Vercher, in *Adaptive Processes in Visual and Oculomotor Systems*, E. L. Keller and D. S. Zee, Eds. (Pergamon, New York, 1985)]. Proprioception in eye alignment and localization of visual targets has been demonstrated in strabismic patients [M. J. Steinbach and D. R. Smith, *Science* **213**, 1407 (1981)], whereas other authors [O. Bock and G. Kommerell, *Vision Res.* **26**, 1825 (1986)] with the same approach, obtained data that supported the outflow model.

5. The computerized system made use of a Summagraphics BITPAD 90 cm by 70 cm digitizing table connected through an electronics interface to an IMS 5000 microcomputer. The S was seated at the table, the head immobilized in a forward tilted angle by a bite bar covered with dental wax. A row of green light-emitting diode (LED) targets was positioned horizontally 60 cm above the table. A glass plate 1.5 mm thick was placed horizontally between the digitizing table and the row of LEDs at such a level that, as seen by the S, through the glass plate, the LEDs appeared as virtual targets exactly on the surface of the digitizing table. Five subjects ranging in age between 25 and 47 years were tested. Virtual targets were used to prevent Ss from using tactile cues to locate the targets. The LED targets had the same light intensity and appeared as green spots, about 2 mm in diameter.
6. Eye position is not consciously sensed [P. A. Merton, *Symp. Soc. Exp. Biol.* **18**, 387 (1964)]. In addition, in our experiments, the S's corneal afferents were desensitized by administration of local anesthetic.
7. The data are presented as "pointing maps" showing on a single graph all the pointing marks directed toward the three targets. The computer calculated the average pointing position for each set of five pointings directed towards a given target. A special representation was selected to visualize the data and provide an estimate of the data dispersion. For this, the regression line passing through each set of data points (five pointings towards a given target, in a given condition) was calculated. A new coordinate reference was defined with the abscissa parallel to the regression line and passing through the mean of the data. The ordinate was defined, perpendicular to the regression line, and centered on the mean of the data points. The location of each point and the data dispersions were calculated in the new coordinate system. An ellipse was drawn centered on the mean position of the points with its principal and minor axes oriented along the new axes. The lengths of the major and minor axes were equal to 2 SD centered about the mean of the data points, as calculated with respect to the new coordinate system. If one assumes a normal, homogeneous distribution for the data points, the probability for a given point to be within the ellipse is close to 36%.
8. Eye pulling could have had an effect on the sensing of hand position [T. A. Easton, *Brain Res.* **25**, 633 (1971); *Expl. Neurol.* **34**, 497 (1972); G. M. Gauthier, J. L. Vercher, F. Mussa-Ivaldi, E. Marchetti, *Exp. Brain Res.* **73**, 127 (1988); G. M. Gauthier, D. Nommay, J. L. Vercher, *J. Physiol. (London)* **406**, 24P (1988); R. C. Miall, D. J. Weir, J. F. Stein, *Neuroscience* **16**, 511 (1985); G. M. Gauthier and F. Mussa-Ivaldi, *Exp. Brain Res.* **73**, 138 (1988)]. No such interaction accounts for the mislocation errors we observed by pulling the covered eye of our Ss.
9. Our data seem in contradiction with the data of A. A. Skavenski, G. Haddad, and R. M. Steinman [*Percept. Psychophys.* **11**, 287 (1972)], since we show, in a somewhat simpler protocol dealing with the localization of a single target, that proprioception was involved. (In Skavenski *et al.*'s protocol, the role of inflow in the localization of target was studied by using a second target presented to the deviated eye, and correction had to be made for phoria and retinal error.) These authors concluded that "perception of direction is directly proportional to the magnitude of the outflow signal." Still, in the data from Skavenski *et al.*, proprioception may well explain the discrepancy between pure and only inflow information and actual data. Our data show that when one eye is deviated, the shift in localization of the target viewed by the other eye is about 16% of the angular deviation of the covered eye. On the average, the error between predicted pure inflow and actual data recorded by Skavenski *et al.* is in the range of error. Therefore, the data of Skavenski *et al.* essentially agree with ours.
10. We thank D. Zee for reviewing the manuscript. Supported by grants from C.N.R.S. Unité Associée 372 and INSERM 896007.

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# Mediation of Cardioprotection by Transforming Growth Factor–β

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**Myocardial ischemia causes heart injury that is characterized by an increase in circulating tumor necrosis factor (TNF), the local production of superoxide anions, the loss of coronary vasodilation (relaxation) in response to agents that release endothelial cell relaxation factor, and cardiac tissue damage. Ischemic injury can be mimicked by TNF. When given before or immediately after ischemic injury, transforming growth factor–β (TGF-β) reduced the amount of superoxide anions in the coronary circulation, maintained endothelial-dependent coronary relaxation, and reduced injury mediated by exogenous TNF. Thus, TGF-β prevented severe cardiac injury, perhaps by alleviating damage mediated by increases in circulating TNF.**

MYOCARDIAL ISCHEMIA AND REperfusion involves a critical sustained reduction in coronary flow followed by restoration of flow to the ischemic region of the heart. However, reperfusion results in dysfunction to the endothelium of the coronary vasculature as well as injury to the cardiac muscle cells (*1, 2*). Among the factors thought to mediate these damaging effects are the release of cytokines [for example, interleukin-1 (IL-1) and TNF] and oxygen-derived free radicals [for example, superoxide anions (*3, 4*)]. These humoral agents are produced by adhering neutrophilic leukocytes or by endothelial cells (*5, 6*) and are released upon reperfusion. Moreover, TNF concentrations are increased in humans after myocardial infarction (*7*). One means of counteracting these deleterious humoral agents is by addition of the naturally occurring growth factor, transforming growth factor–β (TGF-β).

TGF-β is a homodimeric protein with a molecular size of 25 kD originally defined for its ability to reversibly induce a transformed phenotype and anchorage-independent growth of normal fibroblasts (*8–10*). The most common form is TGF-$\beta_1$, which acts as a regulatory protein that modulates a variety of biological actions relating to developmental processes (*11*). Specific cell membrane receptors for TGF-β are present in many cell types. TGF-β also appears to act in a manner opposing actions of the cytokine TNF-α (*12, 13*). TGF-β is present in cardiac myocytes and coronary endothelial cells (*14*), but disappears after myocardial infarction, except at the border zone of injured myocardial tissue, where increased TGF-β occurs (*15, 16*). TGF-β appears to be angiogenic and inhibits neutrophil adherence to endothelial cells (*17*). TGF-β may therefore moderate the damaging consequences of reperfusion after myocardial ischemia. TGF-β could exert cardioprotection during acute myocardial ischemia by preventing endothelial cell–induced myocardial injury and by promoting healing of injured myocytes after their dysfunction.

We therefore tested the ability of recombinant human TGF-$\beta_1$ to prevent the loss of endothelium-dependent relaxation (EDR) in the coronary microvasculature soon after reperfusion of ischemic myocardium and to reduce myocardial injury 24 hours after myocardial ischemia and reperfusion when the infarction process is well under way. Certain vasodilators like acetylcholine (ACh) and adenosine diphosphate exert their vasodilation only in the presence of an intact endothelium, which is stimulated to release a substance termed endothelium-derived relaxing factor (EDRF) (*18*). If the endothelium is injured so that EDRF is not released, no vasodilation occurs to these endothelium-dependent agents. In contrast, several other vasodilators are endothelium-

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independent dilators, including nitroglycerin and nitroprusside, and they dilate blood vessels directly (*19*).

Adult male Sprague-Dawley rats (250 to 330 g) were anesthetized with pentobarbital sodium (35 mg/kg, intraperitoneal); their hearts were isolated and perfused by the Langendorff method at a constant flow (15 ml/min) with oxygenated Krebs-Henseleit solution (*20*). Hearts (seven per group) were subjected to ischemia by reducing coronary infusion to 15% of control flow for 30 min, then reestablishing normal flow (that is reperfusion) for an additional 20 min. Twenty minutes after reperfusion, the coronary vasodilator responses to the endothelium-dependent dilator ACh were 35 ± 6% of initial responses as compared with 98 ± 7% of initial responses in hearts not subjected to ischemia ($P < 0.01$). This shows that endothelium-dependent vasodilators exert a reduced vasodilator response during myocardial ischemia and reperfusion in the rat heart. No such defect in vasodilation occurred in response to the direct vasodilator nitroglycerin. The vasodilator responses to nitroglycerin were 85 ± 9% of initial in hearts subject to ischemia and 96 ± 8% of initial in nonischemic hearts. These responses were not significantly different from each other. TGF-β (10 μg per rat), given 2 or 24 hours before isolation of the heart, prevented the decrease in EDR to ACh; vasodilator responses were 91 ± 10% and 104 ± 12% of the control response at 2 and 24 hours (six hearts per group), respectively. However, TGF-β was only partially effective when given 5 min before isolation of the heart (75 ± 13% of initial), as assessed by analysis of variance with a post hoc *t* test performed with the Bonferroni procedure, the same statistical procedure applied to all data in the figures. Thus, TGF-β significantly preserved endothelium-dependent dilation when given 2 to 24 hours before induction of myocardial ischemia.

To determine the dose dependence of TGF-β in preserving this endothelium-dependent response, we injected rats intravenously with 100 ng, 1 μg, or 10 μg of TGF-β 2 hours before cardiac perfusion (Fig. 1A). Ischemia and then reperfusion caused a marked loss of EDR to ACh infused directly into the coronary vasculature of the perfused rat heart (34 ± 7% of control). However, at 1 and 10 μg of TGF-β, the defect in EDR to ACh was prevented and 100 ng protected almost completely. Thus, the TGF-β effect appeared and was effective over the range of 6 to 600 nM. The defect in the endothelium-dependent responses after ischemia was not because of a generalized impairment of vasodilation (that is, an injury to vascular smooth muscle cells), because the dilator response to the direct vasodilator nitroglycerin was retained (Fig. 1B). These results suggest a defect in endothelial cell formation or release of EDRF, thought to be nitric oxide (NO) (*21*), a labile substance that activates guanylate cyclase to produce cyclic guanosine monophosphate (cGMP) in vascular smooth muscle cells, the second messenger responsible for the vasodilation (*22*).

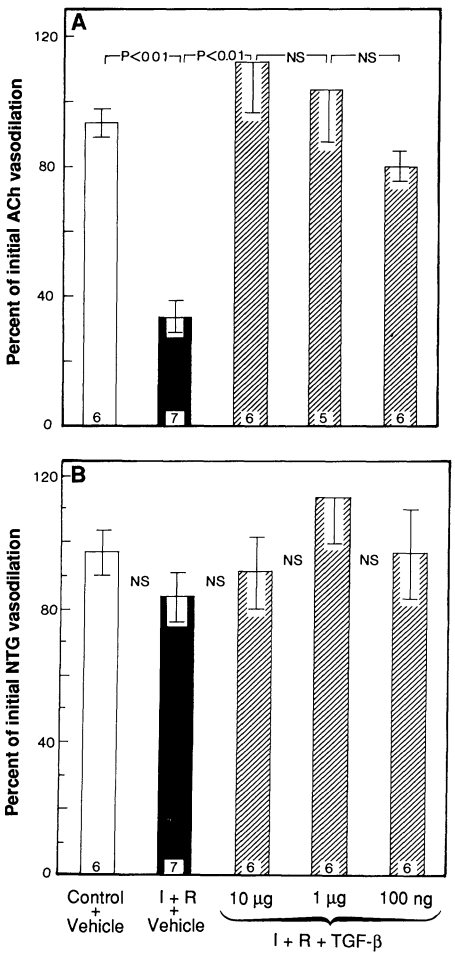



**Fig. 1.** Vasodilator response to ACh (**A**) and nitroglycerin (NTG) (**B**) in isolated perfused control rat hearts and hearts subjected to ischemia for 30 min and reperfusion for 20 min (I + R). The cDNA for human TGF-$\beta_1$ was cloned and expressed in Chinese hamster ovary cells (*31*). Recombinant human TGF-$\beta_1$ was purified to greater than 95% and prepared in solution at 0.8 mg/ml in 20 mM sodium acetate, pH 5.0, and contained <8 EU/ml (endotoxin at 1 pg/μg of protein). Diluent for injection was 20 mM sodium acetate + 0.1% human serum albumin, pH 5.0. A subclone of the cell line Mv 1 Lu (ATCC CCL 64), a mink lung epithelial-like cell, was used to quantify TGF-β activity (*32*). All rats were given either 10 μg of TGF-β or 0.1 ml of acetate buffer intravenously 2 hours before isolation of the heart. Hearts were perfused with Krebs-Henseleit buffer at 37°C under a constant normal flow of 15 ml/min (control) or at ischemic flow (2.2 ml/min). Before ischemia, all hearts were given 0.05 μmol of U-46619 (9,11-methanoepoxy-prostaglandin $H_2$) to constrict the coronary vasculature, followed by 15 nmol of Ach, and then 3 min later, 0.05 μmol of U-46619 followed by 30 nmol of nitroglycerin. Fifty minutes later (after 30 min ischemia and 20 min reperfusion, or 50 min control flow), vasodilator responses to ACh and NTG were again tested. Results are expressed as percent of initial vasodilator response to each vasodilator agent. Bar heights are means, brackets are ±SEM, and numbers of hearts in each group are at the bottom of the bars. TGF-β prevented the reduction in vasodilation in response to ACh but not to NTG in rat hearts subjected to I + R.

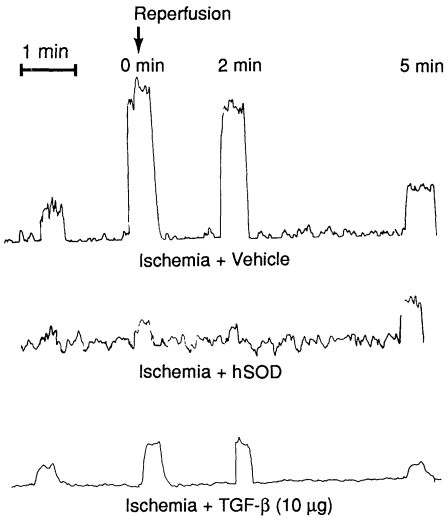
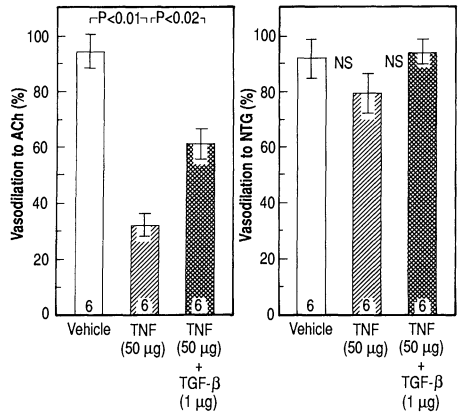



**Fig. 2.** Chemiluminescence recordings of the effluent of isolated perfused rat hearts subjected to ischemia + reperfusion and given either acetate buffer as vehicle (top) or 10 μg of TGF-β. Six untreated ischemic hearts released 571 ± 105 chemiluminescence units (CU) and the five TGF-β–treated rat hearts released 107 ± 36 CU ($P < 0.02$ from untreated hearts). In five ischemic hearts, 5 mg of hSOD was added to the perfusate, which resulted in a chemiluminescence of only 47 ± 15 CU ($P < 0.001$ from vehicle group).

Because NO is inactivated in the circulatory system by superoxide anions (*23*), we tested the ability of TGF-β to protect against superoxide ion formation. Toward this goal, we perfused hearts and processed the effluent through a pulsed flow-through chemiluminescent photometer (Model 800 continuous flow luminescence meter, Chronolog Corporation). Rat hearts were subjected to the same 30-min ischemia, then 20 min of reperfusion. Two hours before isolation of the heart, rats were given TGF-β (10 μg) or an acetate buffer as a vehicle (Fig. 2). The pulsed chemiluminescence of the coronary effluent was recorded; chemiluminescence increased at the time of reperfusion in the untreated rat. The increase was reduced by recombinant human superoxide dismutase (hSOD) (Grunenthal, Aachen, Federal Republic of Germany), indicating that most of this chemiluminescence was

**Fig. 3.** Anti-EDRF action TNF in isolated perfused rat hearts. Recombinant murine TNF-α was produced in *Escherichia coli* by recombinant DNA techniques and had a specific activity of $7 \times 10^7$ U/mg (*33*). TNF activity (units per milliliter) was determined by a standard cytotoxic assay on murine connective tissue-like cells, LM (*34*). TNF (50 μg) was given intravenously 2 hours before isolation of the heart. Hearts were perfused at constant flow (18 ml/min) and preconstricted with 0.1 μM U-46619. All hearts were tested with ACh and NTG. Bar heights are mean responses, brackets are ±SEM, and numbers of hearts are in the bottom of the bars. The ACh dilator response but not that of NTG was significantly inhibited by TNF.



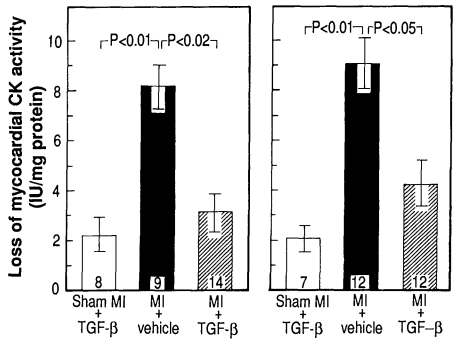




**Fig. 4.** Loss of myocardial CK activity. Rats were subjected to myocardial infarction (MI) by occlusion of the left anterior descending coronary artery and reperfusion 10 min later. CK loss was determined by the difference between septal CK specific activity (IU/mg), which was the control noninfarcted area, and the left ventricular free wall CK activity (infarcted area). Rats were either treated intravenously 24 hours before infarction with TGF-β (10 μg) or acetate buffer control (left) or treated 10 min after occlusion just before reperfusion (right). Hearts were autopsied 24 hours later and CK activity and protein content determined.

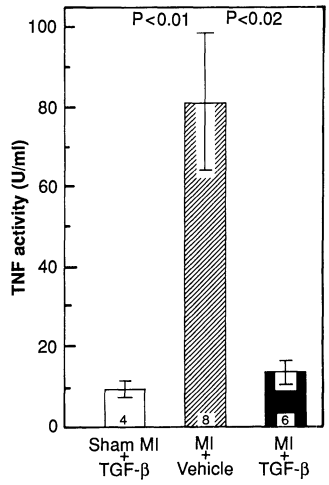



**Fig. 5.** Rats were subjected to acute myocardial ischemia, then reperfusion of the left anterior descending coronary artery 10 min later. Just before reperfusion, either 50 μl of 0.9% NaCl or 10 μg of TGF-β were given intravenously. Rats were bled 24 hours after reperfusion and plasma levels of TNF (mean ± SEM) were determined as described in Fig. 3. In two sham MI rats not given TGF-β, TNF concentrations were 7 U/ml, a value similar to sham MI rats receiving TGF-β.

caused by superoxide ions. TGF-β also eliminated superoxide production in the ischemic coronary circulation. The major site of superoxide production in the rat heart is from xanthine oxidase located in the coronary vascular endothelium, which is activated by ischemia to produce superoxide anions (*24*). TGF-β (1 ng/ml to 1 μg/ml) added to a synthetic mixture of xanthine and xanthine oxidase superoxide-generating system (*25*) did not scavenge any formed superoxide radicals. Thus, TGF-β is not a superoxide anion scavenger. These findings are therefore consistent with an endothelium preserving action of TGF-β.

The cytokine TNF is probably a mediator of ischemia and shock (*26*). TNF can exert actions directly opposing TGF-β, and functionally blocks the release of EDRF in cat and rat blood vessels in the absence of blood cells (*27*). We therefore tested the effect of TNF on vasodilator responses to ACh in isolated perfused control hearts not subjected to ischemia and reperfusion (Fig. 3). In these isolated perfused rat hearts, TNF (50 μg) injected 2 hours before isolation of the hearts produced an impairment of the vasodilator responses to ACh comparable to that observed in ischemia followed by reperfusion (32 ± 6 versus 34 ± 7%, not significantly different). TGF-β (1 μg) given just before the TNF curtailed this TNF-induced impairment in the vasodilator response to ACh. No such alteration in vasodilator response to nitroglycerin occurred with TNF.

We tested the effect of TGF-β administered after the onset of the ischemic process in experimentally induced myocardial infarction in rats. Using ether-anesthetized rats, we occluded the left coronary artery just proximal to the first main branch with a silk ligature that could be removed 10 min later, to allow coronary reperfusion. TGF-β or its vehicle (acetate buffer) were injected intravenously just before reperfusion (10 min after occlusion). Twenty-four hours later, we removed the hearts and studied the loss of creatine kinase from the left ventricle (the infarcted region) and from the interventricular septum (the control nonischemic region). By analyzing the difference in creatine kinase (CK) activities in both regions, we estimated the amount of CK activity lost from the infarcted region as an index of cardiac cellular injury (*28*). TGF-β (10 μg) given either 24 hours before occlusion (24 hours before reperfusion) or 10 min after occlusion (just before reperfusion) curtailed the myocardial CK loss observed in rats subjected to ischemia and reperfusion but given only the vehicle for TGF-β (Fig. 4). Thus, our findings show cardioprotection induced by TGF-β even when given after the onset of ischemia. In this latter series, we also measured plasma TNF activities (Fig. 5). TGF-β blocked the increase in circulating TNF activity observed after ischemia and reperfusion. Thus, TNF may contribute to the myocardial cellular injury occurring during ischemia and reperfusion. TGF-β appears to inhibit the release of TNF into the blood after myocardial ischemia by a mechanism that is not known at present, but may occur at both transcription and translation (*29*). Moreover, because cardiac TGF-β decreases during myocardial infarction, the ischemic injury to the myocardium may represent a functional deficit in endogenous TGF-β at the site of injury. Exogenous TGF-β may correct this defect. This hypothesis is also consistent with the finding that TGF-β deactivates macrophages and thus reduces the inflammatory effects of these white blood cells (*30*). Our findings point to agents such as TGF-β, which preserve endothelial cell function, as potentially useful therapeutic agents in reperfusion injury accompanying myocardial ischemia.

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# An Insulin-Stimulated Protein Kinase Similar to Yeast Kinases Involved in Cell Cycle Control

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**A protein kinase characterized by its ability to phosphorylate microtubule-associated protein-2 (MAP2), is thought to be an early intermediate in an insulin-stimulated phosphorylation cascade and in a variety of other mammalian cell responses to extracellular signals. A complementary DNA that encodes this protein serine-threonine kinase has been cloned, and the protein designated extracellular signal-regulated kinase 1 (ERK1). ERK1 has striking similarity to two protein kinases, KSS1 and FUS3, from yeast. The yeast kinases function in an antagonistic manner to regulate the cell cycle in response to mating factors. Thus, ERK1 and the two yeast kinases constitute a family of evolutionarily conserved enzymes involved in regulating the response of eukaryotic cells to extracellular signals.**

INSULIN AND SEVERAL OTHER EXTRACELLULAR cues initiate signal transduction by activating a tyrosine kinase either intrinsic to or associated with their specific receptors. These activated kinases then trigger phosphorylation cascades consisting of protein serine-threonine kinases, few of which have been identified. MAP2 kinase is rapidly stimulated in response to insulin (*1–4*), and is one of the few serine-threonine protein kinases known to be phosphorylated on tyrosine residues in vivo (*2*). It can phosphorylate an insulin-sensitive ribosomal protein S6 kinase in vitro causing an increase in its activity (*5, 6*). Thus, the MAP2 kinase may be an early intermediate regulating insulin-dependent protein kinase cascades and may be a direct substrate of the tyrosine kinase of the insulin receptor. The finding that phosphorylation on threonine as well as tyrosine residues is required for MAP2 kinase activity (*7, 8*) suggests that it is regulated by multiple phosphorylations. Other kinases poised at critical steps in phosphorylation cascades, such as Raf (*9*) and cdc2$^+$ (*10*), also display this behavior.

In addition to stimulation by insulin, MAP2 kinase activity can be rapidly increased by a variety of other extracellular signals that promote cellular proliferation and/or differentiation (*1–4*). In this regard, MAP2 kinase may be equivalent to pp42 (*11*), a protein whose phosphotyrosine content increases after exposure to growth factors and transformation by viruses (*12*). MAP2 kinase activity is also stimulated in post-mitotic adrenal chromaffin cells in response to signals that induce catecholamine secretion (*13*), and in PC12 cells in response to nerve growth factor–induced neuronal differentiation (*14*). Thus, MAP2 kinase is likely to be involved in many different signal transduction pathways in a wide variety of cell types.

Purified MAP2 kinase consists of one major band with a molecular size of 43,000 daltons (*8*). After tryptic cleavage of this band, amino acid sequences of seven peptides were obtained (*15*). Although none of the peptide sequences are in the GenBank database, consensus sequences characteristic of serine-threonine protein kinases (*16*), GEGAYG (part of the nucleotide binding site) and DLKPSN, were found (*17*). A series of degenerate oligonucleotides corresponding to different regions of the amino acid sequence were used for polymerase chain reactions (PCR) (*18*). Complementary DNA templates prepared from Rat 1 fibroblasts and oligonucleotides corresponding to segments of the peptides that contain the conserved GEGAYG and DLKPSN sequences were used in a PCR and yielded an amplified DNA fragment of an expected size. Based on similarities to other protein kinases, we assumed that of the two sequences GEGAYG is closer to the $NH_2$-terminus of the protein; the two sequences are separated by about 120 amino acids in most protein kinases. PCR with the degenerate oligonucleotide encoding the amino acids QYIGEG (5′-TTCTAGAATTC CA-(A,G) TA(C,T) AT(A,T,C) GG(A,T,C,G) GA(A,G) GG-3′) and the degenerate oligonucleotide corresponding to the noncoding strand for the amino acids DLKPSN (5′-TTCTCGAGTCGAC (A,G)TT (A,T,C,G)-GA (A,T,C,G)GG (C,T)TT (A,T,C,G)-A(A,G) (A,G)TC-3′) yielded an amplified product of ~360 base pairs (bp). The PCR product was sequenced to confirm that it encoded part of a novel protein kinase, and used as a probe to screen a rat brain cDNA library (*19*). A single clone, which hybridized at high stringency, was isolated and contained a 1.9-kb cDNA insert.

A probe made from the cDNA insert identified a 1.9-kb transcript by Northern (RNA) blot analysis, indicating that our cDNA clone contains an insert that is a nearly full-length transcript. The ERK1 transcript was detectable in all tissues and cell lines examined (Fig. 2). The highest concentrations (three to six times higher) are in the central nervous system (brain and

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