Astrocytes have a critical role in the neuronal response to ischemia, as their production of neurotrophic mediators can favorably impact on the extreme sensitivity of nervous tissue to oxygen deprivation. Using a differential display method, a novel putative RNA binding protein, RA301, was cloned from reoxygenated astrocytes. Analysis of the deduced amino acid sequence showed two ribonucleoprotein domains and serine/arginine-rich domains, suggestive of their function as RNA splicing factor. Northern analysis displayed striking induction only in cultured astrocytes within 15 min of reoxygenation and reached a maximum by 60 min after hypoxia/reoxygenation. Immunoblotting demonstrated expression of an immunoreactive polypeptide of the expected molecular mass, 36 kDa, in lysates of hypoxia/reoxygenated astrocytes. Induction of RA301 mRNA was mediated, in large part, by endogenously generated reactive oxygen species, as shown by diphenyl iodonium, an inhibitor of neutrophil-type nicotinamide adenine dinucleotide phosphate oxidase which blocks oxygen-free radical formation by astrocytes. Similarly, increased expression of RA301 in supporting a neurotrophic function of astrocytes was suggested by inhibition of interleukin-6 elaboration, a neuroprotective cytokine, in the presence of antisense oligonucleotide for RA301. These studies provide a first step in characterizing a novel putative RNA binding protein, whose expression is induced by oxygen-free radicals generated during hypoxia/reoxygenation, and which may have an important role in redirection of biosynthetic events observed in the ischemic tissues.
lary acidic protein). Cultures were then replate at a density of 5 × 10^4 cells/cm² in the above medium. When cultures achieved confluence, they were exposed to hypoxia by an incubator attached to an hypoxic chamber which maintained a humidified atmosphere with low oxygen tension (Coy Laboratory Products, Ann Arbor, MI) as described previously (9). Where indicated, after exposure to hypoxia, cultures were returned to the ambient atmosphere (reoxygenation), and hypoxia/reoxygenation was performed by the method described elsewhere (6). In brief, after all reagents were equilibrated in the hypoxic atmosphere, then fixed to the membrane by exposure to UV irradiation prior to hybridization. Filters were washed twice in high stringency buffer (0.3 SSC, 0.5% SDS, and 0.1 M Na acetate) and twice in low stringency buffer (0.3 SSC, 0.1% SDS). Filters were hybridized at 65°C in hybridization buffer (6× SSC, 0.5% SDS, and 0.1 M Na acetate). The membrane was washed twice with 0.3× SSC, 0.1% SDS, and 0.1× SSC, 0.05% SDS at 65°C. The membrane was exposed to x-ray film (Kodak X-OMAT, Eastman Kodak Co.), and sub-jected to autoradiography. Induction of RA301 mRNA was evaluated by comparison with β-actin mRNA. To assess induction of RA301 in astrocytes by hypoxia/reoxygenation, total RNA was extracted from astrocytes exposed to hypoxia/reoxygenation at the indicated time, and Northern blotting was performed as described previously (8). dNTP mixtures were cloned into the pT7BlueT-vector (Novagen). Plasmid DNA was isolated and sequenced by standard procedures. The nucleotide sequence of each RA301 cDNA was determined in both directions using the M13 forward sequencing primer or reverse primer. The cloned cDNA fragments were then sequenced using the dideoxy method (12). The sequence data are available in the EMBL and GenBank™ data bases, and NBRF protein data base. In the present study, one of the cDNA fragments (tentatively named RA301) was subjected to further analysis. By Northern blotting using radiolabeled cDNA probes including U1-70K (human U1 small nuclear RNA), U6-45S (human U6 small nuclear RNA), and U1-70K probe (cDNA fragment probe), both RA301 and sense cRNA probes according to the method described previously (8). In brief, cultured astrocytes were exposed to hypoxia/reoxygenation at the indicated time, and Northern blot analysis was performed using a cDNA probe. The hybridization signals from the same membrane were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) with ImageQuant software. The relative expression of RA301 mRNA was calculated as the ratio of the density of the hybridization signal from RA301 to that from β-actin. The expression of RA301 in ischemic brain—Expression of RA301 antigen was studied by immunohistochemistry. Serum proteins were affinity purified from serum proteins using a column containing the antigen coupled to cyanogen bromide-activated agarose. The affinity-purified antibodies were conjugated with biotin using a kit obtained from Vector Laboratories (Burlingame, CA) under the presence of 35S-UTP (NEG-039H, DuPont NEN). After hybridization, sections were washed in high stringency buffer (0.3 SSC, 0.5% SDS, and 0.1 M Na acetate) and twice in low stringency buffer (0.3 SSC, 0.1% SDS). Filters were washed twice with 0.3× SSC, 0.1% SDS, and 0.1× SSC, 0.05% SDS at 65°C. Culture supernatants were collected 8 h after reoxygenation. IL-6 activity was measured by enzyme-linked immunosorbent assay. After astrocytes (about 1 × 10^6 cells) were exposed to hypoxia/reoxygenation, cell lysates were collected and stored at −80°C until measurement. IL-6 activity in the culture supernatant was measured 8 h after reoxygenation. Sense oligonucleotide (3′-CGA CAG CGA GTA CTG AGG CC-5′) which most effectively inhibited IL-6 release was also added as a control for specificity (each oligonucleotide was added at 10 μg/ml heat-denatured salmon sperm DNA). The membrane was washed twice with 0.3× SSC, 0.1% SDS, and 0.1× SSC, 0.05% SDS at 65°C. Each RNA sample was hybridized with the radiolabeled cDNA probe (20 μg of each) 30 min before reoxygenation. To assess the role of RA301 in the induction of IL-6 after reoxy-genation, sense oligonucleotide (3′-CGA CAG CGA GTA CTG AGG CC-5′) complementary to the antisense structure (Y5-60: 5′-GCT GTC GAT CAT GAC TCC GG-3′) which most effectively inhibited IL-6 release was also added as a control for specificity (each oligonucleotide was added at 10 μg/ml heat-denatured salmon sperm DNA). To assess the role of RA301 in the induction of IL-6 after reoxy geneation, sense oligonucleotide (3′-CGA CAG CGA GTA CTG AGG CC-5′) which most effectively inhibited IL-6 release was also added as a control for specificity (15). Production of Anti-RA301 Peptide Antibody and Western Blotting—To obtain antibody reactive with RA301 antigen, the peptide with the sequence of C-E-N-V-D-A-K-E-A-K-E-R-A-N-G-M-E (in one-letter code, amino acid residues 167–183 (see Fig. 1A), extra cysteine residue was introduced at the N terminus for conjugation) was synthesized and conjugated with bovine serum albumin using a kit obtained from Sigma. Rabbits were immunized by conventional methods and anti-serum was obtained from three rabbits immunized with 100 μg of synthetic peptides followed by the evaluation of titer of the serum by enzyme-linked immunosorbent assay. After astrocytes (about 1 × 10^6 cells) were exposed to hypoxia/reoxygenation, cell lysates were collected and stored at −80°C until measurement. IL-6 activity in the culture supernatant was measured 8 h after reoxygenation. Sense oligonucleotide (3′-CGA CAG CGA GTA CTG AGG CC-5′) which most effectively inhibited IL-6 release was also added as a control for specificity (each oligonucleotide was added at 10 μg/ml heat-denatured salmon sperm DNA). To assess the role of RA301 in the induction of IL-6 after reoxy geneation, sense oligonucleotide (3′-CGA CAG CGA GTA CTG AGG CC-5′) which most effectively inhibited IL-6 release was also added as a control for specificity (each oligonucleotide was added at 10 μg/ml heat-denatured salmon sperm DNA). 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Statistical Analysis—Where indicated, statistical analysis was performed by Student's non-paired t test for the multiple comparison, following analysis of variance.

RESULTS
Viability of Astrocytes Exposed to Hypoxia/Reoxygenation—Oxygen tension in the medium fell to 8 torr within 3–5 h after cultures were transferred to the hypoxia chamber. Cell viability was maintained throughout hypoxia, and following replacement of cultures back to normoxia (reoxygenation), based on lack of lactate dehydrogenase release into culture supernatants, continued trypan blue exclusion, adherence to the culture substrate, and unchanged morphologic features (data not shown).

Isolation of the cDNA for RA301 by Differential Display—Using the primer 5'-CCT TTC CGA CGT-3' for differential display, a cDNA, termed RA301, was amplified from the cDNAs
RNA Binding Protein and Reoxygenation

Induction of RA301 message in cultured rat astrocytes by hypoxia/reoxygenation (A, Northern blot analysis in pooled RNAs; B, time course; and C, effect of cycloheximide and DPI). In Panel A, RNA was extracted and pooled from three different cultures. About 5 μg of RNA from either normoxic (lane N), hypoxic (lane H), or reoxygenated (lane H/R) cultures were subjected to Northern blot hybridization using the RA301 cDNA probe (upper panel) and β-actin probe (lower panel) radiolabeled with [32P]dCTP. In Panel B, total RNA was extracted from astrocytes at the end of hypoxia (0 min after reoxygenation) and the indicated time points (0.5–12 h after reoxygenation). Then, about 5 μg of total RNA were subjected to Northern blot analysis as indicated above. In Panel C, either cycloheximide (10 μg/ml Cx) or DPI (50 μM) was added to the culture 15 min before reoxygenation, and total RNA was applied to Northern blot analysis. The same analysis using RNAs obtained at the end of hypoxia (lane H/R, 0), 15 and 60 min after reoxygenation (H/R, 15 and H/R, 60) from the same batch of experiments is also shown.

Prepared from pooled RNA of hypoxia/reoxygenated astrocytes. The deduced amino acid sequence, based on nucleotide sequencing of the RA301 cDNA (accession no. D49708, in DDBJ, GenBank™, EMBL data bases), revealed several motifs specifically associated with RNA binding factors, including two serine/arginine-rich domains, an RNA recognition motif, and a glycine octamer domain (Fig. 1A). The total number of amino acids expected is 288, and the predicted molecular mass is about 36 kDa. The amino acid sequence of RA301 has greatest homology (38.2%) to transformer-2 (Tra-2), a Drosophila splicing factor (Fig. 1B). The glycine octamer structure (Fig. 1A) is homologous to the glycine-rich domain of several RNA splicing factors, such as SC35 (20) and SF2/ASF (21) (Fig. 1C). Northern analysis of RNA from hypoxia/reoxygenated astrocytes using the full-length cDNA of RA301 as the probe demonstrated a band corresponding to 2 kilobase pairs, suggesting that the cDNA of 2 kilobase pairs was likely to be full-length (Fig. 2A).

Induction of RA301 mRNA in Astrocytes Exposed to Hypoxia/Reoxygenation—Compared with normoxic cultured astrocytes, where no band was detectable, Northern analysis showed a dense band after cultures were exposed to hypoxia, followed by reoxygenation (Fig. 2A). In contrast, hypoxia alone did not up-regulate RA301 mRNA. Maximal expression of the mRNA was observed 30–60 min following replacement of hypoxic astrocytes into normoxia (Fig. 2B), and the increase in levels of RA301 mRNA was evident as early as 15 min after reoxygenation (Fig. 2C). By 4 h after exposing hypoxic astrocytes to normoxia, levels of RA301 mRNA had declined to close to the pretreatment baseline.

Addition of cycloheximide (10 μg/ml) to hypoxic astrocytes simultaneously with their placement in normoxia did not block induction of RA301 mRNA (Fig. 2C), whereas this concentration of cycloheximide blocked incorporation of [3H]leucine into material precipitable in trichloroacetic acid by >90%. In contrast, the neutrophil-type NADPH oxidase inhibitor, DPI, when present at the start of reoxygenation, completely blocked the appearance of RA301 mRNA (Fig. 2C). Under these conditions, DPI prevents generation of oxygen-free radicals by reoxy-
up-regulation of multiple RNA binding factors and related proteins. By Northern analysis, U1-70K, U2AF65, and U2AF35 did not display increased expression at the mRNA level, compared with the results previously obtained with RA301 (Fig. 3).

Expression of RA301 in Astrocytes Exposed to Hypoxia/Reoxygenation: Effect of Antisense/Sense Oligonucleotides—Rabbit antiserum was raised against the synthetic peptide corresponding to residues 167–183 in RA301 immunoblotted a band corresponding to a molecular mass of about 36 kDa (Fig. 4A). This band was not observed in lysates of normoxic and hypoxic astrocytes, but was readily detectable in extracts from hypoxia/reoxygenated cells (Fig. 4A). Immunoreactive RA301 was observed within 2 h of reoxygenation, becoming maximal by 4 h (Fig. 4A). To modulate expression of RA301, antisense oligonucleotides were employed. Antisense oligonucleotide (Y5-60) blocked induction of RA301 antigen in astrocytes exposed to hypoxia/reoxygenation, although sense oligonucleotide at the same concentration was without effect (Fig. 4B). Further evidence of the specificity of Y5-60 for inhibiting expression of RA301 was its lack of effect on induction of HSP72 which also occurs in astrocytes exposed to hypoxia/reoxygenation (Fig. 4C). Consistent with a central role for de novo biosynthesis in the production of RA301 antigen, cycloheximide (10 μg/ml) inhibited its expression (Fig. 4B).

Expression of RA301 mRNA and Antigen in Ischemic Rat Brain—A rat brain ischemia model was employed to assess expression of RA301 in vivo. In situ hybridization showed increased levels of mRNA in the ischemic hemisphere, versus lower levels in nonischemic hemisphere (Fig. 5, A and B). RA301 mRNA was maximally expressed about 12 h after 2-h unilateral occlusion of the middle cerebral artery and remained elevated up to 24 h, returning to baseline levels by about 3 days (data not shown). RA301 mRNA appeared diffusely induced in the ischemic hemisphere (Fig. 5, A and B). Detailed analysis with microautoradiography (Fig. 5, D and E) suggested that astrocytes expressed RA301 mRNA in addition to neurons of the ischemic cerebral cortex. Controls with the sense probe demonstrated no specific staining pattern in ischemic hemisphere (data not shown). Immunoblotting of homogenates derived from ischemic hemisphere demonstrated an approximately 36-kDa band not observed with extracts from nonischemic control brain (Fig. 5C).

Effect of RA301 Expression on Increased Release of IL-6 Activity by Astrocytes Exposed to Hypoxia/Reoxygenation—IL-6 is a neurotrophic cytokine which, in previous studies, has been shown to be synthesized and released in increased amounts by astrocytes subjected to hypoxia/reoxygenation (6). To explore a possible role for RA301 in IL-6 production by astrocytes, antisense oligonucleotide was employed. Y5-60 blocked by >65% elaboration of IL-6 activity into the conditioned medium of astrocytes exposed to hypoxia/reoxygenation (Fig. 6A), under conditions where expression of RA301 was largely blocked (Fig. 4B). Two other antisense oligonucleotides, Y5-59 and Y5-61, did not effectively modulate expression of RA301 antigen (data not shown) and had no effect on astrocyte release of IL-6 activity (Fig. 6B). None of these oligonucleotides affected the activity of exogenous IL-6 added to the HH-50 bioassay.

DISCUSSION

Exposure of cells to a period of oxygen deprivation followed by reoxygenation imposes a major metabolic stress. The period of hypoxia, in which there is a shift to anaerobic glycolysis, is associated with events such as up-regulation of the non-insulin-dependent glucose transporter, activation of NF-IL-6, and transcription of target genes, including IL-6 and tumor necrosis factor-α (22), which we have hypothesized primes the cell for the subsequent phase of reoxygenation. It is also likely that other events, such as activation of AP-1, which is stimulated by antioxidants, will occur during hypoxia, further modifying biosynthetic mechanisms (23, 24). In contrast, during reoxygenation, generation of oxygen-free radicals occurs. This is especially striking in vivo when leukocytes are attracted to reperfused tissues and activated, and their formation of reactive oxygen intermediates is induced. Previous studies have drawn attention to a role for oxygen-free radicals in triggering production of polypeptide mediators relevant to the biology of ischemia; reactive oxygen intermediates appear to initiate expression of IL-1 and IL-8 in mononuclear phagocytes (25, 26).
The presence of RA301 in ischemic regions of rat brain further emphasizes its potential expression in loci where it is likely to impact on ultimate expression of proteins in cellular elements subject to ischemia. The importance of such de novo protein products in the cellular adaptation to hypoxic/reoxygenation is illustrated by the induction of cell death which invariably follows addition of cycloheximide to reoxygenated astrocytes following 8 h of reoxygenation (7). These studies provide a first step in characterizing a gene product of potential importance in the cellular response to ischemia, RA301, and raise multiple questions concerning its potential impact on redirecting essential biosynthetic events in astrocytes, and, potentially, other cells exposed to hypoxia/reoxygenation. The concomitant attenuation of the production of IL-6 by astrocytes exposed to hypoxia/reoxygenation when RA301 expression was blocked by antisense oligonucleotide suggests that RA301 may affect synthesis of critical gene products.

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