rats following KA treatment. Seizures were induced in c-*fos*–*lac*Z transgenic rats by intraperitoneal injection of KA (10 mg/kg). Rats were sacrificed at 2 (*A and B*), 6 (*C and D*), and 24 hr (*E and F*) following treatment. A sustained increase in β-galactosidase activity, with leakage into the cytoplasm, was observed in the pyriform cortex. Magnification: 10X in *A, C,* and *E*; 20X in *B, D,* and *F*.

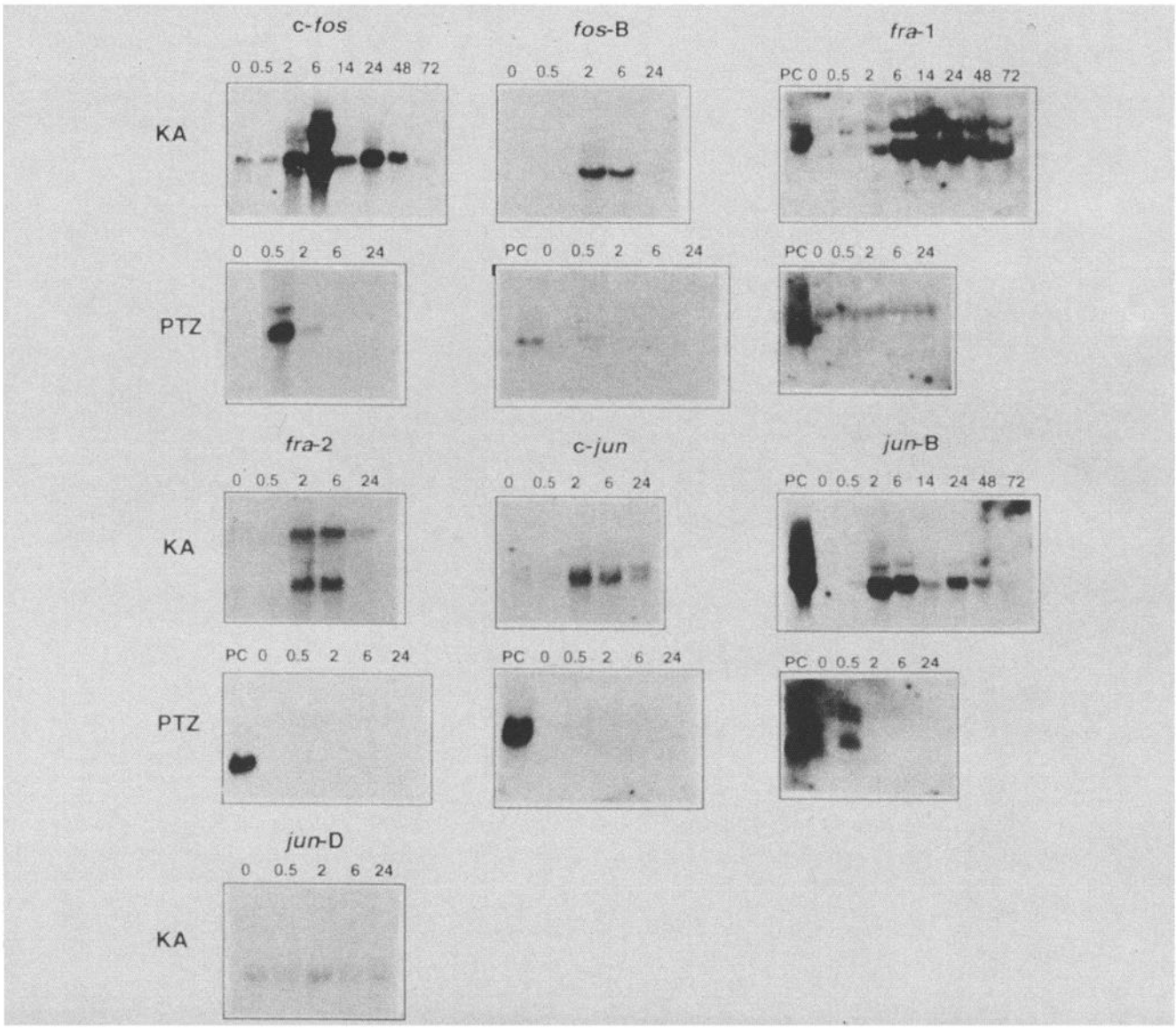

Figure 5. Northern blots comparing immediate-early gene responses to PTZ and KA in the rat hippocampus. Total RNA was isolated from dissected hippocampi of rats treated with either PTZ (55 mg/kg) or KA (10 mg/kg) for various lengths of time. (Times, in hours, following treatment are indicated.) *PC* represents a positive control RNA (liver RNA from a cycloheximide-treated mouse). Blots were probed for c-*fos*, *fos*B, *fra*-1, *fra*-2, c-*jun*, *jun*B, and *jun*D as indicated; 10 μg total RNA/lane was loaded and overall loading and transfer efficiency was monitored by ethidium bromide staining and GADPH levels.

presence of AP-1 DNA binding complexes, gel retardation analyses were performed on crude nuclear extracts from control and KA-treated rat hippocampi.

After administration of KA, there was an increase in AP-1 binding activity present in hippocampal nuclear extracts (Fig. 6). Increased binding was evident by 2 hr, peaked at 6 hr and was still above baseline 3 d later. Interestingly, two distinct AP-1 bands displayed different kinetics of activation. Binding specificity of both bands was shown by competition by the cognate AP-1 oligonucleotide but not by a mutated oligonucleotide that does not have AP-1 activity (Fig. 6). The more rapidly migrating complex persisted for at least 3 d in the hippocampus of KA-treated rats while the slower migrating species returned to baseline levels between 1 and 2 d posttreatment. The rapidly migrating band is reminiscent of the persistent AP-1 activity observed by Nestler and colleagues in rats that had received repeated administration of cocaine (Hope et al., 1992). These results confirm and extend the Northern blot data and demonstrate that among the sequelae of kainic acid treatment is a protracted increase in components of AP-1 DNA binding complexes.

DNA damage in hippocampal neurons induced by KA treatment

KA is known to cause the death of neurons in the rat hippocampus (Nadler et al., 1978; Represa et al., 1987). This is confirmed by the loss of cytotechtonic order in the hippocampi of KA-treated transgenic rats (Fig. 3). Thus, the presence of Fos-lacZ in the cytoplasm of CA1 and CA3 neurons appears to be a marker of their impending demise. However, the nature of the relationship between Fos-lacZ expression and death is still unknown. Furthermore, the differential expression of some clEGs

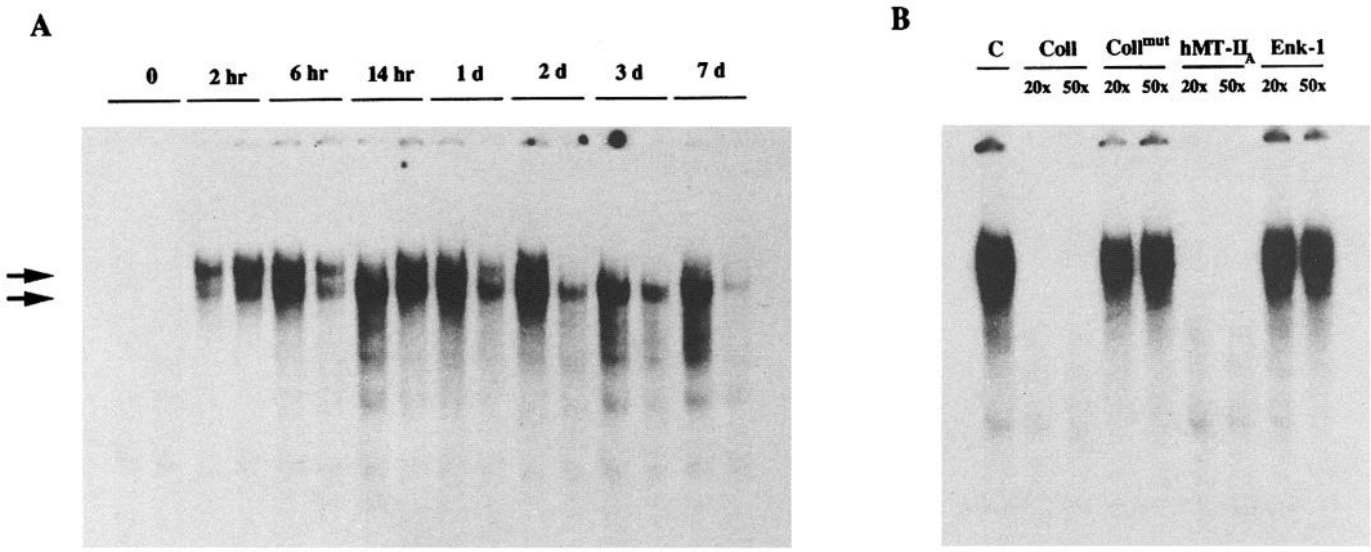

Figure 6. Increase in AP-1 binding in hippocampal nuclear extracts following kainate-induced seizures. *A*, Rats were sacrificed at the indicated times after an intraperitoneal injection of kainic acid (10 mg/kg). Each time point shows results from two different rats. The two bands representing AP-1 DNA-protein complexes are indicated by the arrows. B) The effects of adding a 20- or 50-fold molar excess of cold oligonucleotide containing either the collagenase AP-1 site (*lanes 2, 3*), a mutated collagenase AP-1 site (*lanes 4, 5*), an hMT-II_A site (*lanes 6, 7*), or an Enk-1 site (*lanes 8, 9*) on AP-1-like binding in nuclear extracts from a 6 hr poststimulated hippocampus.

in a biphasic manner raises the additional issue of whether both periods of gene activation have the same functional relationship to death. In an attempt to address these questions we have determined the spatial and temporal pattern of cell death and Fos-lacZ expression in the KA-treated rat brain. As independent indicators of cell death, nuclear morphology and DNA integrity were assessed by Hoechst 33258 staining (Oberhammer et al., 1993) and the terminal transferase biotinylated-UTP nick end labeling (TUNEL) procedure (Gavrieli et al., 1992), respectively.

TUNEL-positive neurons first became apparent at 24 hr following administration of KA (data not shown). Thereafter, the number of TUNEL-positive cells rose dramatically to peak at 48 hr posttreatment (Fig. 7) and declined back to near basal values by 72 hr after administration of KA. These affected neurons were located predominantly in the pyriform cortex and layers CA1 and CA3 of the hippocampus (Fig. 7). During the same time period, Fos-lacZ accumulated to maximal levels between 6 and 24 hr posttreatment and declined back to baseline by 72 hr although it was still readily detectable at 48 hr in brain regions that were TUNEL-positive (Figs. 3, 4, 7). Regions of the CNS that were resistant to KA toxicity, such as the dentate gyrus, remained TUNEL negative throughout the experimental time course and did not show any protracted Fos-lacZ expression (data not shown).

While we have been unable to make the TUNEL procedure compatible with β-galactosidase histochemistry, a number of points are clear. First, since essentially all neurons in CA1 and CA3 express Fos-lacZ in the acute phase, all of the TUNEL-positive cells in these regions must have experienced a cIEG response hours or days prior to DNA damage becoming detectable. Second, more neurons are Fos-lacZ positive than go on to be TUNEL-positive during the ensuing 48–72 hr. Therefore, the mere presence of Fos-lacZ in the acute phase is not an absolute indicator of subsequent cell death. However, these experiments do not establish whether the delayed period of cIEG expression, which is spatially confined to vulnerable neuronal populations and characterized by cytoplasmic Fos-lacZ, is associated with dying cells. That is, is the presence of cytoplasmic Fos-lacZ an indication of the loss of nuclear integrity and a collapse of protein trafficking a prelude to death?

To address this issue we have combined β-galactosidase histochemistry with the DNA stain, Hoechst 33258, since the morphological changes that accompany cell death, such as condensation of chromatin and nuclear fragmentation, have been visualized using this reagent (Oberhammer et al., 1993). In Hoechst stained sections of rat hippocampus, abnormal nuclei are first observed at 48 hr post KA treatment (Fig. 8*b*); a time that is coincident with the peak of TUNEL labeling (Fig. 7). These abnormal nuclei are characterized as being shrunken and occasionally fragmented (Fig. 8*b*, arrowheads). At both 1 and 2 d following KA treatment, Fos-lacZ-positive cells in CA3 appeared to have normal nuclei based upon the above criteria (Fig. 8). Thus, even though Fos-lacZ had leaked into the cytoplasm of many neurons in CA3, the nuclei of these cells were not detectably compromised. Those cells that had condensed nuclei did not express Fos-lacZ (Fig. 8*b*, arrowheads). There were some weakly Fos-lacZ-positive structures in CA1/CA3 that were not associated with any Hoechst fluorescence (Fig. 8*b*, open arrows). It is assumed that these apparently anuclear structures represent Fos-lacZ in dendrites of neurons whose soma was not in the section as well as the remnants of Fos-lacZ positive neurons.

While it is possible that the Fos-lacZ positive neurons observed at 48 hr subsequently go on to die, the lack of TUNEL-staining at later times suggests that these cells might survive. Indeed, since the neurons that show a protracted period of Fos-lacZ expression appear to have intact nuclei at 48 hr posttreatment it may be that this highly unusual component of the cIEG response is directed at counteracting excitotoxicity.

In summary, those neurons that are sensitive to KA are induced for c-*fos* expression and Fos-lacZ appears with a delay in the cytoplasm of the most vulnerable populations. The presence

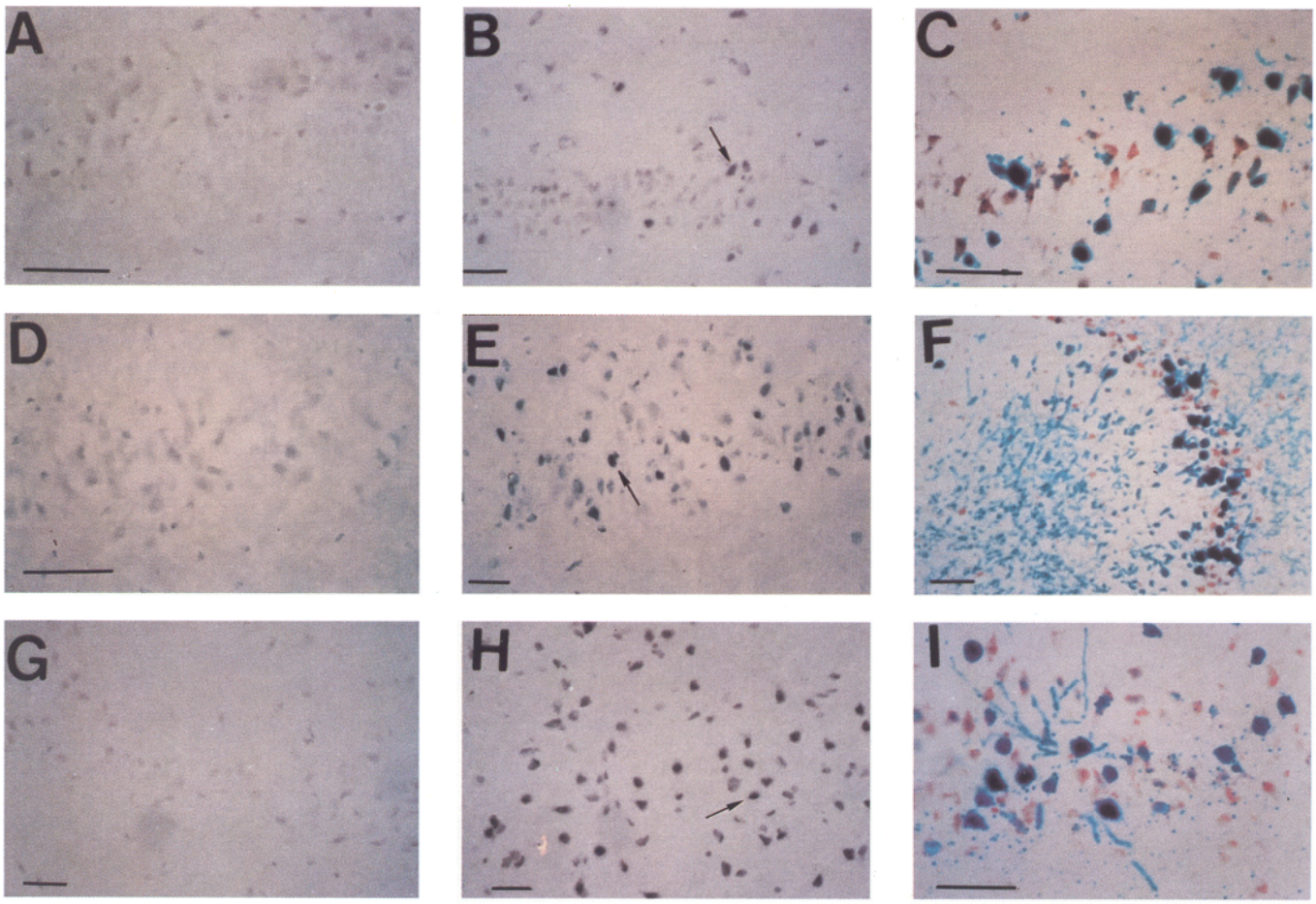

Figure 7. Kainic acid induces DNA damage in vulnerable neurons. Seizures were induced in c-*fos*–*lacZ* transgenic rats by intraperitoneal injection of KA (10 mg/kg). Rats were sacrificed at 48 hr following treatment. *A, D,* and *G* are from control, untreated rats. Sections are shown from hippocampal areas CA1 (*A–C*) and CA3 (*D–F*) and the pyriform cortex (*G–I*). In *A, B, D, E, G,* and *H* sections were stained by the TUNEL procedure in order to detect fragmented DNA. *Arrows* indicate TUNEL-positive neurons. *C, F,* and *I* show sections stained for Fos-lacZ expression. Scale bars, 105 μm.

of Fos-lacZ in the cytoplasm precedes any overt morphological sign of nuclear damage. Since all CA3 neurons expressed Fos-lacZ and many subsequently died, the presence of cytoplasmic Fos-lacZ was thought to be an early harbinger of neuronal death. However, the apparently normal nuclear morphology of Fos-lacZ-positive cells at later times suggests that it is a reaction to the processes that lead to death. Therefore, while cytoplasmic Fos-lacZ may in some instances be an effective marker for death, it is not an absolute predictor and is not required for death. Besides c-*fos*, we establish that there is a complex and unique pattern of gene expression that accompanies excitotoxicity. These gene products may contribute to the biological processes associated with neuronal damage.

Discussion

A strain of c-*fos*–*lacZ* transgenic rats has been characterized in which β-galactosidase activity can be used as a simple and unambiguous measure of c-*fos* expression. Since the fusion gene utilizes murine c-*fos* genomic sequences, it is quite remarkable that this transgene accurately recapitulates both basal and inducible patterns of cognate c-*fos* expression in the rat brain. This indicates that the murine c-*fos* gene contains all of the transcription regulatory elements that are required for correct expression in the rat brain. Furthermore, similar signaling pathways must underlie c-*fos* induction in the various situations examined in this study.

While grossly similar patterns of Fos-lacZ expression were evident in transgenic rats and mice, some differences were detected. One notable example is in the caudatoputamen following administration of indirect dopamine agonists (data not shown). In general, these discrepancies were quantitative in nature and appeared to reflect species differences in c-*fos* expression. The c-*fos*–*lacZ* transgenic rat should prove to be a useful model for several neurobiological applications since it is more amenable to behavioral and physiological analyses than the mouse and it yields sufficient material for parallel biochemical and molecular genetic studies.

The observations made in the c-*fos*–*lacZ* transgenic rats raise several questions concerning the role of the cIEG response in neuronal death. For example, the induction could simply reflect a breakdown in intracellular signaling pathways. Alternatively, one or more cIEG product could fulfill a causative role in neuronal death, perhaps by regulating the expression of "suicide" genes. On the other hand, the induction of cIEGs may represent a cellular stress response that is mounted to counteract the processes set in train by the excitotoxin. Finally, the different responses may be attributable to the distinct excitatory mechanisms of KA and PTZ, rather than neuronal death per se. At this

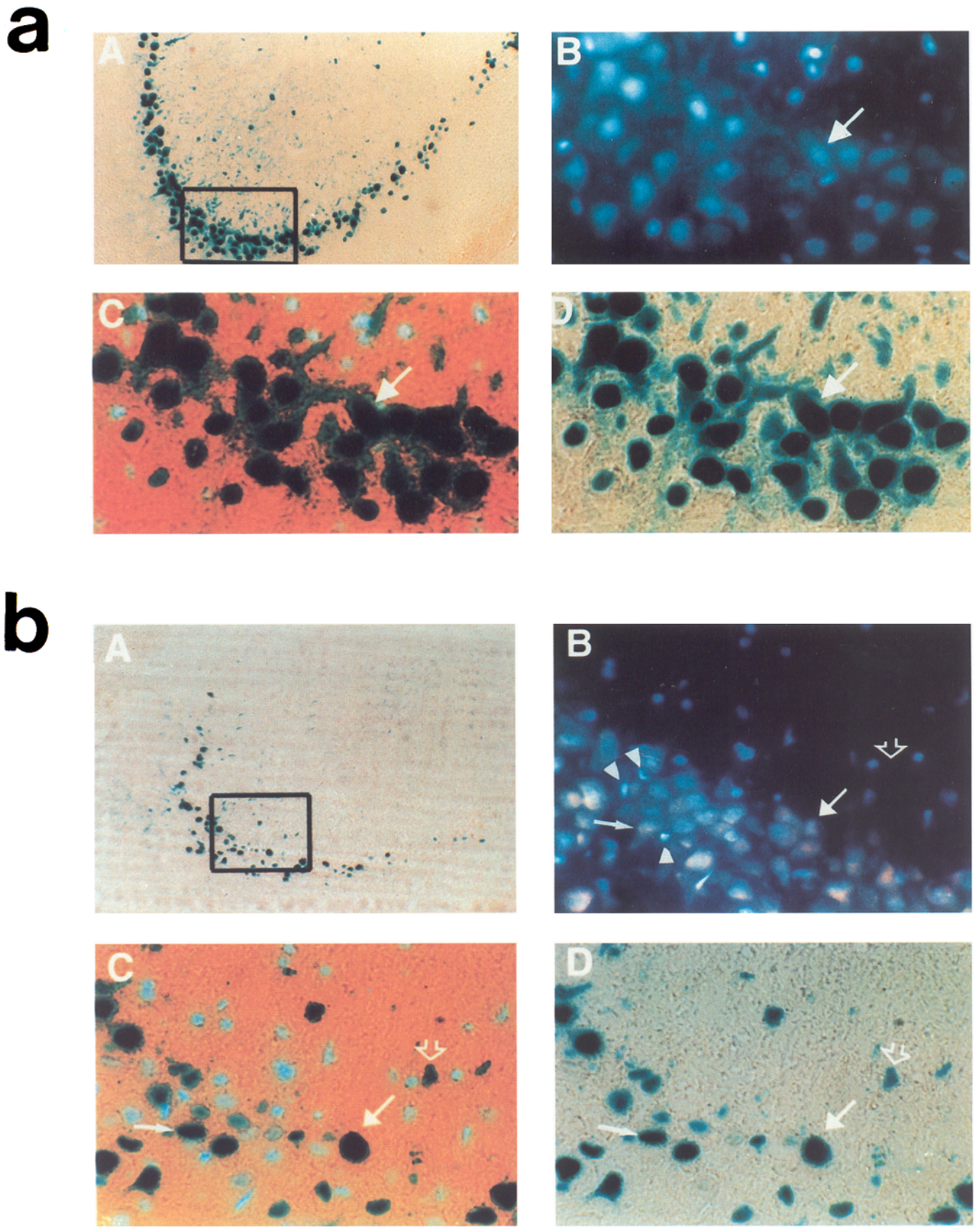

Figure 8. Morphological evaluation of Fos-lacZ positive cells using Hoechst 33258. Tissue sections from c-*fos–lacZ* transgenic rats 1 d (*a*) and 2 d (*b*) following KA treatment were first stained with Hoechst 33258 and then subsequently stained for Fos-lacZ. *A* from both sets shows the Fos-lacZ staining in area CA3 (magnification is 10×). Higher magnification images (40×) of the *boxed region* are shown in subsequent panels. *B* shows the Hoechst stain prior to β-galactosidase histochemistry; *C* and *D* show the Fos-lacZ expression in the same area. *C* was photographed under low

level of analysis it is impossible to resolve the relationship among gene expression, mechanism of seizure activity and neuronal death, since the latter two are inextricably intertwined. Indeed, it is probable that these differences are what causes KA to be neurotoxic while PTZ is not.

In addition to the data shown here, a number of studies have pointed to an association between cIEG expression and cell death. This includes expression in many sites of programmed cell death in the developing and adult organism (Gonzales-Martin et al., 1992; Smeyne et al., 1993a,b; Marti et al., 1994). Besides these natural sites of cell death, cIEG expression has been observed in cells that have been triggered to die. For example, hormone deprivation leads to a bimodal induction of cIEGs in the prostate gland that is associated with cell death (Buttyan et al., 1988). In the nervous system, c-*jun* is persistently induced by treatments that result in neuronal damage such as hypoxia and status epilepticus (Dragunow et al., 1993) while focal cerebral ischemia induces c-*fos*, c-*jun*, and *jun*B (An et al., 1992). In addition, DNA damaging agents such as UV irradiation and topoisomerase inhibitors lead to an induction of c-*fos* and c-*jun* (Devary et al., 1991; Smeyne et al., 1993a). This widespread association of cIEG expression with terminal differentiation and death would argue that the process is common to many cell types. Furthermore, since cIEG expression is detected hours or even days before overt signs of cell death appear (e.g., DNA damage) and is retained in neurons that appear to survive KA treatment (e.g., normal nuclei), it is highly unlikely that the response is caused by a catastrophic failure of intracellular signaling or homeostasis.

While the induction of the cIEG response prior to death is unlikely to be an irrelevant consequence of the collapse of signaling pathways, the association alone does not establish causation. The evidence supporting a direct involvement of cIEG products in the death process comes from experiments in which their activity was blocked. Antisense oligonucleotides to c-*fos* and c-*jun* inhibited apoptosis in lymphoid cells induced by growth factor deprivation (Colotta et al., 1992). Also in lymphoid cells, antisense oligonucleotides or transdominant suppressers to NGFI-B inhibited apoptosis (Liu et al., 1994; Woronicz et al., 1994). These studies indicated that the combined function of Fos and Jun or NGFI-B were required for apoptosis. However, some of these results do not appear compatible with the finding that there is no overt disruption of programmed cell death in *fos*-null mice (Johnson et al., 1992, Wang et al., 1992). Furthermore, the cIEG response is triggered in many situations having nothing to do with cell death. These points, as well as the observation of persistent expression of Fos-lacZ in neurons that appear to survive KA treatment, led us to consider the possibility that cIEG components are involved in a cellular stress response.

One common feature that could link the many situations in which the cIEG response is triggered is metabolic stress. For example, 2-deoxyglucose uptake, an indicator of metabolic demand, and Fos-like immunoreactivity often identify the same regions of the nervous system (Sagar et al., 1988; White and Price, 1993). This relationship would account for the induction of a cIEG response by seizures and the many agents that activate particular neuronal populations. Similar arguments can be forwarded for the many instances in which cIEGs are activated in parallel with the recruitment of quiescent cells back into the cell cycle (Greenberg and Ziff, 1984; Muller et al., 1984; Chavrier et al., 1988). However, there are other consequences of elevated metabolic demand, or compromised metabolism, such as oxidative stress, that can result in pathological conditions.

Oxidative stress is characterized by the formation of reactive oxygen intermediates that can result in damage to DNA, lipids, and proteins (reviewed in Olanow, 1993). While classically this was observed following ionizing radiation, oxidative stress is also known to occur during neurodegeneration, including that triggered by glutamate receptor agonists (Raley-Susman et al., 1992; Bondy and Lee, 1993; reviewed in Olanow, 1993). Indeed, the demonstration of DNA cleavage in hippocampal neurons following KA treatment could be one indication that these cells have suffered free radical damage.

Recently, it was shown that the binding of Fos-Jun heterodimers to DNA is sensitive to reduction-oxidation (redox; Abate et al., 1990a,b). A single cysteine residue that is conserved in the DNA binding domain of many basic-zipper superfamily members must be in a reduced, sulfhydryl, form for dimer binding to take place (Abate et al., 1990a). The predominant molecule carrying out the reduction of this cysteine is a bifunctional enzyme, Ref-1, that is also a DNA repair enzyme possessing apurinic/apyrimidinic (AP) endonuclease activity (Xanthoudakis and Curran, 1992; Xanthoudakis et al., 1992). In fact, Ref-1 can regulate the DNA binding activity of several transcription factors, including Fos-Jun, NFκB, Myb, and members of the ATF/CREB family (Xanthoudakis et al., 1992). Thus, the same enzyme that is responsible for repairing DNA damaged by free radicals is also capable of regulating the ability of some cIEG products to bind to DNA. It is possible that Ref-1 plays a role in oxidative stress by coordinating both transcriptional responses to oxidative insult and the repair of damaged DNA. Both of these processes may occur during KA excitotoxicity (Raley-Susman et al., 1992; Bondy and Lee, 1993).

Several genes have been discovered that could conceivably be targets of cIEG function following oxidative stress. For example, superoxide dismutase is involved in the defense against oxygen toxicity, and mutations in one form of this gene have been linked to familial amyotrophic lateral sclerosis (ALS; Olanow, 1993). Other potential target genes include those encoding enzymes involved in glutathione metabolism, some of which contain AP-1 binding sites (Morrow et al., 1990; Diccianni et al., 1992).

There are some features of the cIEG response that seem unique to situations involving excitotoxicity. First, the cIEG response has two phases, an acute, transient, induction that is common to all seizures and a second, protracted, period of expression that seems unique to vulnerable neurons. Second, the spectrum of genes expressed under conditions that result in seizures and neuronal death are distinct. For example, the induction of *fra*-1 and *fra*-2 was only observed in kainic acid treated animals. The functional properties of Fra-1 and Fra-2 have been less ex-

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light to allow covisualization of Hoechst stain and β-galactosidase. Since the Fos-lacZ staining quenches the Hoechst fluorescence, one must look at *B* to see the Hoechst staining in Fos-lacZ positive cells. *Solid arrows* point to examples of a Fos-lacZ-positive cells with normal nuclei. *Arrowheads* point to condensed nuclei which appear at 2 d following KA treatment. *Open arrows* indicate faint Fos-lacZ positive structures that were not associated with any Hoechst fluorescence.

tensively studied; however, they do form AP-1 complexes with Jun and Jun family members as well as with other basic-zipper superfamily transcription factors (Hai and Curran, 1991). Therefore, it is possible that there may be a unique complement of AP-1 complexes associated with processes leading to, or counteracting, neuronal death.

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