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Localization of the protein product of the immediate early growth response gene, *Egr-1,* in the kidney after ischemia and reperfusion

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***Egr-1* is an "immediate early" gene that is induced by growth factors and agents that induce differentiation and encodes a protein with a "zinc-finger" motif. This protein is believed to be involved in transcriptional regulation. Because the fate of the kidney, and hence the organism, after an ischemic insult is dependent upon cellular repair, differentiation, and proliferation, we examined whether there was expression of the *Egr-1* protein after an ischemic insult to the rat kidney. We have previously reported that *Egr-1* mRNA accumulates to high levels in mouse kidneys after 30 min of ischemia and 1 h of reperfusion. In the present study, performed in rats, we show that *Egr-1* mRNA transiently accumulates to very high levels after 40 min of ischemia and 1 h of reperfusion, is decreased by 3 h, and is nondetectable by 24 h of reperfusion. Reperfusion is required for *Egr-1* protein accumulation to occur. The *Egr-1* protein was localized by immunohistochemical techniques primarily to the nuclei of the thick ascending limbs and principal cells of the collecting ducts in the cortex and medulla. The subcellular localization was exclusively nuclear. There was some staining of the glomerular tuft and staining was particularly prominent in the parietal epithelial cells. In parallel to the accumulation of *Egr-1* mRNA, the expression of the protein was transient and was no longer apparent after 5 h of reperfusion. The *Egr-1* protein may play an important role in regulation of the response to ischemia of those segments of the nephron that are highly susceptible to oxygen deprivation and have a high level of intrinsic plasticity. It is possible that this protein may modulate cellular processes important for the ultimate ability of these critical nephron segments to recover from an ischemic insult.**

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Introduction

Renal ischemia is characterized by alterations in epithelial cell polarity (Molitoris *et al.,* 1988), tissue damage, and associated tubular cell death, which leads to abnormal epithelial function and organ failure (Bonventre *et al.,* 1988). When the insult is reversible, regeneration of epithelial cells and recovery of epithelial and organ function follows. The ability to restore differentiated function and regenerate epithelial structure and function distinguishes the kidney from the heart and brain, two organs in which ischemic cell death leads to permanent tissue and organ dysfunction because of an inability to regenerate cardiac myocytes or neurons. The cellular mechanisms responsible for this recovery process in the kidney are poorly understood. An understanding of the molecular mechanisms used by epithelial cells in response to the stress of prolonged oxygen deprivation followed by restoration of oxygen delivery is critical to the development of strategies directed toward enhancing the recovery process.

We have previously reported that the mRNAs of two "immediate-early" response genes, *Egr-1* and c-*fos,* accumulate to high levels in mouse kidney after 30 min of ischemia and 1 h of reperfusion (Ouellette *et al.,* 1990). In this report, however, the kidney cell population expressing the gene was not identified. The *Egr-1* gene (Sukhatme *et al.,* 1987; Sukhatme *et al.,* 1988), also termed NGFI-A (Milbrandt, 1987), Krox 24

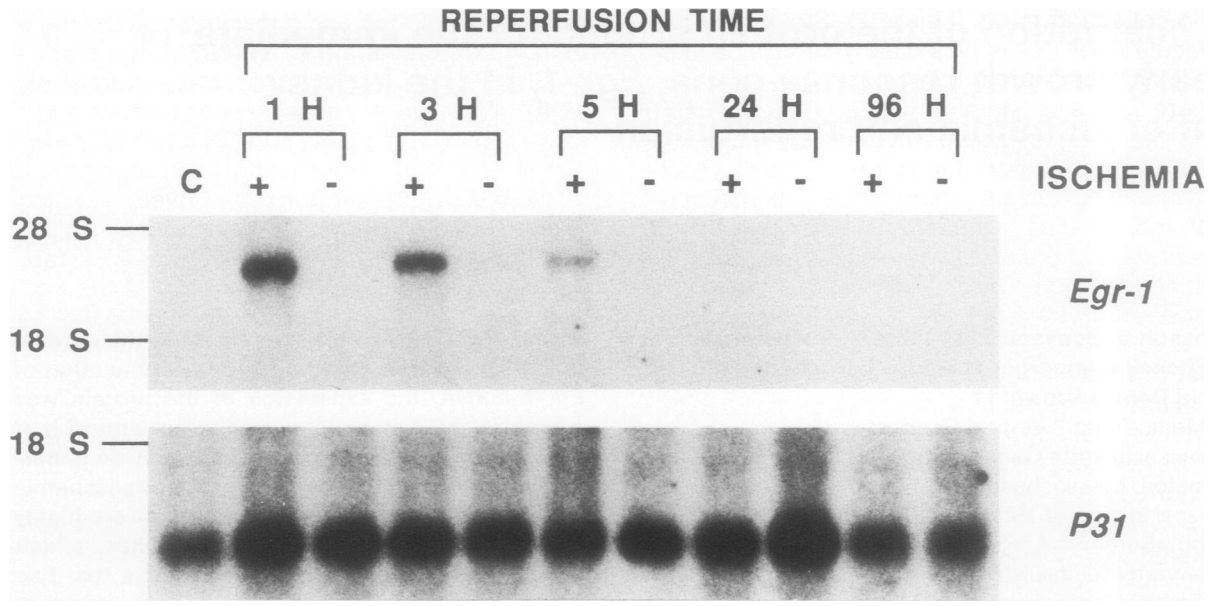

Figure 1. **Northern blot analysis of *Egr-1* mRNA accumulation in the rat kidney after 40 min of ischemia and varying periods of reperfusion.** Both postischemic (+) and contralateral kidneys (−) were removed. Lanes contain RNA from individual kidneys. Blots were hybridized with the *Egr-1* cDNA probe. To establish that total RNA in each lane was comparable, the blot was also hybridized with the P31 cDNA which encodes a housekeeping protein. Bars denote rRNA markers of 4618 (28 S) and 1864 (18 S) nucleotides.

(Lemaire *et al.,* 1988), Zif/268 (Christy *et al.,* 1988), and TIS 8 (Lim *et al.,* 1987) codes for a protein with a predicted "zinc-finger" motif. It is induced in many cell types in the absence of de novo protein synthesis by growth-stimulating agents (Christy *et al.,* 1988; Sukhatme *et al.,* 1988) and under many conditions of cellular differentiation (Milbrandt, 1987; Sukhatme *et al.,* 1988). The conditions under which the gene is induced, together with the zinc-finger motif, suggests that the *Egr-1* protein has an important regulatory role in transcription. The fact that the *Egr-1* protein is a substrate for phosphorylation (Cao *et al.,* 1990), has DNA binding activity (Christy and Nathans, 1989), and functions as a transcription factor (Lemaire *et al.,* 1988; Patwardhan *et al.,* 1990; manuscript submitted) is strong evidence for a role as a regulatory protein.

As an immediate-early gene, *Egr-1* is a member of a group of genes that are believed to serve as nuclear messengers, controlling the activation of genes that are expressed later, thus acting to couple external stimuli to long-term changes in the cell (Sheng and Greenberg, 1990). In the nervous system these genes may serve as critical intermediates, activated during the first hour of training or stimulation, and responsible for long term morphological and biochemical changes (Barzilai *et al.,* 1989; Goelet *et al.,* 1986). *Egr-1* is preferentially activated under conditions that induce long-term potentiation of the hippocampal perforant path-granule cell synapse (Cole *et al.,* 1989). By analogy, *Egr-1* may serve as an important link between the early events occurring postischemia and the later events of cell differentiation and cell division. In the current study immunocytochemical techniques were used to localize the *Egr-1* protein in post-ischemic rat kidney.

Results

mRNA levels in post-ischemic kidney

Egr-1 mRNA levels in kidneys taken after 40 min of ischemia and varying periods of reperfusion are shown in Figure 1. mRNA levels are highest at 1 h of reperfusion, clearly increased at 3 h of reperfusion, barely detectable at 5 h, and nondetectable after 24 and 96 h of reperfusion. Kidney *Egr-1* mRNA levels were nondetectable in control sham operated kidneys and contralateral kidneys at each of the time points tested.

Immunocytochemical localization of the Egr-1 gene product

In the cortex, after 40 min of ischemia and 1 h of reperfusion, the *Egr-1* gene product was localized primarily to the thick ascending limbs

and collecting ducts (Figure 2). Staining of the macula densa cells was clearly seen. The subcellular localization was exclusively nuclear. There was also staining of the glomerular tuft and the staining was particularly prominent in the glomerular parietal epithelial cells. There was no detectable staining of the proximal convoluted tubules. The staining intensity was greatest and the most widespread 1 h after the initiation of reperfusion. Staining was no longer apparent at 5 h and 24 h of reperfusion (Figure 2, B and C). This temporal pattern paralleled that of total kidney *Egr-1* mRNA accumulation.

In both the outer and inner stripes of the outer medulla, staining was also localized to the nuclei of the thick ascending limbs and collecting ducts (Figure 3, A and B). There was no staining of the S_3 segments of the proximal tubule. Although all of the cell nuclei of the thick ascending limbs were stained, staining in the cortical and medullary collecting ducts was heterogeneous within a given collecting duct (Figure 4). When a monoclonal antibody raised against the 31-kDa subunit of a proton pumping ATPase (Brown *et al.,* 1987) was used to identify intercalated cells in a double-staining procedure, it was clear that presence of the *Egr-1* protein product was more pronounced in the principal cells when compared with intercalated cells (data not shown).

In the inner stripe the staining of thick ascending limbs and collecting ducts was pronounced and relatively uniform from tubule to tubule. Staining in the inner medulla was present in the collecting ducts as well as in some thin limbs of Henle.

To evaluate whether reperfusion was necessary for the appearance of the *Egr-1* protein, kidneys were perfusion fixed immediately after releasing the vascular clamp, subsequent to 40 min of ischemia. There was no significant amount of staining in the outer medulla (Figure 5) or in any other region of the kidney.

When the contralateral kidney was examined after 40 min of ischemia and 1 h of reperfusion, there was a small amount of staining in the thick ascending limbs and collecting ducts (Figure 6A). Kidneys from normal, control rats showed no nuclear staining with the anti-Egr-1 antibody (Figure 6B). No staining was seen when preimmune serum was substituted for the antibody to the *Egr-1* gene product in the first incubation step in either control or postischemic kidneys.

Discussion

The *Egr-1* gene has been generally found to be coregulated with c-*fos* in many conditions of growth stimulation and differentiation (Herschman, 1989; Sukhatme *et al.,* 1988). It encodes an 80-kDa nuclear phosphoprotein (Cao *et al.,* 1990). We have previously reported that there is an ~30-fold increase in mRNA levels of *Egr-1* after 30 min of ischemia and 1 h of reperfusion in mouse kidney. c-*fos* mRNA levels are also markedly enhanced in this model system. In our previous studies, however, it was not established whether the protein product of the *Egr-1* gene was expressed in the kidney. Because of the important heterogeneity of structure and function, as well as differential susceptibility to ischemic injury, it is important to localize the protein to the individual segments of the kidney. In addition, because of the putative role of this protein as a transcription factor, it was important to identify the subcellular localization of the protein.

The present results indicate that *Egr-1* mRNA accumulation reaches a peak after 1 h after reperfusion of the ischemic rat kidney. This observation is similar to our findings in the mouse kidney in which the ischemic period was 30 min rather than the 40 min of ischemia to which the rat kidney was exposed. The present study also clarifies the kinetics of mRNA accumulation, demonstrating the rapid return of mRNA levels to normal kidney nondetectable levels. Furthermore, our results indicate that the protein coded by the *Egr-1* gene is present primarily in the nuclei of the thick ascending limbs and collecting ducts. It is also present in the parietal epithelial cells of the glomerulus and in some of the cells of the glomerular tuft after ischemia and reperfusion. It is not, however, detectable in proximal tubules after ischemic injury. The glomerular tuft staining may be due to mesangial cell staining since these cells in culture express the gene in response to calcium, phorbol esters, and mitogenic agents (Bonventre *et al.,* 1989; Sellmayer *et al.,* 1991).

The high level of induction of the *Egr-1* gene product, at 1 h of reperfusion, is similar to the time response of induction of the protein following serum stimulation of quiescent mouse fibroblasts, where the protein reaches maximal levels at 1 h (Cao *et al.,* 1990). Since the *Egr-1* gene does not require protein synthesis for enhanced expression, mRNA levels may increase earlier in the postischemic period, when protein synthesis may be impaired. This potentially provides a set of early responses that may be important to limit damage and/or initiate repair processes.

Reperfusion, after an ischemic period, is necessary for expression of the protein product.

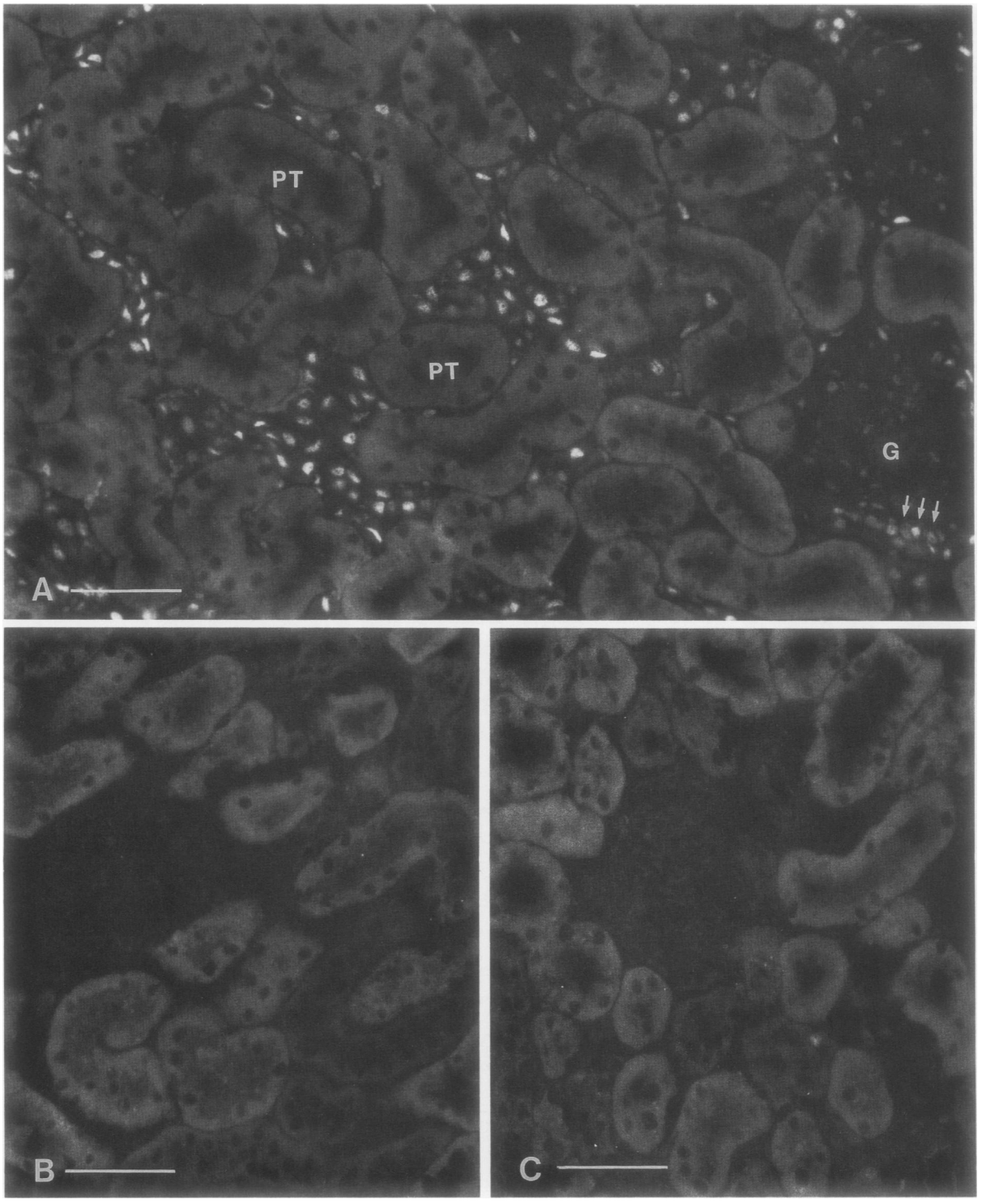

Figure 2. **Cryostat sections of cortex from ischemic kidneys fixed after different times of reperfusion and incubated with anti-*Egr 1* antibodies followed by goat antirabbit FITC.** **(A)** Staining pattern after 1 h of reperfusion. Nuclei are brightly fluorescent in thick ascending limbs and collecting ducts. In addition, nuclei of parietal glomerular epithelial cells of Bowman's capsule, and some nuclei in the glomerular (G) tuft (probably mesangial cells) are stained. Cells of the part of the thick ascending limb forming the macula densa are also positive (arrows). In contrast, proximal tubule (PT) cell nuclei are unstained. **(B)** Staining pattern after 5 h of reperfusion. No positive staining is detectable in any cell type. Compare this negative staining with the dramatic reduction of *Egr-1* mRNA that is shown at this time point in Figure 1. **(C)** Staining pattern after 24 h of reperfusion. All nuclei are unstained, corresponding to the absence of message shown by Northern analysis at this time point (see Figure 1). Bar = 50 μm.

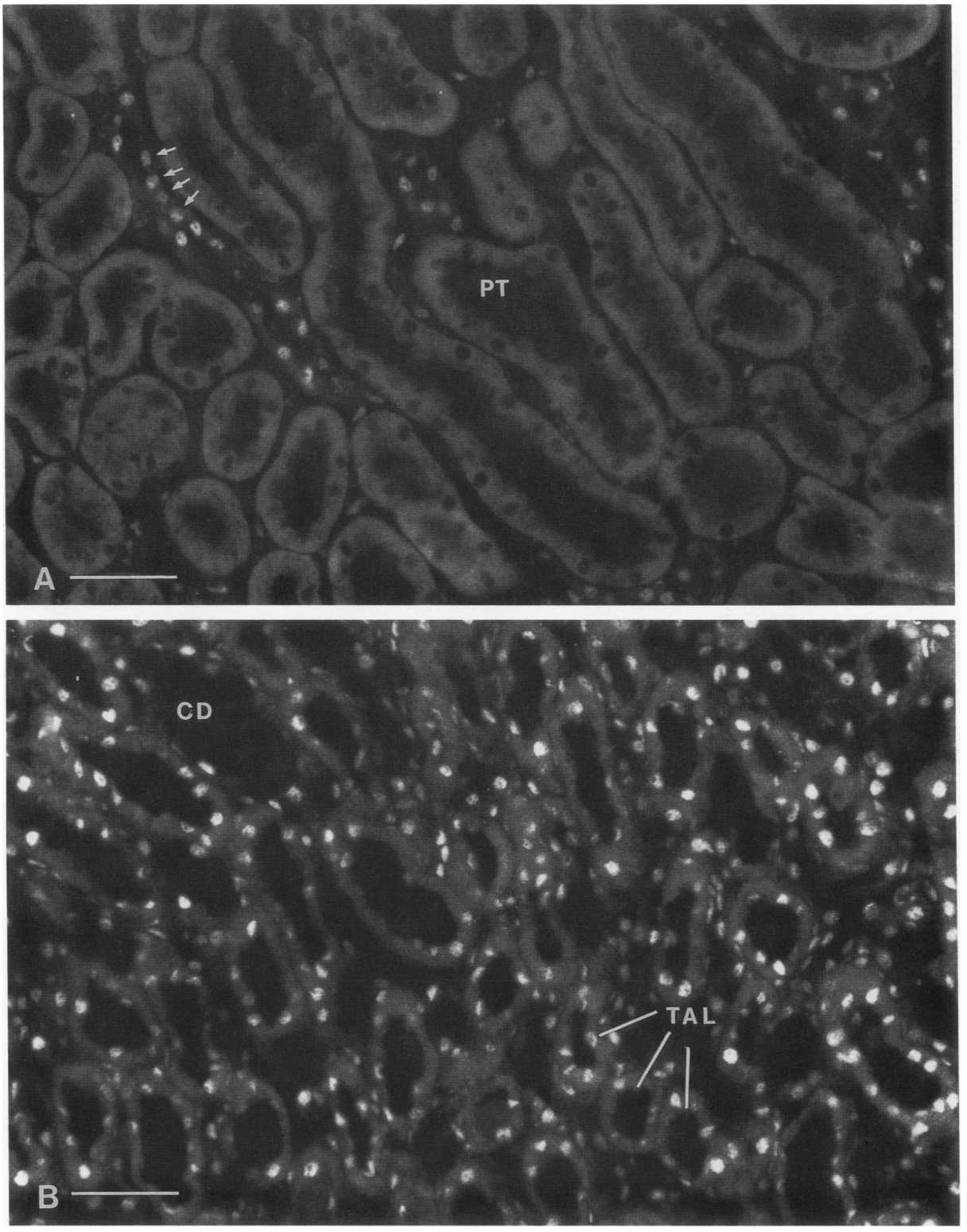

Figure 3. **Cryostat sections of outer medulla of ischemic rat kidney after 1 h of reperfusion.** **(A)** Outer stripe of outer medulla showing positive staining of nuclei in thick ascending limbs (arrows) and collecting ducts. The nuclei of straight proximal tubules (PT) in the outer stripe are not stained. **(B)** Inner stripe of outer medulla showing heavy nuclear staining of thick ascending limbs (TAL) and collecting ducts (CD). In addition, the nuclei of some thin limbs and capillaries appear to be fluorescent. Bar = 50 μm.

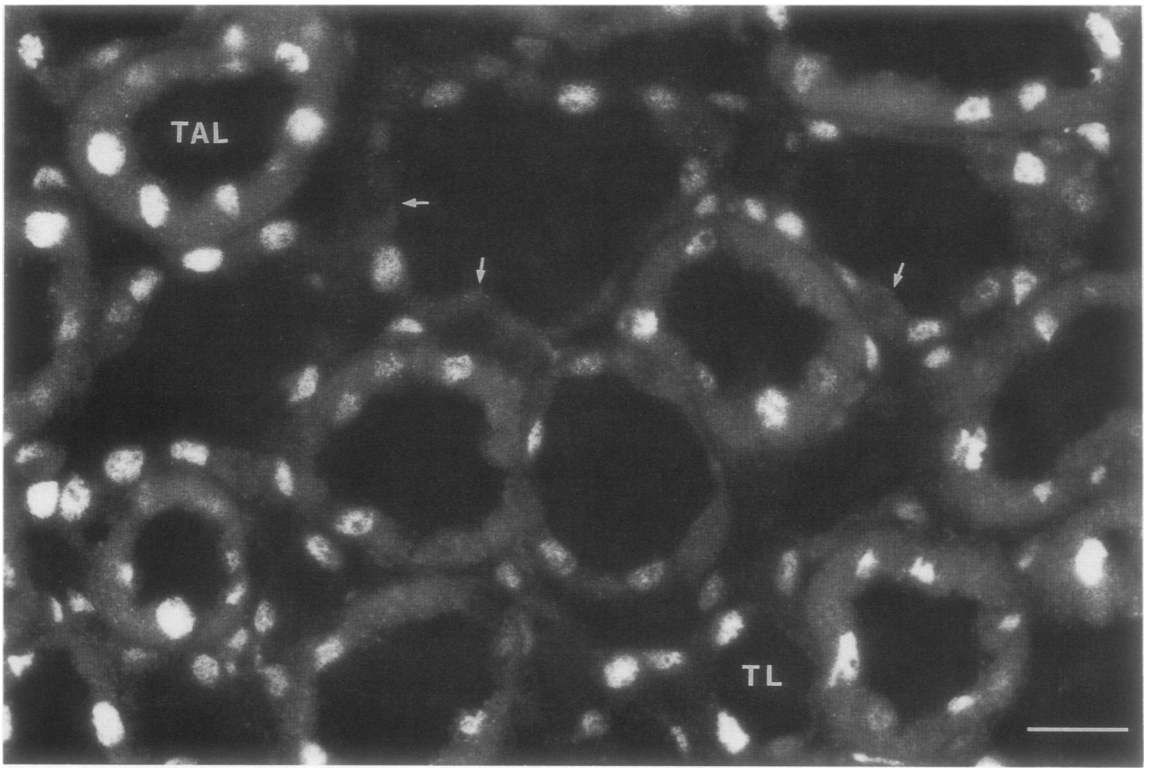

Figure 4. **Higher magnification showing the Egr-1 staining pattern in the inner stripe of the outer medulla after 1 h of reperfusion.** Whereas all nuclei in the thick ascending limbs (TAL) appear to be stained, the nuclei of intercalated cells in the collecting ducts are unstained (arrows). We have identified these cells as intercalated cells by double immunostaining with antiproton pumping ATPase antibodies. Staining in some thin limb (TL) segments is apparent in this micrograph. Bar = 20 μm.

This finding is consistent with our prior observation in the mouse that reperfusion is necessary for accumulation of *Egr-1* mRNA (Ouellette *et al.,* 1990).

It is interesting that the thick ascending limbs are sites of intense *Egr-1* gene product expression. This segment of the nephron has been proposed to be the most sensitive to ischemic injury under conditions where glomerular filtration rate and delivery of solute to the segment are maintained in the isolated perfused rat kidney (Brezis *et al.,* 1984). Focal necrotic lesions are sometimes seen in the thick ascending limb in the human kidney in acute renal failure (Oliver *et al.,* 1951) and marked cellular and mitochondrial swelling, as well as nuclear heterochromatin clumping, has been observed in response to a hypotensive insult in the rat thick ascending limb (Kreisberg *et al.,* 1976). It has been proposed that the corticomedullary countercurrent exchange of O_2 results in marginal O_2 supply to the thick ascending limbs, where the cells are mitochondria-rich and transport large amounts of solute by active processes. The arguments, however, may not be directly relevant to the experimental condition of complete occlusion of the renal artery. With the possible exception of cells close to the renal capsule, most cells in the kidney would be expected to be equally hypoxic when renal blood flow was completely interrupted. It is possible that, upon reflow, medullary congestion might interfere with oxygen delivery to the thick ascending limbs. This does not, however, explain the selective appearance of the *Egr-1* protein in the cortical thick ascending limb, including the macula densa. This pattern of localization suggests that the severity of anoxia is not the only factor in expression of the protein. Furthermore, in the clamp model of ischemic injury in the rat, the S_3 segment is at least as susceptible to ischemia/reperfusion damage in the outer medulla as is the thick ascending limb and more susceptible than the cortical thick ascending limb (Venkatachalam *et al.,* 1978). In addition, the early part of the S_1 tubule segment is also very sensitive to oxygen deprivation (Shanley *et al.,* 1986), yet does not express the *Egr-1* protein.

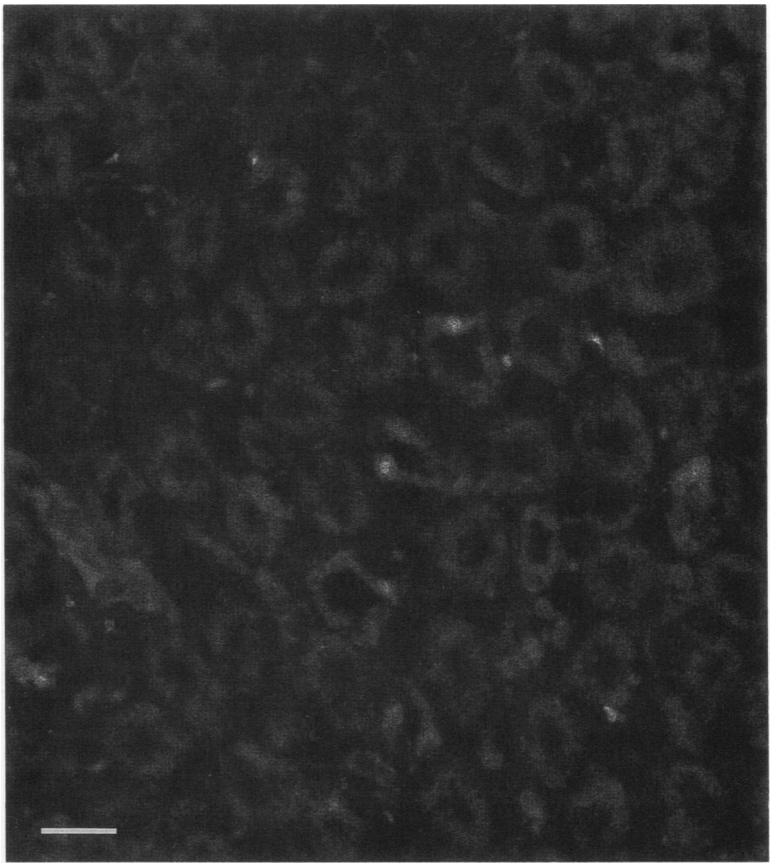

Figure 5. **Cryostat section of inner stripe of the outer medulla from kidney that was ischemic for 40 min and perfusion-fixed with PLP immediately upon release of the vascular clamp.** Very few nuclei show positive staining indicating that reperfusion is required to attain the high levels of protein accumulation shown in Figures 3B and 4. Bar = 50 μm.

Thus *Egr-1* protein expression does not directly correlate with the degree of injury in this ischemic model.

This preferential response of the thick ascending limb to express the *Egr-1* protein may reflect the plasticity of this segment of the nephron, which responds to a number of physiological and pathophysiological stimuli with cellular hypertrophy, atrophy, or enzymatic induction. For example, the decrease in overall nephron size observed with hypothyroidism is particularly pronounced in the thick ascending limb (Bentley *et al.,* 1985). Hypertrophy of the kidney induced by a high-protein diet is associated with preferential hypertrophy of the thick ascending limb (Bouby *et al.,* 1987), and this is associated with increased Na^+K^+ ATPase activity in this segment (Bouby and Bankir, 1988). Furthermore vasopressin induces selective hypertrophy of thick ascending limb cells in the Brattleboro rat (Bouby *et al.,* 1985).

The *Egr-1* mRNA accumulation and the expression of the *Egr-1* protein product soon after the induction of renal ischemia and reflow may reflect a cellular response to the dedifferentiating influences of ischemic injury. With ischemia there is a partial loss of renal cell polarity with Na^+K^+ ATPase, which is normally localized to the basolateral membrane, found in the brush border, and some brush border membrane proteins, such as leucine aminopeptidase, found in the basolateral membrane (Molitoris *et al.,* 1988). This loss of polarity represents a significant stress to the cell. If the cell is damaged significantly, it may not survive, and proliferation of adjacent cells may be necessary to restore epithelial integrity and function. If the damage is not severe enough to kill the cell, however, the cell may respond to the insult with a "differentiation" response, because establishment of epithelial polarity is one of the earliest events in epithelial cell differentiation. Indeed, the *Egr-1* gene is induced under conditions when differentiation is induced in heart and nerve cell cultures (Sukhatme *et al.,* 1988). *Egr-1* is also induced by epidermal growth factor in cell culture (Sellmayer *et al.,* 1991), and Humes has shown that epidermal growth factor enhances renal tubule cell regeneration and repair (Humes *et al.,* 1989). Perhaps the fact that the thick as-

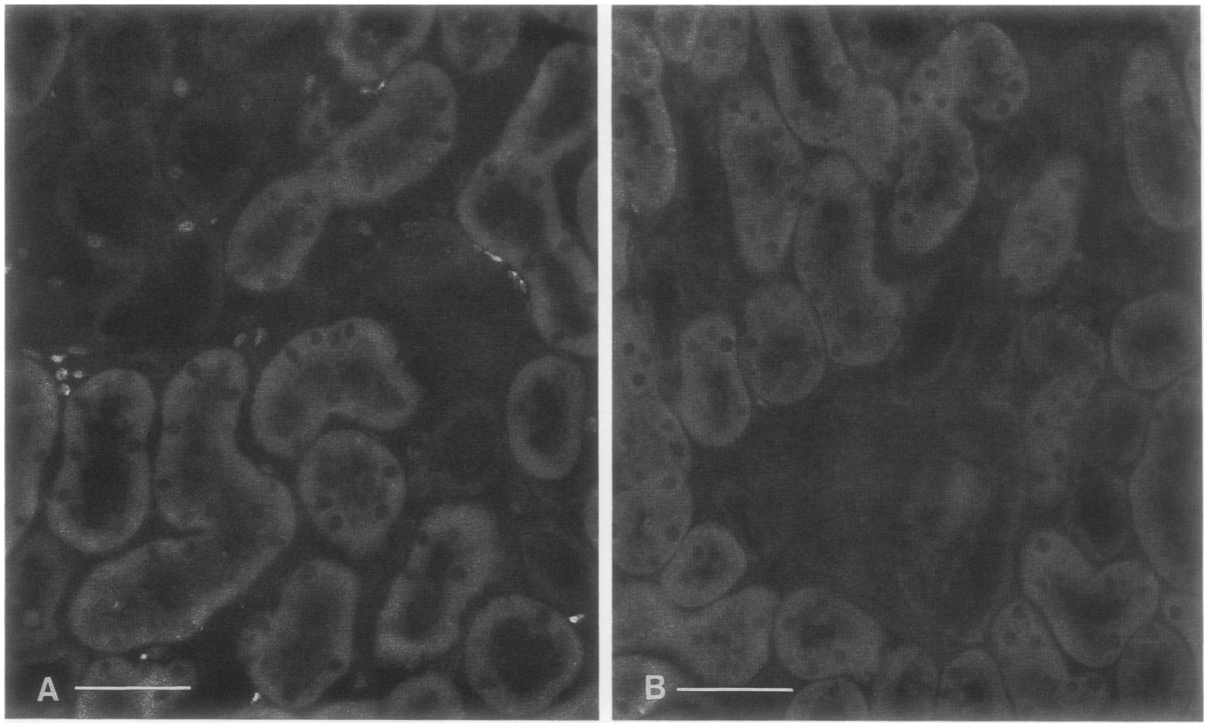

Figure 6. **Cryostat section of cortex from (A) contralateral nonischemic and (B) normal kidney.** (**A**) Cryostat section of contralateral, nonischemic kidney from rat after 1 h reperfusion. There is some staining of nuclei in thick ascending limb and collecting duct segments, but this staining is much less than in the ischemic kidney (see Figure 2A). (**B**) Cryostat section from normal kidney of a nonoperated rat showing absence of staining with anti-*Egr-1* antibodies. Bar = 50 μm.

cending limb can accumulate the *Egr-1* protein represents an adaptive response with protective consequences for cell and nephron segment integrity.

The presence of the *Egr-1* protein in collecting duct cells may also be related to marginal outer medullary reoxygenation. Collecting duct function has not been well studied in ischemia, but Wilson and Honrath (1988) have reported that impaired collecting duct function is an important factor in the elevation of urinary sodium concentration and decrease in urinary osmotic pressure characteristic of postischemic acute renal failure.

In conclusion, renal ischemia and reperfusion result in a transient marked accumulation of mRNA of the immediate-early gene, *Egr-1.* The protein product of this gene has been localized to the thick ascending limb and principal cells of the collecting ducts. The preferential response of these distal nephron segments may reflect enhanced plasticity of this part of the nephron. *Egr-1* protein accumulation represents an immediate response to the stress of ischemia which may be important to cell survival, epithelial repair, and the preservation of organ function.

Methods

Animals

Sprague-Dawley rats (225–335 g), purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA), were maintained under alternating 12-h cycles of light and dark. Animals were anesthetized with an intraperitoneal injection of pentobarbitol (6.5 mg/100 g body wt). Twenty units of heparin (heparin sodium injection, U.S. Pharmacopea) were injected intraperitoneally. A catheter was placed into the right jugular vein and 0.9% NaCl was infused at a rate of 4.1 ml/h or 8–10 ml of 0.9% NaCl was injected intraperitoneally without intravenous hydration with no difference in results. A left flank incision was made and the renal pedicle clamped with an aneurysm clamp (Roboz Surgical Instrument Co., Washington, DC). The incision was closed temporarily until 40 min later when the clamp was removed. The incision was then sutured closed and after the stated time of reperfusion, both kidneys were removed for extraction of RNA or were fixed in situ for immunocytochemistry (see below).

Northern blot analysis

Samples of RNA (10 μg) in 50% formamide and 6% formaldehyde (Rave *et al.*, 1979) were heated at 65°C for 10 min and electrophoresed at 30 V for 18–24 h in 2% agarose gels in buffer containing 40 mM 3-(*N*-morpholino) propanesulfonic acid, pH 7.0; 10 mM sodium acetate; 1 mM EDTA; and 6% formaldehyde. RNA was transferred by capillarity to GeneScreen (DuPont, Wilmington, DE) Plus membranes using 10× standard saline citrate (1× SSC = 150 mM NaCl,

10 mM sodium citrate) as the transfer buffer. Membranes were prehybridized at 42°C in a solution of 50% formamide, 1% sodium dodecyl sulfate (SDS), 1 M NaCl, 10% dextran sulfate, and denatured herring sperm DNA (100 μg/ml). Probes were labeled in vitro with Klenow fragment after priming with random hexamers (Feinberg and Vogelstein, 1983, 1985) using [^{32}P]dCTP (3000 Ci/mmol, DuPont New England Nuclear Products, Inc., Boston, MA). After hybridization, membranes were washed twice with 2× SSC at room temperature for 5 min, twice at 65°C with 2× SSC, 1% SDS for 60 min, and twice at room temperature with 0.1× SSC for 30 min. The *Egr-1* clone was OC3.1 (Sukhatme *et al.,* 1988). A cDNA clone encoding P31, a housekeeping protein (Theodor *et al.,* 1985) was used to evaluate relative amounts of RNA in each lane of the Northern blot.

Antibodies

The *Egr-1* antibody was raised in rabbits against a fusion protein produced in *Escherichia coli.* The fusion construct consisted of a 591 bp bovine recombinant DNA fragment, a 46 bp oligonucleotide linker, and a 1655 bp *Egr-1* DNA fragment (Cao *et al.,* 1990).

Immunocytochemistry

Rat kidneys were perfusion-fixed via the left ventricle with a paraformaldehyde-lysine-periodate (PLP) fixative (McLean and Nakane, 1974). After an initial 10-min fixation, the kidneys were kept in the fixative overnight at 4°C, rinsed with phosphate-buffered saline (PBS), and kept in PBS containing 0.02% sodium azide until prepared for cryosectioning. For preparing 5-μm frozen sections, tissue pieces were equilibrated for at least 1 h at room temperature in PBS containing 0.6 M sucrose, embedded in optimal cutting temperature medium (Miles Scientific, Naperville, IL), frozen in liquid nitrogen, and sectioned using a Reichert Frigocut (Leica, Deerfield, IL) cryostat. Sections were placed on poly-L-lysine-coated glass slides and were either used immediately or kept at 4°C until staining.

Tissue sections from ischemic and contralateral control kidneys, as well as kidneys from normal, nonoperated rats, were incubated for 10 min in PBS containing 1% bovine serum albumin (BSA), then for 1 h with the specific anti-*Egr-1* antiserum diluted 1:500 in PBS/1% BSA. Preimmune serum was used as a control. Sections were then washed three times for 5 min in PBS and then incubated for 30 min with 1:75 dilution (in PBS containing 1% BSA) of fluoresceinated goat antirabbit immunoglobulin antibody (Calbiochem, San Diego, CA). The sections were finally washed three times for 5 min each in PBS and mounted in 50% glycerol, 2% *n*-propyl gallate in 0.2 M tris(hydroxymethyl)aminomethane (Tris)-HCl pH 8.0. Sections were examined with a Nikon FXA photomicroscope equipped for epifluorescence (DonSanto Corp., Natick, MA) and photographed using Kodak TMAX 400 film pushed to ASA 1600.

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