Differential action of AGCF2 upon cell type-dependent expression of human angiotensinogen gene

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Received 13 May 1997; revised version received 16 June 1997

Abstract To investigate the regulatory mechanisms of human angiotensinogen (ANG) gene expression in the brain, we analyzed the 1.3-kb promoter by transfection studies and gel shift assays. The region from −106 to +44 was sufficient for promoter activity in glioblastoma cells, and multiple nuclear factors including AGCF2 (human ANG core promoter binding factor 2) bound within this 150-bp region. The mutations within AGCF2-binding elements decreased the transcriptional activity in glioblastoma cells but rather increased it in hepatoma cells. These results indicate that AGCF2 has a differential function between these cells and contributes to the glia-dependent angiotensinogen promoter activity.

Key words: Angiotensinogen; Hypertension; Gene expression; Promoter; Transcription factor

1. Introduction

Angiotensinogen (ANG), the unique substrate of renin in the renin-angiotensin system (RAS), is the precursor of the octapeptide hormone, angiotensin II (AII), which plays an important role in the regulation of blood pressure and electrolyte homeostasis [1,2]. Until recently, AII was believed to be exclusively a circulating hormone, but since components of the RAS have been demonstrated in a variety of tissues, such as adrenal glands, kidney, heart, and also in the brain, the various tissue RAS is considered to play a role in local tissue regulation [3,4].

It is well established that the injection of AII into the brain causes significant physiological responses, including increases in blood pressure, drinking behavior, and vasopressin release [5–8]. In addition to these effects, several lines of evidence have suggested that AII exerts specific actions according to the developmental changes in relative expression of the receptor subtypes in the brain [9–11]. Recently, Ichiki et al. [12] and Hein et al. [13] reported that mice lacking AT_2 receptor have an impaired drinking response to water deprivation as well as a reduction in spontaneous movement. These results support the notion that the brain RAS plays an important role independently of circulating one.

ANG is one of the important regulators in the brain RAS, because there is a 28% greater expression of the ANG gene in the brain of the spontaneously hypertensive rat (SHR) as compared with the Wistar-Kyoto rat [14], and hypertension is significantly reduced by the intracerebroventricular injection with the synthetic antisense oligodeoxynucleotides of ANG mRNA to normotensive levels in SHR [15]. Furthermore, ANG mRNA levels were developmentally regulated in the rat brain, where its major production sites are glial cells [16–18], as a transient peak of its expression is the embryonic day 17 [19]. However, little is known about the regulatory mechanism of the human ANG promoter activity in the brain because of the limited availability of human materials. In the present study, we identified the potential DNA elements of the human ANG promoter functioning in a human glioblastoma cell line (A172) which endogenously expresses ANG mRNA [20] by in vitro transfection analyses.

2. Materials and methods

2.1. Plasmid constructions

A promoter-less plasmid, SV0Ecat was constructed by inserting the SV40 enhancer to the unique *Bam*HI site of pUCSV0cat [21]. The human ANG promoter-chloramphenicol acetyltransferase (CAT) chimeric constructs were made as follows: 13cat, DM4cat, DM7cat, DM7.8cat, DM8cat, DM9cat, DM10cat, DM10.5cat, DM11cat, DM12cat contained 1266 bp (−1222 to +44), 791 bp (−747 to +44), 560 bp (−516 to +44), 388 bp (−344 to +44), 356 bp (−312 to +44), 287 bp (−243 to +44), 150 bp (−106 to +44), 118 bp (−74 to +44), 103 bp (−59 to +44), and 76 bp (−32 to +44) fragments, respectively, and these DNA fragments were subcloned into the *Bgl*II/*Hin*dIII sites of SV0Ecat. DM10cat was used as a template to construct internal deletion of ALE and mutations in 5′-AGCE2, AGCE1, and AGCE2 by oligonucleotides-directed mutagenesis [22]. After the internal deletion and the site-directed mutations were obtained and confirmed by sequencing, the altered 140-bp or 150-bp fragments were subcloned into the *Bgl*II/*Hin*dIII sites of SV0Ecat for A172 or SV0cat for HepG2 cells.

2.2. Cell culture and transient expression assays

A172 and HepG2 cell lines were maintained as described previously [20]. The cells were plated at a density of 1×10^6 cells/60-mm dish and transfected 24 h later by calcium phosphate co-precipitation [23] using 6 μg plasmid DNA. After 48 h of culture, cell extracts were prepared and protein concentration was determined by the Bio-Rad protein assay kit. Extracts containing equal amounts of 40 μg of protein were used in CAT assays. The extent of conversion of chloramphenicol to its acetylated form was measured with a Bio-imaging analyzer (Model BAS2000; Fujix, Tokyo, Japan). All experiments were independently performed at least six times for each construct.

2.3. Preparation of nuclear extracts

Nuclear extracts from A172 cells were prepared using the protocol of Dignam et al. [24]. The final protein concentration was about 5 μg/μl.

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Abbreviations: bp, base pair; CAT, chloramphenicol acetyltransferase; ANG, angiotensinogen; RAS, renin-angiotensin system; AGCF1, human ANG core promoter binding factor 1; AGCE1, human ANG core promoter element 1; EMSA, electrophoretic mobility shift assay; C/EBP, CCAAT enhancer-binding protein; CREB, cyclic adenosine monophosphate response element-binding protein; ATF, activating transcription factor; ALE, ATF-like element

PII S0014-5793(97)00802-8

2.4. Electrophoretic mobility shift assays (EMSA)
EMSAs were performed as described previously [25].

3. Results

3.1. Identification of the minimal region sufficient for promoter activity in human ANG-producing glioblastoma cells

To identify potential elements that contribute to the human ANG gene expression in the brain, we analyzed its promoter coupled to the CAT gene in transiently transfected A172 cells. This was considered to be suitable model for our purpose, since it was a human glioblastoma cell line which endogenously expressed the ANG mRNA [20]. The 1.3-kbp promoter region functioned in A172 cells (Fig. 1). Deletion of the sequences from −1222 bp to −107 bp did not essentially affect the promoter activity; however, there was a drastic reduction in CAT activity when the upstream sequence was eliminated further up to −74 bp. These results suggested that the proximal promoter region from −106 bp to +44 bp was sufficient for the human ANG promoter activity in A172 cells.

3.2. The human ANG promoter contains multiple binding sites for sequence-specific nuclear factors

To investigate the binding activities of nuclear factors to the human ANG promoter, EMSA was performed using the dissected DNA fragments that cover with the proximal promoter region, as the probes. First, we analyzed the core promoter region surrounding the transcription start point (Fig. 2). Incubation of AGCE1 (human ANG core promoter element 1) fragment with the nuclear extracts prepared from A172 cells produced the previously reported complex, AGCF1 (AGCE1-binding factor 1), which was ubiquitously expressed [25] (data not shown). Incubation of B fragment with A172 nuclear extracts produced retarded complexes which represented a sequence-specific interaction between each fragment and nuclear factors, since the formation of these complexes was specifically reduced with molar excess of unlabeled competitors (Fig. 2B, lanes 1 to 4). On the contrary, incubation of C fragment with the nuclear extracts produced no specific complex (data not shown). To determine the subregion responsible for nuclear factor binding within B fragment, EMSA was conducted using A172 nuclear extracts with the end-labeled B fragment, 200-fold molar excess of unlabeled D, E, and AGCE2 fragments. Only AGCE2 fragment could dissociate the specific complexes (Fig. 2B, lanes 5 to 9). Moreover, double-stranded oligonucleotides containing the consensus binding sequences for C/EBP, ATF, Spl, AP-1, AP-2, AP-3, and NF-I/CTF

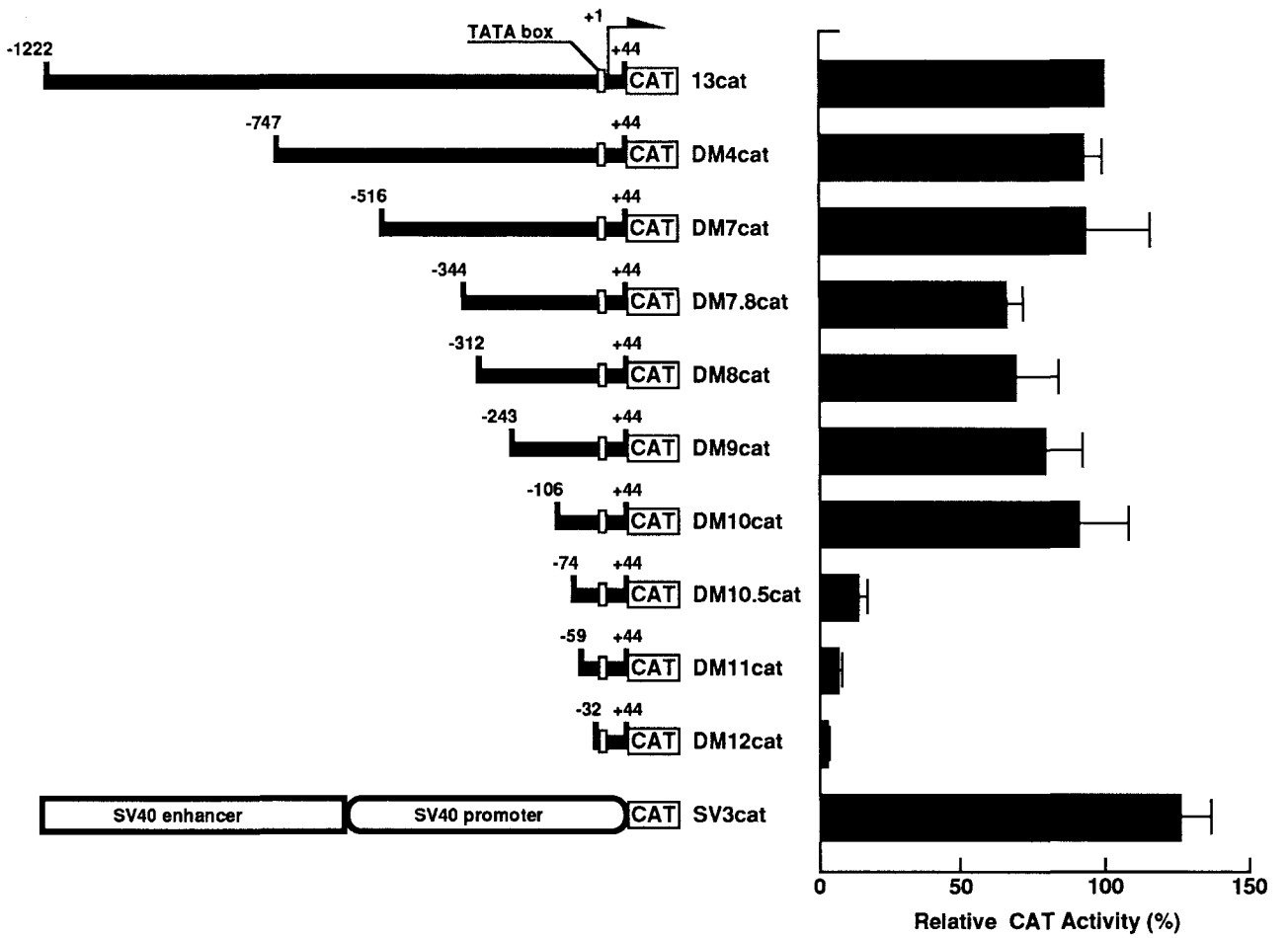

Fig. 1. 5′-Deletion analysis of the human angiotensinogen promoter. Left: 5′-deletion constructs of the human ANG promoter-CAT hybrid genes. Thick horizontal lines and open boxes marked by CAT represent the promoter sequences and CAT vector sequences, respectively. Names of the constructs used are listed to the right. Right: A172 cells were transfected with 6 μg of the indicated CAT vector. After 48 h of culture, extracts containing equal amounts of 40 μg of protein were used in CAT assays. The CAT activities are presented relative to that of the 13cat construct (hatched columns). Thin lines represent the S.E.; $n = 8$ to 10.

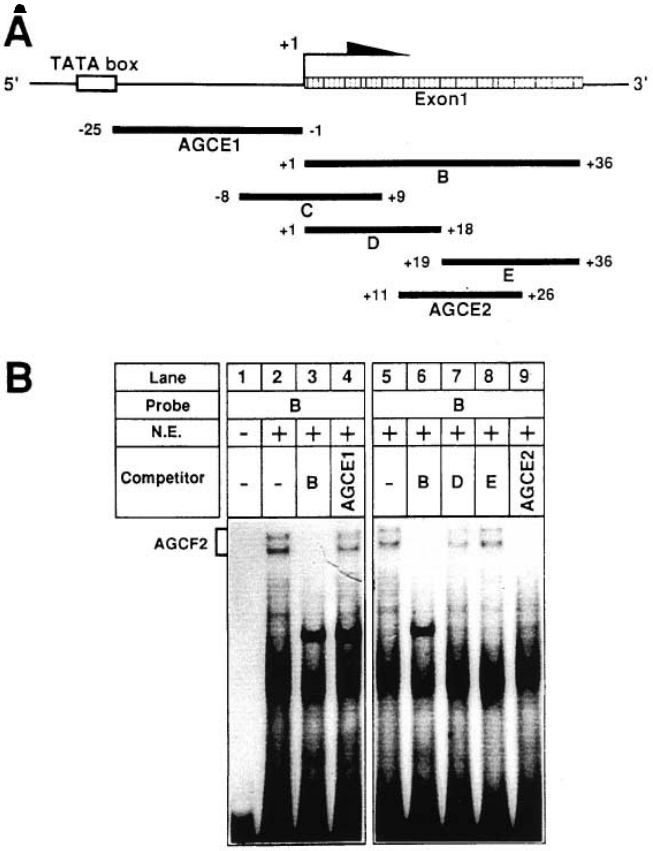

Fig. 2. Identification of specific binding nuclear factors using different portions of the core promoter sequences. (A) On the top, near the core promoter region of human ANG gene is represented. Below, the oligonucleotides used to detect specific interactions between the core promoter sequences and nuclear factors are indicated by thick horizontal lines. (B) EMSA. The indicated double-stranded oligonucleotides were end-labeled with T4 polynucleotide kinase using [γ-^{32}P]ATP. Five μg of A172 nuclear extract (N.E.) were incubated with 0.5 ng of ^{32}P-labeled probe. In a competition assay, 200-fold molar excess of the unlabeled oligonucleotides, as indicated for each lane, were added to the reaction mixture. Binding reactions were resolved by 4.5% acrylamide, 1×TBE electrophoresis. A specific nuclear factor, AGCF2, is indicated. The others are non-specific nuclear factors.

failed to compete with AGCF-2-binding activities (data not shown).

We next performed competition assays to examine the effects of the mutation in AGCE2 on the nuclear factor binding activities (Fig. 3). The DNA-protein complexes formed by AGCF2 binding to AGCE2 could be inhibited by molar excess of non-labeled AGCE2, but AGCE2m only partially replaced this binding (Fig. 3B, lanes 1 to 4). Furthermore, we analyzed the upstream region which contained ATF-like element (ALE) and AGCE2-like sequences (Fig. 4A). Incubation of ALE fragment with the nuclear extracts produced several retarded complexes including CREB/ATF family and novel ones (manuscript in preparation). Incubation of CT+P fragment produced no specific complex (data not shown). Finally, incubation of 5′-AGCE2 fragment with the nuclear extracts produced low mobility complexes as observed in AGCE2 fragment, and these complexes had the same sequence specificity as AGCF2 (Fig. 4B). These results suggest that AGCF2 binds to both 5′-AGCE2 and AGCE2.

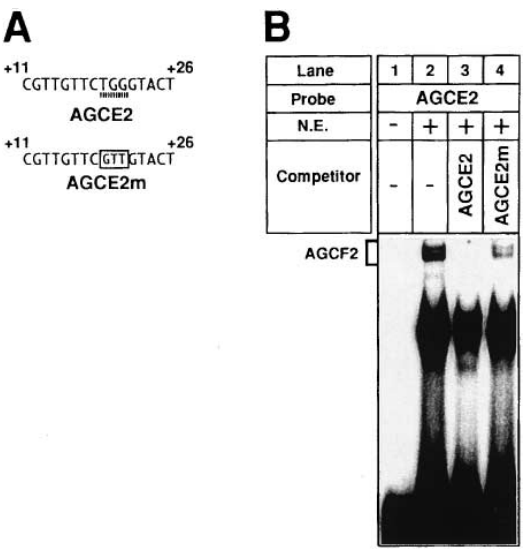

Fig. 3. Effect of AGCE2 mutation on the binding activities. (A) Oligonucleotides comprising wild type (AGCE2) and mutant (AGCE2m) are shown. The positions of base substitutions are underlined. The mutations are indicated by boxed nucleotides. Double-stranded versions of the indicated sequences were used in competition experiments. (B) EMSA was performed as described in the legend to Fig. 2B. In a competition assay, 200-fold molar excess of unlabeled oligonucleotides, as indicated for each lane, were added to the reaction mixture. Binding reactions were resolved by 4.5% acrylamide, 1×TBE electrophoresis. A specific nuclear factor, AGCF2, is indicated. The others are non-specific nuclear factors. N.E., nuclear extract.

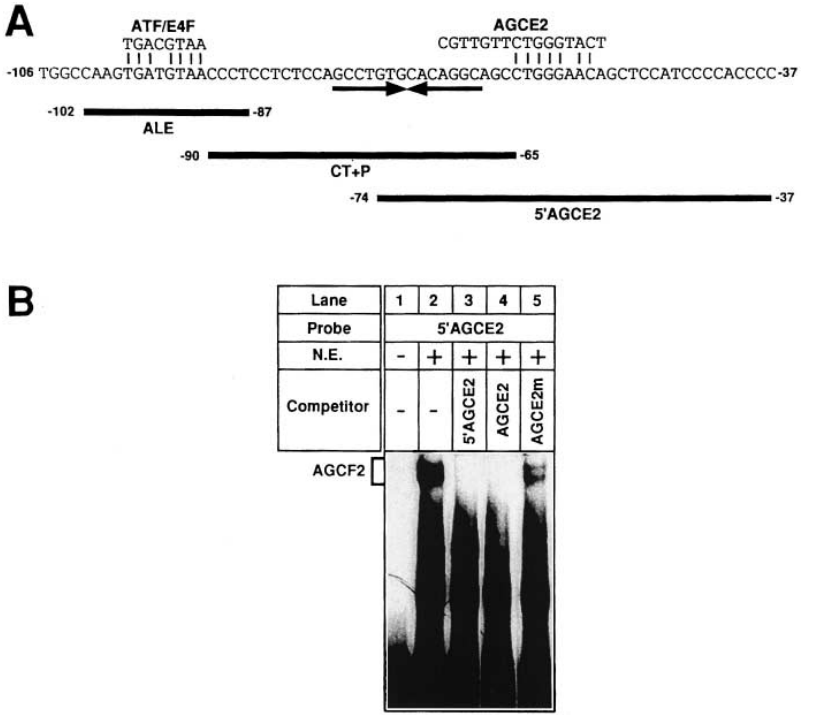

Fig. 4. Identification of specific binding nuclear factors using different portions of the upstream promoter sequences. (A) On the top, the upstream promoter sequences of hAG gene, ATF-like motif and AGCE2-like motif are represented. Below, the oligonucleotides used to detect specific interactions between the upstream promoter sequences and nuclear factors are indicated by thick horizontal lines. (B) EMSA was performed as described in the legend to Fig. 2B. In a competition assay, 200-fold molar excess of unlabeled oligonucleotides, as indicated for each lane, were added to the reaction mixture. Binding reactions were resolved by 4.5% acrylamide, 1×TBE electrophoresis. A specific nuclear factor, AGCF2, is indicated. The others are non-specific nuclear factors. N.E., nuclear extract.

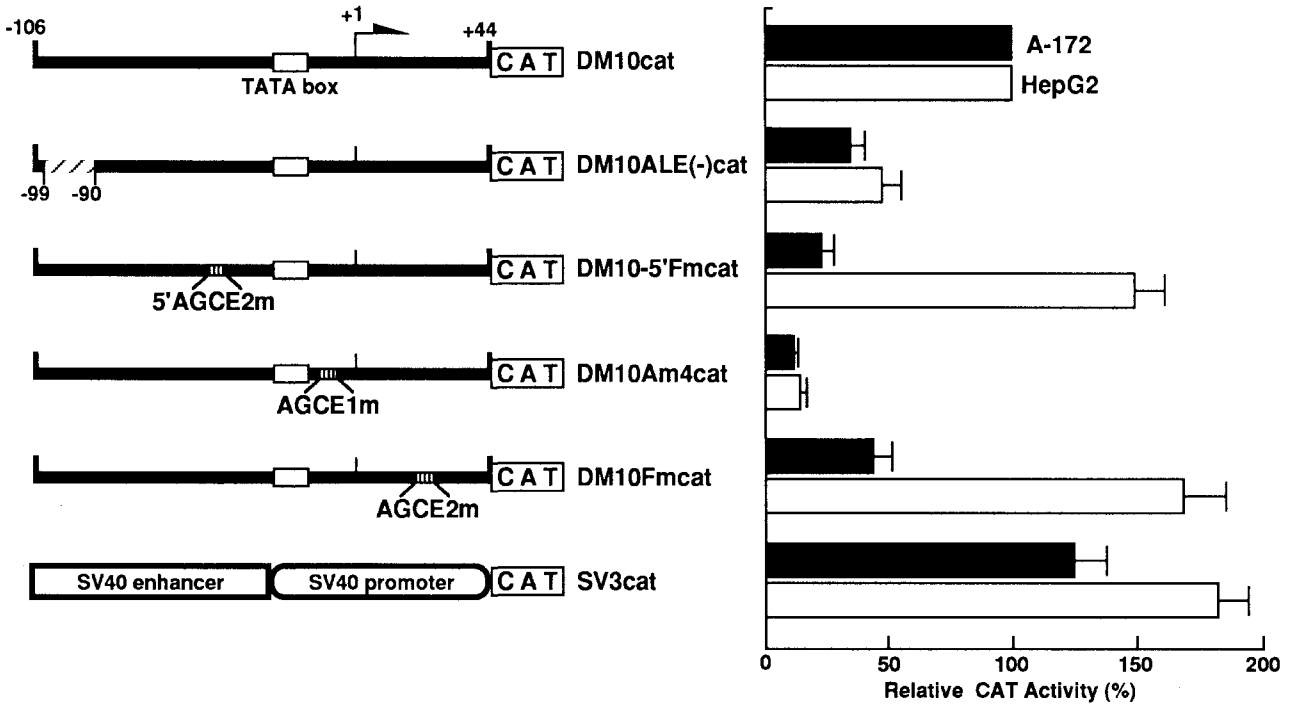

Fig. 5. Effects of mutations within ALE, 5′-AGCE2, AGCE1, and AGCE2 on the native promoter activities. Thick horizontal lines represent wild-type promoter sequences, open boxes marked CAT represent CAT vector sequences, unmarked open boxes represent TATA box, white vertical lines represent mutations (5′-AGCE2m, AGCE1m, and AGCE2m), and hatched line represents internal deletion whose positions are indicated below the line. Names of the constructs are listed to the right. Right, A172 were transfected with the indicated CAT vectors, and CAT assays were performed as described in Fig. 1. The CAT activities are presented relative to that of the DM10cat construct (hatched columns). Thin lines represent the S.E.; $n = 8$ to 10.

3.3. ALE, 5′-AGCE2, AGCE1, and AGCE2 function as critical components of the human ANG promoter

We previously reported that the proximal promoter region from −106 to +44 was important for the human ANG transcription in human hepatoma cell line (HepG2) [21]. Furthermore, AGCF1-, ALF-, and AGCF2-binding activities were also observed in HepG2 cells (data not shown and [25]). To assess the role of ALF, AGCF1, and AGCF2 for the human ANG constitutive transcription, the mutations that affect nuclear factor-binding activities (Figs. 3 and 4 and [25]) were introduced into the promoter, and then those CAT activities were analyzed in both A172 and HepG2 cells. In the case of ALF, the internal deletion of its binding site was used in order to avoid the generation of the other CREB/ATF family binding sequences. As shown in Fig. 5, the deletion of ALE and the mutations within AGCE1 decreased the transcription activity in both HepG2 and A172 cells. Interestingly, the mutations within 5′-AGCE2 and AGCE2 decreased the transcriptional activity in A172 cells but rather increased its activity in HepG2 cells. Taken together, these results suggested that ALE, 5′-AGCE2, AGCE1, and AGCE2 were essential for the efficient human ANG promoter activity in A172 cells, and AGCF2-binding sites differentially functioned in a cell type-dependent manner.

4. Discussion

The position of AGCE1 which is located in the region between TATA box and the transcription initiation site is very unique. Recently, we reported that AGCF1 is an authentic regulator of human ANG transcription by mediating the responsiveness to upstream and downstream element-binding factors in HepG2 cells [25]. Although the human ANG downstream enhancer core elements could not work in A172 cells [26,27], ALE functions in both cell lines (Fig. 5). In A172 cells, AGCE1 may also mediate the responsiveness to ALE-, 5′-AGCE2-, and AGCE2-binding factors. This would be supported by the observation that both ALF- and AGCF2-binding elements efficiently activated the human ANG promoter, but had very weak effects on the heterologous herpes simplex virus thymidine kinase promoter which had no AGCE1-like sequence in A172 cells (data not shown).

Cell-specific cofactors have been identified and discovered to give a cell-type specific function to ubiquitously expressed sequence-specific transcription factors. Oct-binding factor 1 (OBF-1) expressed only in B cells has no intrinsic DNA-binding activity and interacts with ubiquitously expressed Oct-1 on promoter DNA. It is suggested that OBF-1 can give a B cell-specific activation function to Oct-1 [28]. Furthermore, Krebs et al. [29] reported that papovavirus JC core promoter functions in a glial cell-specific manner, and suggested the requirement of a glial cell-specific coactivator. We indicated the differential action of AGCF2-binding elements upon the human ANG promoter activity between A172 and HepG2 cells, although AGCF2-binding activities were detected in all the cell lines tested, regardless of whether human ANG is expressed or not (data not shown). These results provide the possibility that AGCF2 may have an important function in the glia-dependent ANG expression through either the interaction of a cell-type specific cofactor or a specific modification or both. Further study will be necessary to define, by means of cloning the cDNA encoding AGCF2, the molecular mechanism by which they can maintain the promoter activity in the brain.

Acknowledgements: This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan, Special Research Project on Circulation Biosystems at the University of Tsukuba, Uehara Memorial Foundation, Kanae Foundation of Research for New Medicine, The Inamori Foundation, and Asahi Glass Foundation. We gratefully acknowledge all our laboratory members for their helpful discussion and encouragement.

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