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Retrovirus-mediated transfer of an angiotensin type I receptor (AT_1-R) antisense sequence decreases AT_1-Rs and angiotensin II action in astroglial and neuronal cells in primary cultures from the brain

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ABSTRACT **The AT_1-R has been implicated in many cellular and physiological actions of angiotensin II (AII) in the brain. A retrovirus vector (LNSV) containing an AT_{1B}-R antisense sequence (AT_{1B}-AS) (termed LNSV-AT_{1B}-AS) was constructed and used to determine the feasibility of using viral-mediated gene transfer to control AT_1-Rs and AII actions in astroglial and neuronal cells in primary cultures from rat brain. Briefly, a 1.26-kb antisense sequence corresponding to nt −132 to +1128 of AT_1-R cDNA was cloned into the LNSV vector, the vector was transfected into PA317 cells, and transfected cells were selected in G418. Incubation of brain cells with culture medium containing LNSV-AT_{1B}-AS viral particles showed that AT_{1B}-AS was integrated into the genome and transcribed in brain cells. This was associated with a significant decrease in AT_1-Rs and in the AII-stimulated increase of c-fos mRNA, a measure of AT_1-R function. These observations show that the AT_{1B}-AS gene can be transferred into astroglial cells in culture by LNSV and that such a transfer inhibits AT_1-Rs and the AII stimulation of cellular activities. In addition, the usefulness of this approach to study AII-dependent pathophysiology in primary neuronal cultures from brain, in particular, is established.**

The brain angiotensin system plays a key role in the control of blood pressure (BP) (1, 2). This and many other physiological actions of angiotensin II (AII) are mediated by activation of the AT_1-R subtype in specific areas of the brain (2). The physiological importance of the brain AII system in the control of BP is further heightened by observations that the spontaneously hypertensive (SH) rat, a genetic model for essential hypertension, exhibits hyperactivity of the brain AII system, including an increased level of AT_1-R mRNA (1, 3, 4). Furthermore, intervention of the hyperactivity of the brain AII system decreases BP in the SH rat (5).

An *in vitro* brain cell culture model system has been established in our laboratory to elucidate the cellular and molecular mechanisms of AII action in the brains of normal and SH rats. Previous studies with this model have shown that neurons from the hypothalamus/brain stem of the SH rat show an increased expression of the AT_1-R gene (2, 6), a decreased level of endogenous AII, and an increased ability of AII to stimulate hydrolysis of inositol phospholipids (7). These observations suggest that the hyperactivity of the brain AII system in the adult SH rat is also expressed in neuronal cultures *in vitro*. In addition, these findings indicate that the increase in brain AT_1-R gene expression in the SH rat is probably genetically linked and not the result of the animal being persistently exposed to high BP.

It is evident from the above discussion that increased expression of AT_1-R is a key step that contributes to hyperactivity in the brain AII system of the SH rat. In fact, various pharmacological approaches have been used to control the AT_1-R in an attempt to demonstrate this view (5, 8, 9). Although these approaches have been able to lower the BP and delay the development and establishment of hypertension in this model, they are temporary measures. The objective of this study was to examine the feasibility of utilizing the retrovirus-mediated transfer of an AT_1-R antisense sequence (AT_1-AS) *in vitro* into the brain cells to control AT_1-R expression and function and thus to develop a gene transfer technology to control AII-dependent hypertension; such an approach would be long lasting. Our observations demonstrate that AT_{1B}-AS can be transferred into astroglial and neuronal cells in primary culture and results in decreased levels of AT_1-R and the attenuation of AII-stimulated cellular action.

MATERIALS AND METHODS

Construction of LNSV-AT_1-AS, a Retroviral AT_1-AS Recombinant, by Reverse Transcription (RT)–PCR of AT_{1B}-R cDNA. AT_{1B}-R cDNA was generated by RT–PCR essentially as described (6). In brief, the protocol is as follows. A pair of AT_{1B}-R-specific primers were synthesized containing a *Hin*dIII restriction site at the 5′ end: sense, 5′-CCA AGC TTG TGT CAG AGA GCA ATT CAC CTC ACC-3′; antisense, 5′-CCA AGC TTG GTA GTG AGT GAG CTG CTT AGC CCA-3′. The RT reaction was performed with 5 μg of total RNA from hypothalamus/brain stem neuronal cultures and 50 pmol of AT_{1B}-R antisense primer as described (6). The PCR mixture contained 50 pmol of AT_{1B}-R sense and antisense primers and a PCR of 30 cycles (94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min) was used. PCR products were electrophoresed, and the DNA band of 1.26 kb was excised and purified (10). Further characterization of the 1.26-kb PCR product was carried out by *Cla* I and *Fok* I restriction enzyme analysis and sequencing.

Recombination of AT_{1B}-AS with LNSV. The retroviral vector LNSV was kindly provided by Geoffrey C. Owens (University of Colorado Health Sciences Center, Denver). The rationale for the choice of this vector was essentially based on reports of its ability to deliver genes to cells of neural origin (11, 12). AT_{1B}-R cDNA (100 ng) and LNSV vector (50 ng) were digested with *Hin*dIII for 3 h at 37°C, followed by phenol/chloroform extraction and ethanol precipitation. The fragments were then mixed with 1× standard ligase buffer (50 mM Tris·HCl, pH 7.5/7 mM $MgCl_2$/1 mM dithiothreitol/1 mM ATP) and 10 units of bacteria T4 DNA ligase and incubated overnight at 12°C. Recombinants from the ligation

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Abbreviations: AT_1-R, angiotensin type I receptor; AII, angiotensin II; AT_1-AS, AT_1-R, antisense sequence; BP, blood pressure; SH, spontaneously hypertensive; RT, reverse transcription; Sar, sarcosine.

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were transformed into competent HB101 bacterial cells by the heat-pulse method (13, 14). To select AT_{1B}-R-positive recombinants, >50 bacterial colonies were picked from a transformed LB plate and suspended in 100 μl of water. After boiling for 1 min, 1 μl of each sample was subjected to PCR with AT_{1B}-R sense and antisense primers as described above. The colonies that produced the 1.26-kb PCR product were then grown in LB medium with ampicillin (100 μg/ml), and recombinant DNA was purified by using the Qiagen-tip (Qiagen, Chatsworth, CA) method according to the protocols provided by the company. LNSV has a neomycin-resistance gene for selection driven by a long terminal repeat promoter (Fig. 1*A*). An AT_{1B}-AS corresponding to nt −132 to +1128 in the coding region of the AT_{1B}-R was cloned in the LNSV. Fig. 1*B* shows that *Hin*dIII digestion gave the anticipated 1.26-kb band (lane 1) that corresponds to nt −132 to +1128 in the AT_{1B}-R sequence. A *Cla* I restriction fragment of ≈536 bp was observed (lane 3), and *Cla* I/*Sal* I digestion gave two bands (lane 2) of 1171 bp and 536 bp. Sizes of these fragments are similar to those anticipated from the location of these restriction sites. *Fok* I digestion of the 1.26-kb band (lane 1) gave two bands of 952 bp and 308 bp (Fig. 1*B*, lane 4), which is consistent with the location of *Fok* I restriction site in AT_{1B}-AS. In addition, sequence analysis confirmed the identity of these fragments. Constructs with a reverse-oriented AT_{1B}-R insert (LNSV-AT_{1B}-AS) were further amplified and purified by using the Qiagen-tip method.

Preparation of Retroviral Medium Containing LNSV-AT_{1B}-AS Particles. Exponentially growing PA317 packaging cells (American Type Culture Collection) were replated at 5×10^5 cells per 100-mm tissue culture dish. After 24 h, 50 μg of LNSV-AT_{1B}-AS recombinant DNA was used to transfect these cells as described (15). After transfection, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum and G418 (800 μg/ml) continuously for 4 weeks for selection and extension. At this time, medium containing G418 was replaced with DMEM without G418 and cells were incubated for an additional 48 h to produce retrovirus for infection. The titer of this retrovirus preparation was routinely measured by infecting NIH 3T3 cells (15).

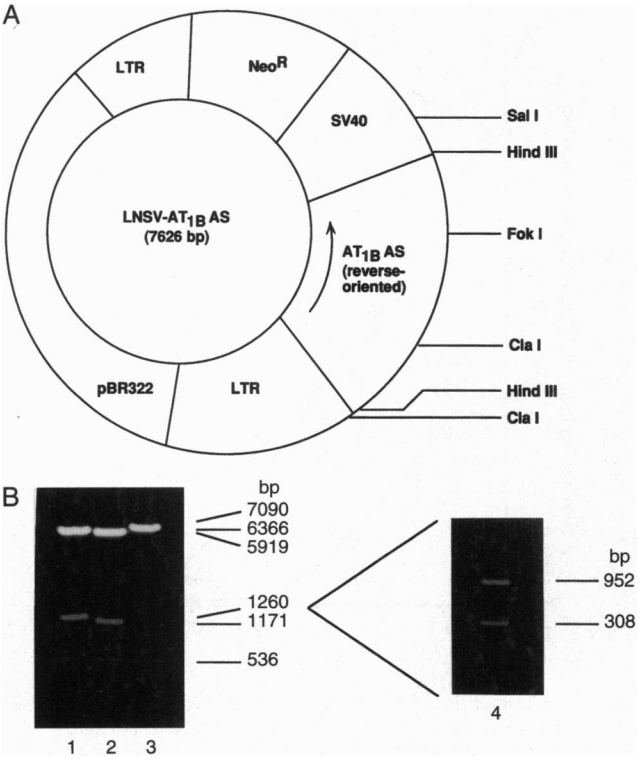

FIG. 1. (*A*) Map of LNSV-AT_{1B}-AS. LTR, long terminal repeat; Neo^R, neomycin-resistance gene. (*B*) Restriction enzyme analysis of LNSV-AT_{1B}-AS. Lanes: 1, *Hin*dIII digestion; 2, *Cla* I/*Sal* I digestion; 3, *Cla* I digestion; 4, *Fok* I digestion.

LNSV-AT_{1B}-AS Infection of Astroglial and Neuronal Cells in Primary Culture. Protocols for infection of astroglia and neurons were distinct. Primary cultures of astroglial cells from the hypothalamus/brain stem areas of Wistar Kyoto (WKY) normotensive rats were established essentially as described (16). Cells from primary cultures were replated in either 35-mm or 100-mm tissue culture dishes at 6400 cells per cm^2 (16). These secondary cultures were grown for 3 days before the growth medium was replaced with LNSV-AT_{1B}-AS-containing medium (4×10^5 colony-forming units/ml) and incubation was continued for an additional 2 days. Viral medium was removed and cultures were subjected to G418 selection in DMEM containing 10% fetal calf serum and G418 (800 μg/ml) (15) for 14 days prior to the experiments. Parallel control cultures were also prepared that underwent the same treatment except for infection and G418 selection. In addition, a similar retrovirus construct containing β-galactosidase instead of AT_1-AS was used as a control to demonstrate that the changes in the expression of AT_1-R are not the result of retroviral infection or due to the effect of G418.

Neuronal cultures from the hypothalamus/brain stem area of 1-day-old WKY rats were established on poly(L-lysine)-coated 8-well slides (Nunc InterMed, Grand Island, NY) as described (7, 17). After 4 days in culture, cells were infected by adding virus particles (4×10^5 colony-forming units/ml) in culture medium and incubating the resulting culture for 48 h at 37°C. Cultures were fixed and subjected to RT-*in situ* PCR analysis as described below.

Southern and Northern Blot Hybridization. Genomic DNAs from the LNSV-AT_{1B}-AS-infected and noninfected control astroglial cultures were digested with *Hin*dIII for 3 h at 37°C and electrophoresed on a 1% agarose gel (18). After denaturing, DNA was transferred to GeneScreen membrane and probed with a PCR-generated AT_{1B}-R-specific probe randomly labeled with [^{32}P]dCTP by the Klenow fragment of DNA polymerase I, essentially as described (19, 20).

Total RNA (20 μg) from infected and noninfected astroglial cultures was electrophoresed, transferred to a GeneScreen membrane, and subjected to Northern blot hybridization analysis with a [^{32}P]dCTP-random-labeled AT_{1B}-R-specific probe, essentially as described (19–21).

Measurement of AT_1-R and AII Stimulation of the Level of c-fos mRNA. LNSV-AT_{1B}-AS-infected and noninfected astroglial cells were grown in 35-mm tissue culture dishes for binding experiments. Competition inhibition of ^{125}I-labeled [Sar^1, Ile^8]AII (Sar, sarcosine) with losartan (DUP-753, DuPont/Merek) was carried out to measure levels of AT_1-R essentially as described (17, 19). The effect of 100 nM AII on the level of c-fos mRNA in astroglial culture was measured essentially as described (21).

RT-*in Situ* PCR Detection of AT_{1B}-AS. RT-*in situ* PCR was carried out essentially as described by Boehringer Mannheim, with slight modifications adapted for brain cells, and all regents for it were purchased from Boehringer Mannheim. Brain cells grown in 8-well slides (Nunc InterMed) were fixed in neutral buffered 10% (vol/vol) formalin for 18 h at 4°C and treated with trypsin (10,000 N^{α}-benzoyl-L-arginine ethyl ester units/mg) for 45 min at room temperature and with 10 units of RNase-free DNase at 37°C overnight. RT was done with 1 μM AT_{1B} sense primer and 1 unit of avian myeloblastosis virus reverse transcriptase (6). After RT, cells were covered with 25 μl of 1× PCR buffer containing 4.5 mM $MgCl_2$, all four dNTPs (each at 200 μM), 0.08% bovine serum albumin, 16 μM digoxigenin-11-dUTP, and AT_{1B} sense and antisense primers

(each at 0.8 μM). Slides were placed on the heating block of a thermal cycler (Perkin–Elmer/Cetus) and a PCR of 18 cycles was done as described (6). Slides were washed with xylene for 10 min, dehydrated with ethanol, and air-dried.

Immunodetection was carried out as follows. Cells were incubated with a 1:100 dilution of anti-digoxigenin Fab fragment conjugated with alkaline phosphatase in 0.1 M Tris·HCl, pH 7.5/0.15 M NaCl at room temperature for 60 min. Slides were washed in the same buffer for three 10-min periods and, finally, with 0.2 M Tris·HCl, pH 9.5/0.15 M NaCl/0.01 M $MgCl_2$ for 5 min. The color reaction used 5-bromo-4-chloro-3-indole phosphate and 4-nitroblue tetrazolium chloride (22).

RESULTS

Effect of LNSV-AT_{1B}-AS on Astroglial Culture. The first series of experiments was conducted with astroglial cultures to optimize conditions to infect and influence AT_1-R in primary brain cell cultures. Astroglial cultures were incubated with LNSV-AT_{1B}-AS (4×10^5 colony-forming units/μl) for 48 h, followed by G418 selection and growth in DMEM containing G418 and 10% fetal calf serum. Southern and Northern blot hybridizations were carried out to determine whether AT_{1B}-AS was incorporated and transcribed in these cultures. Fig. 2*A* shows a *Hin*dIII restriction analysis of genomic DNA from LNSV-AT_{1B}-AS-infected and noninfected astroglial cultures. Two restriction endonuclease fragments of ≈2.8 kb and 3.2 kb were observed in both infected and noninfected cells. However, a fragment of ≈1.3 kb was seen in only LNSV-AT_{1B}-AS-infected cells. Northern blot hybridization analysis (Fig. 2*B*) also showed a band of ≈1.3 kb in infected cells. In addition, an ≈2.3-kb band corresponding to the reported size of the AT_1-R mRNA was seen in noninfected and infected cells (6, 7, 19). RT-*in situ* PCR followed by chemical detection of AT_{1B}-AS in DNase-treated LNSV-AT_{1B}-AS-infected cells showed intense staining in cytoplasm of astroglia cells (Fig. 3*b*), confirming the Northern blot hybridization data. The staining appears to be specific since cells subjected to a similar analysis that did not contain reverse transcriptase during the RT reaction failed to show any staining (Fig. 3*c*). In addition, the positive control where cells were not treated with DNase showed highly localized staining in the nuclei as anticipated (Fig. 3*a*). Further confirmation that the AT_{1B}-AS transcript was specifically expressed in LNSV-AT_{1B}-AS-infected cells was provided by our observations that noninfected astroglial cells subjected to an identical RT-*in situ* PCR protocol failed to show any staining (data not shown).

Next, we studied the effect of AT_{1B}-AS expression on the binding of ^{125}I-labeled [Sar^1, Ile^8]AII. Fig. 4 shows a competition inhibition of binding of ^{125}I-labeled [Sar^1, Ile^8]AII by losartan in LNSV-AT_{1B}-AS-infected and noninfected astroglial cultures. Although a dose-dependent inhibition of ^{125}I-labeled [Sar^1, Ile^8]AII was observed by losartan, the levels of binding in LNSV-AT_{1B}-AS-infected cells was only 20–30% of

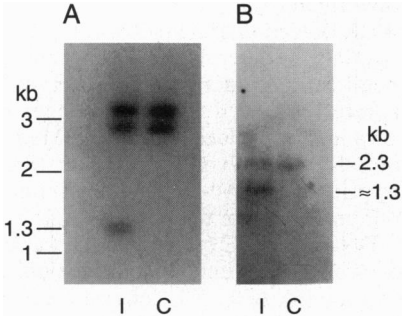

FIG. 2. Southern (*A*) and Northern (*B*) blot analyses of noninfected (lanes C) and LNSV-AT_{1B}-AS-infected (lanes I) astroglial cultures.

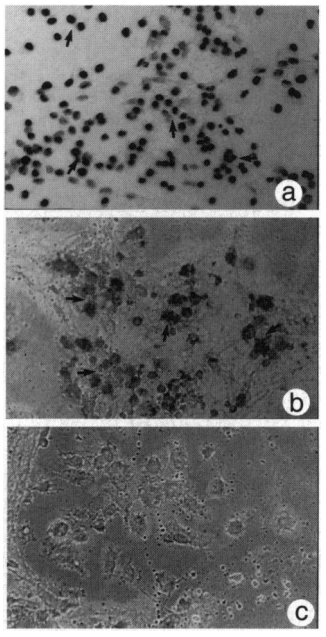

FIG. 3. Detection of AT_{1B}-AS in LNSV-AT_{1B}-AS-infected astroglial cultures by RT-*in situ* PCR. *a*, Positive control demonstrating the staining in the nucleus (arrows); *b*, presence of AT_{1B}-AS mRNA in the cytoplasm (arrows); *c*, control RT reaction without reverse transcriptase. (×200.)

that in noninfected cells. This was the result of a decrease in the B_{max} (10 fmol/mg of protein in infected cells vs. 36 fmol/mg of protein in noninfected cells, $n = 2$) rather than the K_d (0.43 nM infected vs. 0.57 nM noninfected cells, $n = 2$). Previous studies (2, 21) have shown that AII stimulates the level of c-fos mRNA and that this stimulation may be involved

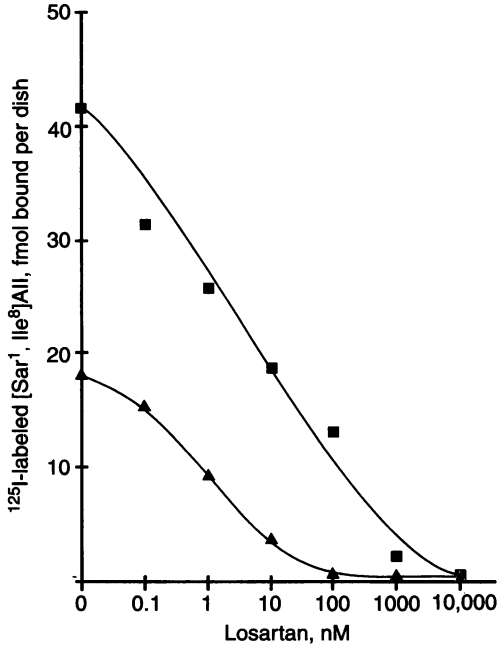

FIG. 4. Competition for ^{125}I-labeled [Sar^1, Ile^8]AII binding by losartan in LNSV-AT_{1B}-AS-infected and noninfected astroglial cultures. Astroglial cultures from WKY rat brain were infected with LNSV-AT_{1B}-AS and subjected to G418 selection. Competition inhibition of ^{125}I-labeled [Sar^1, Ile^8]AII by losartan in infected (▲) and noninfected (●) astroglial cultures was carried out to determine K_d and B_{max} values. The data are mean of triplicate determinations and are representative of two experiments (17, 19).

in AII-mediated activities in brain cells. Thus, similar experimental conditions were used to determine the effect of 100 nM AII on c-fos mRNA and to determine whether the decrease in the numbers of AT_1-R was accompanied by a decrease in the AII responsiveness of LNSV-AT_{1B}-AS-infected cells. Fig. 5 shows that 100 nM AII caused a significant stimulation of the level of c-fos mRNA in noninfected astroglial cultures. In comparison, only a 15% increase in c-fos mRNA was observed in LNSV-AT_{1B}-AS-infected cells upon AII stimulation.

Effect of LNSV-AT_{1B}-AS on Neuronal Cultures. Since neuronal cultures from 1-day-old rat brain undergo a limited degree of cell multiplication, RT-*in situ* PCR was used to determine whether LNSV-AT_{1B}-AS can be used to infect these cultures and influence AII action similar to that observed for astroglial cultures. Fig. 6 shows that a significant number of neurons express AT_{1B}-AS in cultures infected with LNSV-AT_{1B}-AS for 48 h. No such transcript was detected in noninfected neuronal cultures. Incubation of noninfected neuronal cultures with 100 nM AII for 20 min at 37°C caused a significant increase in c-fos mRNA (Fig. 7). The stimulatory effect of AII was attenuated in neuronal cultures infected with LNSV-AT_{1B}-AS.

DISCUSSION

The present study demonstrates that AT_{1B}-AS can be delivered into astroglial and neuronal cells established in primary cultures from the rat brain. This is associated with a significant decrease in AT_1-R and AII stimulation of the level of c-fos mRNA, an early event associated with the cellular actions of AII in both astroglial and neuronal cells (2, 7, 23). The mechanism of this decrease in AT_1-R and AII action is speculative at present, although data suggest that integration of AT_{1B}-AS into the genome and its subsequent transcription may be involved. The decrease in AT_1-Rs and AII action in LNSV-AT_{1B}-AS-infected cells appears to be specific for the

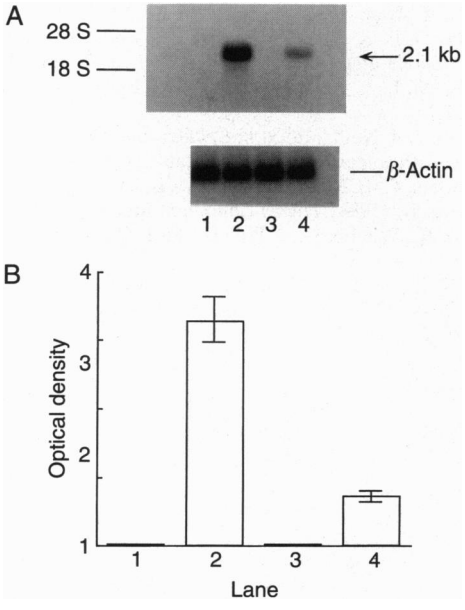

FIG. 5. AII stimulation of c-fos mRNA in LNSV-AT_{1B}-AS-infected and noninfected astroglial cultures. Noninfected (lanes 1 and 2) and infected (lanes 3 and 4) astroglial cultures were treated with 100 nM AII (lanes 2 and 4). Untreated cultures (lanes 1 and 3) were used as controls. Experimental conditions were as described (21). Total RNA was isolated and subjected to Northern blot hybridization analysis (21). (*A*) Representative Northern blot. (*B*) Quantitation of radioactive bands essentially as described (21). Data were normalized to β-actin mRNA and are the mean ± SEM (n = 2).

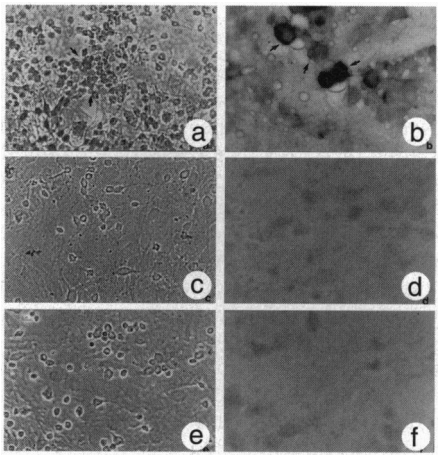

FIG. 6. Detection of AT_{1B}-AS in LNSV-AT_{1B}-AS-infected neuronal cultures by RT-*in situ* PCR. Neuronal cells (1×10^5 cells) were plated in poly(L-lysine)-coated 8-well slides. Cultures were grown for 4 days and then infected with LNSV-AT_{1B}-AS (*a–d*) for 48 h at 37°C. Noninfected cultures (*e* and *f*) were used as control. Cultures were fixed and subjected to RT-*in situ* PCR to detect AT_{1B}-AS. (*a* and *b*) Presence of AT_{1B}-AS (arrows). (*c* and *d*) Control RT reaction without reverse transcriptase. (*a* and *c–f*, ×80; *b*, ×200.)

AT_1-R system. (*i*) Infection of astroglial cells with a similar construct, where β-galactosidase cDNA was substituted for AT_{1B}-AS, followed by selection, failed to show any decrease in AT_1-R. This also suggests that the decrease in AT_1-R could not be a result of G418 selection of astroglial cells after LNSV infection. (*ii*) LNSV-AT_{1B}-AS-infected cells continued to express astroglial characteristics as shown by their ability to be stained with the glial fibrillary acidic protein antibody. In addition, forskolin treatment of infected cultures results in a stellation pattern similar to that seen in noninfected cells. This stellation, induced by the increase in intracellular cAMP, is a property associated with differentiated astroglial cells (16, 24). (*iii*) LNSV-AT_{1B}-AS infection of astroglia results in attenuation of the AII stimulation of the level of c-fos mRNA. This effect is specific for AII since norepinephrine stimulation of c-fos mRNA is not affected in LNSV-AT_{1B}-AS-infected astroglial cells. (*iv*) The mRNA for AT_2 receptors is not altered

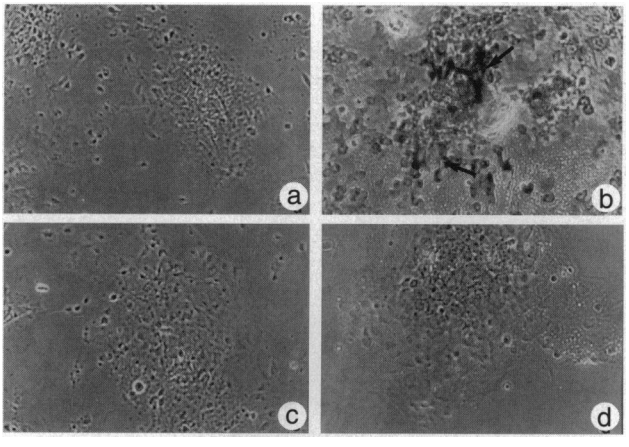

FIG. 7. AII stimulation of the levels of c-fos mRNA in noninfected and LNSV-AT_{1B}-AS-infected neuronal cultures. Neuronal cultures were grown and infected with LNSV-AT_{1B}-AS (*c* and *d*) essentially as described in Fig. 6. Noninfected cultures (*a* and *b*) were used in parallel as control. Cultures were incubated without (*a* and *c*) or with (*b* and *d*) 100 nM AII for 20 min. RT-*in situ* PCR used the following c-*fos* primers: sense, 5′-AGG AGG GAG CTG ACA GAT A-3′; antisense, 5′-CCT GGC TCA CAT GCT ACT A. (×80.)

in LNSV-AT_1R-AS-infected neurons compared with uninfected neurons.

In the present study, we used an AT_{1B}-AS to determine the effect of AT_1-R on AII-mediated cellular action. We believed that an antisense cDNA to either AT_{1A}- or AT_{1B}-R would inhibit translation of AT_1-R as a result of the high degree of homology between these receptor subtypes. Experiments presented in this study confirm this conclusion. The antisense cDNA for AT_{1B}-R subtype alone causes a significantly larger decrease in the AT_1-R and AII response than could be accounted for by inhibition of only the AT_{1B}-Rs. Thus, it is tempting to suggest that, although they are products of two distinct genes (25), the expression of AT_{1B}-AS in neuronal and astroglial cultures influences both AT_{1A}-R and AT_{1B}-R subtypes. This would be consistent with the reported 95–98% homology in the amino acid and nucleotide sequences between the two receptor subtypes (25–27).

Finally, these observations are potentially significant since they provide a means to manipulate cellular and physiological actions of AII at the genetic level. In fact, a similar approach that influences AII-mediated actions has been reported (28, 29). Gyurko *et al.* (28) have shown that direct injections of an antisense oligonucleotide to AT_1-R into the brain decreases BP in the SH rat, and Sakai *et al.* (29) have shown attenuation of brain AII function with a similar approach. Although both approaches appear to be effective, the virus-mediated AT_1-R gene transfer, as reported in our study, has the added advantage that it may be used to control AII function on a long-term basis. Thus, it is reasonable to conclude that the virus-mediated transfer of AT_1-AS into physiologically relevant AII-sensitive organs could provide an alternative approach to the long-term control of an AII-dependent hypertensive state.

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