Original Article

No Involvement of the Nerve Growth Factor Gene Locus in Hypertension in Spontaneously Hypertensive Rats

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Sympathetic hyper-innervation and increased levels of nerve growth factor (NGF), an essential neurotrophic factor for sympathetic neurons, have been observed in the vascular tissues of spontaneously hypertensive rats (SHRs). Such observations have suggested that the pathogenesis of hypertension might involve a qualitative or quantitative abnormality in the NGF protein, resulting from a significant mutation in the gene's promoter or coding region. In the present study, we analyzed the nucleotide sequences of the *cis*-element of the NGF gene in SHRs, stroke-prone SHRs (SHRSPs), and normotensive Wistar-Kyoto (WKY) rats. The present analyses revealed some differences in the 3-kb promoter region, coding exon, and 3' untranslated region (3'UTR) for the NGF gene among those strains. However, the observed differences did not lead to changes in promoter activity or to amino acid substitution; nor did they represent a link between the 3'UTR mutation of SHRSPs and elevated blood pressure in an F2 generation produced by crossbreeding SHRSPs with WKY rats. These results suggest that the NGF gene locus is not involved in hypertension in SHR/SHRSP strains. The present study also revealed two differences between SHRs and WKY rats, as found in cultured vascular smooth muscle cells and in mRNA prepared from each strain. First, SHRs had higher expression levels of *c-fos* and *c-jun* genes, which encode the component of the AP-1 transcription factor that activates NGF gene transcription. Second, NGF mRNAs prepared from SHRs had a longer 3'UTR than those prepared from WKY rats. Although it remains to be determined whether these events play a role in the hypertension of SHR/SHRSP strains, the present results emphasize the importance of actively searching for aberrant *trans*-acting factor(s) leading to the enhanced expression of the NGF gene and NGF protein in SHR/SHRSP strains. (*Hypertens Res* 2005; 28: 155–163)

Key Words: spontaneously hypertensive rats, nerve growth factor, rat chromosome 2, linkage analysis, AP-1

Introduction

Spontaneously hypertensive rats (SHRs) and their substrain,

stroke-prone SHRs (SHRSPs), exhibit sympathetic hyper-innervation of resistant vascular tissues and hypertrophy of the sympathetic ganglia, unlike their normotensive control, Wistar-Kyoto (WKY) rats (1–5). It is thought that this

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Fig. 1. A: A schematic representation of the NGF gene and mRNAs. B: A restriction enzyme map of the 5'UTR of the NGF gene. C: Structure of the region around exons 3B and 4 of the NGF gene. The exons (Ex) are indicated by boxes; specifically, prepro and mature protein-coding regions are indicated by hatched and filled boxes, respectively. The sideways arrows indicate the positions of the primers for PCR. The downward-pointing arrows in B and C indicate the positions at which differences were found in terms of nucleotide sequences among tested rat strains. The letters in parentheses correspond to the alphabetized panels in Fig. 2.

increased sympathetic function contributes to the pathogenesis of hypertension in SHR-derived strains.

Nerve growth factor (NGF) has more potent neurotrophic effects on sympathetic neurons than do other neurotrophins such as brain-derived neurotrophic factor and neurotrophin-3 (6). Interestingly, neonatal sympathectomy with anti-serum against NGF has been shown to prevent the development of hypertension in SHRs (7). Brock *et al.* further demonstrated that sympathectomy in SHRs with established hypertension reduced innervation by sympathetic nerves and led to a decrease in resting blood pressure (8). Additionally, examination of NGF levels in the vasculature of developing rats revealed that NGF protein and NGF gene expression were higher in SHR than in WKY tissues (9–11). These findings led to the hypothesis that increased NGF expression results in abnormal sympathetic neuronal function and morphology, leading to elevated blood pressure in SHR-derived strains. Kapuscinski *et al.* reported that the NGF gene locus was linked to blood pressure in genetic crosses of SHRs and nor-

motensive Donryu rats based on a restriction fragment length polymorphism (RFLP) analysis of the NGF gene between these strains (12). Furthermore, Charchar *et al.* reported that increased NGF gene expression also depended on the number of NGF alleles of the SHR genotype (13). These findings led us to search for mutations within the *cis*-element, especially in the promoter region of the NGF gene in SHRs and SHR-SPs.

In the present study, we looked for possible mutations in the *cis*-element of the NGF gene that might contribute to abnormal NGF function, including its increased expression in hypertensive rats.

Methods

Animals

WKY/Izm, SHR/Izm, and SHRSP/Izm strains were supplied by the Disease Model Cooperative Research Association,

Japan. Wistar and Sprague-Dawley (SD) rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan). WKY/NIH and SHRSP/NIH strains were supplied by the National Institutes of Health (NIH), Bethesda, USA.

Cloning of 5' and 3' Untranslated Regions (UTRs) and Coding Exon of the NGF Gene

Prior to this study, the only nucleotide sequence determined in the rat NGF gene was an approximately 650-bp-long 5' upstream region of exon 1B, as shown in Fig. 1A (14). Therefore, we first determined the nucleotide sequence of an approximately 4.3-kb-long *Sac*I fragment (Fig. 1B) that was cloned from an SD rat genomic library (Clontech, Palo Alto, USA). Based on this nucleotide sequence, the following primers for the polymerase chain reaction (PCR) of genomic DNA were synthesized in order to determine the nucleotide sequences of the 5' region of the NGF gene in the rat strains tested here: NGFP2 (5'-TTGCATGCCAGAGCTGAGATGAAGTAGTCCTT-3') and NGFPR (5'-TTGTCGACTGACTTTGGGACTCCTGGTCATCCT-3'). The underlined sequences were artificially added to create the *Sph*I (NGFP2) or *Sal*I (NGFPR) sites. The fragments of genomic DNA amplified by PCR were cloned into pGEM-3Zf(+). The genomic DNAs prepared from 3–5 rats per strain and multiple clones per strain were used to determine the nucleotide sequences.

To amplify the 3'UTR of the NGF gene by PCR of genomic DNA, NGF3'-F (5'-GCGTTGACAACAGACGACAA-3') and NGF3'-R (5'-TGCTGAGCAGTGTCTGAAAC-3') were used as primers, as shown in Fig. 1C. The nucleotide sequence of the 3'UTR was determined by the direct sequencing analysis method using the NGF3'-R primer. Genomic DNAs prepared from 3–5 rats per strain were used in this analysis.

The region encoding the prepro-NGF protein of the tested rat strains was cloned by reverse transcription–polymerase chain reaction (RT-PCR), as described previously (15). The sources of the first-strand cDNAs were the total hippocampal RNAs. The primers were 5'-AAGGATCCTGGACCCAAGCTCACCTCA-3' (forward; NGF-F) and 5'-GAGTCGACTTACAGGCTGAGGTAGGGA-3' (reverse; NGF-R), as shown in Fig. 1C. The underlined sequences were artificially added to create the *Bam*HI (forward primer) or *Sal*I (reverse primer) site. The fragments amplified by RT-PCR were cloned into pGEM-3Zf(+). Total RNAs were prepared from 3–5 rats per strain, and multiple clones per strain were used to determine the nucleotide sequences.

Assay of CAT Activity

To construct plasmids containing the NGF promoter-CAT reporter gene, two fragments (2-R and 3-R) were generated from the genomic DNAs of the SHRSP/Izm and WKY/Izm strains by PCR using the primer set NGFP2 and NGFPR or

the set NGFP3 (5'-TTGCATGCAGACACAGCTCCATCTTCAACATC-3'; the underlined sequence was artificially added to create the *Sph*I site) and NGFPR, as shown in Fig. 1B. These fragments were inserted between the *Sph*I and *Sal*I sites located at the 5' side of the CAT gene in the pCAT-Basic vector (Promega, Madison, USA). COS-1 cells, which are a simian fibroblast cell line, were seeded at a density of 3×10^5 cells per 60-mm dish and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in 5% CO₂. After 24 h, the cells were transfected with 1 µg of the plasmid containing the NGF promoter-CAT reporter gene, and with *c-fos* and *c-jun* expression vector DNAs, which we constructed by insertion of human *c-fos* and *c-jun* genes, respectively, into the 3' side of Rous sarcoma virus LTR, by using Lipofectin[®], reagent (Gibco BRL, Gaithersburg, USA). After 60 h, the transfected cells were harvested and the extracts were prepared using a Reporter Lysis Buffer (Promega). The CAT activity in 25 µg protein of the cell extract was measured by the CAT Enzyme Assay System (Promega) using 1-deoxy[dichloroacetyl-1-¹⁴C]chloramphenicol (25 kBq/ml) (Amersham Life Sciences, Arlington Heights, USA). Results obtained from the thin-layer chromatography (TLC) assay were analyzed using a MacBAS2000 imaging system (Fuji Film, Tokyo, Japan).

Linkage Analysis

We studied 108 F2 rats (male: 52; female: 56) generated from reciprocal crosses of the WKY/NIH and SHRSP/NIH strains, both of which were maintained at the University of Glasgow. Systolic blood pressure was determined by radiotelemetry in 16-week-old rats at rest in their home cages. These protocols have been described previously (16). The DNAs of these rats were used to determine their genotypes. The genotypes were determined by nucleotide sequence analysis based on direct sequencing methods following PCR, in which the primer sets NGF3'-F and NGF3'-R were used.

Northern Blot Analysis

Vascular smooth muscle cells (VSMCs) were isolated from the thoracic aortic media of 14-week-old SHR/Izm and WKY/Izm rats, as described previously (17). Cells were grown nearly to confluence in DMEM supplemented with 10% FCS, and then the cells were serum-starved in DMEM containing 0.5% FCS. After 48 h, the cells were treated with FCS at a final concentration of 20%. Northern blot analysis was performed using the total RNA prepared from the cells treated with serum. The hybridization was analyzed using a BAS2000 system (Fuji Film).

Determination of the Nucleotide Sequence of the 3'-Terminal Region in NGF mRNA

Total RNAs were prepared from SHR/Izm and WKY/Izm

	-2670	-2660	-2650	-2640	-2630 (at WKY/Izm)
WKY/Izm	GCCAGAACACCTGGAGGAAGGAGTGC	GGCAGAGGCAGGCCAGGAGGTCGG			
WKY/NIH	-----	-----	-----	G-----	
SHR/Izm	-----	-----	-----	A-----	
SHRSP/NIH	-----	-----	-----	A-----	
SHRSP/Izm	-----	-----	-----	A-----	
Wistar	-----	-----	-----	A-----	
SD	-----	-----	-----	A-----	

-1550 -1540 -1480 -1470 (at WKY/Izm)

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AGAGCATGTGCAAA (CAAAA)_x (CAA)_y (A)_z CCTCACTTTTTTCTT

 CAAAA repeat CAA repeat A cluster

	x	y	z
WKY/Izm	5	6	10
WKY/NIH	5	7	8
SHR/Izm	6	4	17
SHRSP/NIH	6	4	17
SHRSP/Izm	6	4	17
Wistar	6	4	18
SD	6	4	19

	177th					182th					
	Thr	Lys	Cys	Arg	Ala	Pro	Asn	Pro	Val	Glu	Ser
	ACC	AAG	TGC	CGA	GCC	CCA	AAT	CCT	GTA	GAG	AGT
WKY/Izm	---	---	---	---	---	--A	---	---	---	---	---
WKY/NIH	---	---	---	---	---	--A	---	---	---	---	---
SHR/Izm	---	---	---	---	---	--G	---	---	---	---	---
SHRSP/NIH	---	---	---	---	---	--G	---	---	---	---	---
SHRSP/Izm	---	---	---	---	---	--G	---	---	---	---	---
Wistar	---	---	---	---	---	--G	---	---	---	---	---
SD	---	---	---	---	---	--G	---	---	---	---	---

poly A signal		
WKY/Izm	<u>ATTAAA</u> TTTCAAAGCATCCTGTGTACCCACACTTGAGTC	A TTTCTCTCAATAAGAG
WKY/NIH	-----	A -----
SHR/Izm	-----	C -----
SHRSP/NIH	-----	C -----
SHRSP/Izm	-----	C -----
Wistar	-----	C -----
SD	-----	C -----

Fig. 2. Differences between nucleotide sequences among the tested rat strains. The alphabetized panels correspond with the positions indicated by the downward-pointing arrows shown in Fig. 1B and C.

VSMCs 8 h after stimulation with serum. The RNA was then converted into cDNA with Oligo(dT)₁₈-*NotI* as a primer. Then, the 3'UTR of NGF mRNA was amplified by PCR using the NGF3'-F and Oligo(dT)₁₈-*NotI* as primers. The amplified fragments were digested by restriction enzymes, *HincII* and *NotI*, and then were subcloned into the BlueScriptII plasmid vector. Finally, the nucleotide sequences of those fragments were determined in multiple clones.

Results

Nucleotide Sequence of the Promoter Region of the NGF Gene in SHR, SHRSP, and Normotensive Strains

The NGF gene is composed of five exons, the last of which encodes prepro-NGF (Fig. 1A) (18). Although four alternative transcripts are already known, three of them include exon 1A or 1B (18). Therefore, it has been postulated that a major functional promoter exists in the vicinity of exons 1A and 1B. The nucleotide sequence of an NGFP2-NGFPR fragment of

Fig. 3. Comparison of the promoter activities of the NGF genes derived from SHR/SHRSP and WKY rats. The promoter activities were estimated using extracts prepared from normal COS-1 cells (lane 1), transfectants with the WKY-derived 2-R fragment-CAT reporter gene (lane 2), transfectants with the SHR/SHRSP-derived 2-R fragment-CAT reporter gene (lane 3), transfectants with the WKY-derived 3-R fragment-CAT reporter gene (lane 4), and transfectants with the SHRSP-derived 3-R fragment-CAT reporter gene (lane 5). Cm, chloramphenicol; bCm, butyrylated chloramphenicol.

approximately 3.1 kb (shown in Fig. 1B) was determined in the following strains: SHR/Izm, SHRSP/Izm, SHRSP/NIH, WKY/Izm, WKY/NIH, Wistar, and SD rats. The results revealed two differences in the upstream region of exon 1, as shown in Fig. 2A and B. The first difference was a point mutation found approximately 2.7 kb upstream from exon 1. This sequence was a G in the WKY/Izm and WKY/NIH strains, and it was an A in the SHR/Izm, SHRSP/Izm, SHRSP/NIH, Wistar, and SD strains. The other difference was a (CAAAA)_x(CAA)_y(A)_z sequence located approximately 1.5 kb upstream from exon 1. The *x* and *y* values in the SHR-derived strains were identical with those in the normotensive strains, Wistar rats and SD rats. The *z* value was identical among the SHR and SHRSP strains, whereas the *z* values in the normotensive strains differed from each other and also from that in the SHR and SHRSP strains. The *y* and *z* values in the WKY/Izm strains differed from those in the WKY/NIH strains. Therefore, the observed differences did not appear to depend on either the hypertensive or the normotensive trait.

Nucleotide Sequences of Exon 4 and the 3'UTR of the NGF Gene in SHR, SHRSP, and Normotensive Strains

We also analyzed the nucleotide sequence of exon 4, which encodes the prepro-NGF, and the 3'UTR sequence approximately 200 bp downstream from the putative poly A signal (ATTAAA) for NGF mRNA (Fig. 1C). In the coding region for the mature NGF protein, a point mutation was found with

Fig. 4. Relationship between genotype of the NGF gene and blood pressure in the F2 generation of SHRSP/NIH $\times$ WKY/NIH rats. The genotypes of the F2 rats were determined by nucleotide sequence analysis of a position approximately 30 bp downstream from the putative poly A signal, as shown in Fig. 2D.

respect to the third letter of the 182nd codon (proline) of prepro-NGF, as shown in Fig. 2C. However, no amino acid substitution resulted from this mutation. In the 3'UTR, there was a point mutation approximately 30 bp downstream from the putative poly A signal: A in the case of the WKY/Izm and WKY/NIH strains, and C in the case of the SHR/Izm, SHRSP/Izm, SHRSP/NIH, Wistar, and SD strains, as shown in Fig. 2D. Therefore, these differences did not appear to depend on either the hypertensive or the normotensive trait.

Comparison between the Promoter Activities of the SHR/SHRSP and WKY NGF Genes

The promoter activity of the SHR/SHRSP NGF gene was compared with that of the WKY gene. Two constructs containing two different fragments (2-R and 3-R) (Fig. 1B) were used for this series. Although these constructs were at first introduced solely into COS-1 cells, their CAT activity remained at a very low level (data not shown). The AP-1 transcription factor binding site (TRE; TPA response element), located in the first intron of the NGF gene (Fig. 1B), has been reported to be essential for the transcriptional control of this gene (19). Therefore, we measured the CAT activity in these cells after co-transfection with the expression vectors for the *c-fos* and *c-jun* genes, both of which encode components of AP-1 transcription factor (20). The results demonstrated that

Fig. 5. Expression of the NGF, *c-fos*, and *c-jun* genes in cultured aortic VSMCs prepared from SHRs or WKY rats. Total RNA from the VSMCs were prepared as described in Methods. The expression levels of these genes were determined at the onset of serum starvation (–48 h), 48 h after serum starvation (0 h), and at 0.5, 1, 2, 4, 8, 12, and 24 h after serum treatment, by Northern hybridization using ^{32}P -labeled probes specific to these genes and the β -actin gene, which served as a standard control. Similar results were obtained from three independent experiments.

the CAT activity associated with the two constructs did not differ significantly between the SHR/SHRSP and WKY genes (Fig. 3). We concluded that the differences between nucleotide sequences found in the promoter regions of the SHR/SHRSP and WKY NGF genes could not account for the abnormal regulation of NGF gene expression in these SHR/SHRSP strains.

Linkage Analysis of the NGF Gene Locus and Blood Pressure

The genotypes of the NGF genes in F2-generation rats produced by crossing SHRSP/NIH $\times$ WKY/NIH rats were determined by analyzing the mutation in the 3'UTR. Figure 4 shows the relationship between the genotype of the NGF gene and blood pressure. The average blood pressures of the WKY, SHRSP/WKY, and SHRSP genotype F2 rats were 173.8 ± 25.2 , 168.7 ± 4.86 , and 175.7 ± 13.79 mmHg, respectively. There was no significant correlation between the genotype of the NGF gene and blood pressure.

Expression of the NGF, *c-fos*, and *c-jun* Genes in SHR and WKY VSMCs

The abovementioned results suggested that there is no associ-

ation between hypertension in SHR/SHRSP strains and the *cis*-element of the NGF gene. Therefore, the enhancement of the expression of the NGF gene in SHR/SHRSP strains might have resulted from an aberration in the AP-1 transcription factor. In the present study, we compared the expression of the NGF, *c-fos*, and *c-jun* genes in cultured VSMCs prepared from the SHR/Izm strain with those in VSMCs from WKY/Izm rats after stimulation with serum. The results are shown in Fig. 5. NGF gene expression in the SHR cells was lower than that in the WKY cells until 0.5 h. However, in SHR cells, the level of NGF gene expression was approximately twice that in WKY cells at 2–4 h, when expression peaked in both strains. After the peak values were observed, the reduction of NGF gene expression was more rapid in SHR cells than in WKY cells. The expression of the *c-fos* and *c-jun* genes in both types of VSMCs was super-induced from 0.5 to 1 h after treatment with serum, as previously reported in studies using a number of different cultured cell types (21). As regards the expression of the *c-fos* gene, at 0.5 h, the observed levels were approximately 2.4 times higher in SHR cells than in WKY cells. The level of expression of the *c-jun* gene at 0.5 h was approximately 1.4 times higher in SHR cells than in WKY cells. These results indicated that the abnormal regulation of *c-fos* and *c-jun* gene expression is most likely due to an enhancement of NGF gene expression in the case of the SHR/SHRSP strains.

Nucleotide Sequences of the NGF mRNA 3'-Terminal Region in SHR and WKY VSMCs

The nucleotide sequences of the NGF mRNA 3'UTRs were determined using the RNAs prepared from the VSMCs of the SHR/Izm and WKY/Izm strains. The sequences adjacent to the poly A tail were shown to have particular patterns, and the proportion of long-mRNA species tended to be higher in the SHRs than in the WKY rats, as shown in Fig. 6; e.g., 6 and 11 clones in SHRs and WKY rats, respectively, had an asterisked sequence of mRNA, whereas 9 and 5 clones in SHRs and WKY rats, respectively, had a double-asterisked mRNA longer than the asterisked mRNA.

Discussion

The main aim of the present study was to search for possible mutations in the *cis*-element of the NGF gene that might contribute to disturbances of its expression or function, as found in SHR/SHRSP strains (9–11). Here, we analyzed the nucleotide sequences of an approximately 3-kb region upstream from exon 1, the coding exon, and the 3'UTR for the NGF gene in SHR, SHRSP, and normotensive strains. Four differences, all between the WKY and SHR/SHRSP strains, were found in these regions. However, with respect to three of these differences (the exception being $(\text{CAAAA})_n(\text{CAA})_m(\text{A})_z$, located in the 5' upstream region), the nucleotide sequences of the SHR and SHRSP strains were identical with those of

Genome	5' -ATTATTTATTAATTTTCAAAGCATCCTGTGTACCCACACTTGAGT-3'		
		▼▼▼▼▼▼▼	
mRNA	*ATTATTTATTAATTTTCAAAGCA.....	WKY	SHR
	ATTATTTATTAATTTTCAAAGCAT.....	11	6
	ATTATTTATTAATTTTCAAAGCATC.....	1	1
	ATTATTTATTAATTTTCAAAGCATC.....	1	0
	ATTATTTATTAATTTTCAAAGCATCCTG.....	0	1
	**ATTATTTATTAATTTTCAAAGCATCCTGTGTA.....	5	9
	ATTATTTATTAATTTTCAAAGCATCCTGTGTACCCA.....	1	1
	ATTATTTATTAATTTTCAAAGCATCCTGTGTACCCACA.....	1	2

Fig. 6. Nucleotide sequence of NGF mRNA 3'UTR. The 3'UTRs of NGF mRNA in SHR/Izm and WKY/Izm VSMCs were cloned, and these nucleotide sequences were analyzed as described in Methods. Seven patterns were found in the region adjacent to the poly A tail (represented by dots) from the sequence analyses of 20 clones in each strain. The top line (Genome) is a nucleotide sequence near the putative poly A signal (underlined sequence) in the genomic DNA of the NGF gene. The numerals in the column to the right of the seven mRNA sequences represent the number of clones derived from WKY or SHR NGF mRNA with the corresponding sequence.

the normotensive strains, Wistar rats, and SD rats (Fig. 2). As regards (CAAA)_n(CAA)_n(A)_n, the number of repeats were identical among the SHR and SHRSP strains, whereas the number of repeats in the normotensive strains differed from each other and also from the hypertensive strains (Fig. 2B). Therefore, it is highly likely that these differences are polymorphisms among strains, and are not genetically responsible for the hypertension observed in SHR/SHRSP strains.

To further evaluate the abovementioned hypothesis, we measured the promoter activity of the NGF gene and estimated the association between the NGF gene locus and the elevation in blood pressure. We showed that there was no difference in promoter activity between the SHR/SHRSP and WKY genes, at least in the presence of AP-1 (Fig. 3). One report has suggested that the NGF gene had a second promoter proximal to exon 3 (22). Therefore, in future studies, it might be necessary to analyze the *cis*-element of the NGF gene in a region other than that studied here. However, our analysis of the link between blood pressure and the NGF gene locus showed no significant association between increased blood pressure and the NGF allele of the SHRSP genotype (Fig. 4). Moreover, no significant association with sex was revealed by our analysis (data not shown). Judging from a comprehensive view of the present analyses, we considered a significant aberration within the *cis*-element of the NGF gene to be unlikely.

The region around the NGF gene locus is known to be a quantitative trait locus (QTL) that exerts influence on blood pressure and is located on rat chromosome 2 (16, 23–25). The Glasgow strain used in our study has also been used to demonstrate the importance of this QTL (16). Therefore, our linkage analysis is expected to provide important information with respect to narrowing the field of candidate QTLs. However, in their analysis of gene-segregating rats from a cross of SHR with normotensive Donryu rats, Harrap and co-workers suggested that the NGF gene locus of SHRs is linked to high blood pressure and increased NGF gene expression (12, 13);

thus, their results are in conflict with ours. At this point in time, the reason for this contradiction remains unclear. To reach a final conclusion regarding the absence of a linkage between the NGF gene locus and hypertension, it might be essential to analyze the gene in the SHR strain used by Harrap *et al.* and to evaluate gene expression in the Glasgow strain used in our own study.

It is highly possible that there are some aberrations in the transcriptional factor for NGF gene expression and/or in the mechanism of the posttranscriptional regulation of protein expression from this gene, provided that a lack of any aberration within the *cis*-element of the NGF gene does in fact lead to an enhancement of NGF protein expression. In the present study, we also took note of the expression patterns of the *c-fos* and *c-jun* genes, both of which encode components of the AP-1 transcription factor, because this factor is believed to be centrally involved in NGF gene expression (19). The induction of these genes by stimulation with serum in SHR VSMCs was found to be more enhanced than that in WKY VSMCs, as was also the case for the NGF gene (Fig. 5). Touyz *et al.* reported that the enhanced expression of the *c-fos* gene by angiotensin II is higher in SHR VSMCs than in WKY VSMCs and that the overexpression of this gene is due to an extracellular signal-regulated protein kinases-dependent pathway (26). Therefore, it will still be necessary to elucidate the mechanism for the transcriptional control of the *c-fos* and *c-jun* genes in SHR/SHRSP strains in consideration of these kinases. In the present experiment, we also found that the basal level of NGF mRNA in SHR cells was lower than that in WKY cells (–48 h to 1 h in Fig. 5), a finding that agreed with that reported by Sherer *et al.* (27). In addition, after 8 h, when NGF mRNA expression had passed its peak, the levels in SHR cells were still lower than those in WKY cells. These levels may also reveal an aberration of a *trans*-acting factor for NGF gene expression in SHR/SHRSP strains. We further determined the nucleotide sequences of the NGF mRNA 3'UTRs and found that the proportion of the long-mRNA spe-

cies tended to be higher in SHR than in WKY rats (Fig. 6). The mechanism for the production of immature NGF RNA just before the addition of the poly A tail in SHR might be different from that in WKY rats. The 3'UTR of mRNA is often involved in the stabilization of mRNA as well as in posttranscriptional regulation. The possibility of an increase in NGF mRNA stability and elevated translational efficiency has been reported in this context (27). As shown in the present study, the production of long NGF mRNA might account for these characteristics, although such production must be validated by further analysis of a larger number of clones.

The present study indicated that there is no linkage of the NGF gene locus to the hypertensive trait of SHR/SHRSP strains. If the enhanced expression of NGF mRNA and its protein is important in the pathogenesis of the hypertension of SHR/SHRSP strains, then there must be some type of abnormality in the transcriptional and/or posttranscriptional regulation of NGF expression, apart from the abnormality in the *cis*-element of the NGF gene in SHR/SHRSP strains. Enhanced *c-fos* and *c-jun* gene expressions by serum-stimulation were determined here; moreover, relatively high levels of long-mRNA species were observed in SHR VSMCs. Although it remains to be determined whether these events are associated with the hypertension observed in SHR/SHRSP strains, we suggest here that a locus regulating transcription and/or posttranscription might be a relatively promising site to consider for further searches for mutations that might lead to enhanced expression of NGF in rats exhibiting hypertension.

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