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Human Act and AR1 Sequences Differentially Regulate Murine and Human D_{1A} Dopamine Receptor Promoters

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The D_{1A} dopamine receptor gene underlies complex transcriptional regulation in order to achieve the tissue-specific expression. Transcription in the D_{1A} genes proceeds from two distinct promoters utilized for the tissue-specific regulation of these genes. Furthermore, analysis of the human D_{1A} dopamine receptor gene has revealed that the region between nucleotides -1173 and -1136 (ActAR1) of the gene might be important for its neural cell-specific expression. To investigate the function of D_{1A} dopamine receptor promoters in the brain cell-specific expression of transgenes, we analyzed the regulatory patterns of two distinct protein-binding regions of ActAR1, i.e., an Act sequence (-1174/-1154) and an AR1 sequence (-1154/-1136), toward murine and human D_{1A} promoters. Transient expression analyses using various chloramphenicol acetyltransferase constructs revealed that Act could not activate murine or human D_{1A} promoters, and that AR1 could effectively stimulate these promoters in a cell type-nonspecific manner. Only ActAR1, a combination of Act and AR1, could activate murine and human D_{1A} promoters in a prominent cell type-specific manner. Abundant protein binding to Act was detected by gel mobility shift assay using nuclear extracts from SK-N-MC, NS20Y, OK, and C6 but faint protein binding using nuclear extracts from HepG2. Furthermore, strong protein binding to AR1 was detected using nuclear extracts from SK-N-MC, NS20Y, HepG2 but faint protein binding from C6 extracts and no detectable protein binding from OK extracts. These observations suggest that the tissue-specific expression of the D_{1A} gene is due, at least in part, to the differential expression of these activator proteins that bind to Act and AR1.

Keywords: Cell Type-specific Manner; Chloramphenicol Acetyltransferase; D_{1A} Gene; Dopamine Receptor; Promoter; Tissue-specific Regulation; Transcription; Transfection.

Introduction

The control of the expression of tissue-specific genes is necessary to make functional and structural change during the development stage and in the adult organism. Expression of tissue-specific genes may be controlled by alternate promoters (Falvey *et al.*, 1995; Finley *et al.*, 1995), alternative transcriptional initiation (Lee *et al.*, 2001), alternative splicing (Lee *et al.*, 2001), different polyadenylation sites (Ranganathan *et al.*, 1995), or stage-specific and/or tissue specific enhancer or silencer elements (Babola *et al.*, 1995; Mandolesi *et al.*, 2002; Park *et al.*, 2002; Youn *et al.*, 2002). Many of these genes possess more than one promoter that might be controlled by different transcription factors (Ambrosio *et al.*, 2002; Logette *et al.*, 2003).

The D_{1A} dopamine receptor gene possesses more than one promoter (Lee *et al.*, 1996), and in humans and mice the D_{1A} genes are transcribed from two distinct promoters, one located immediately upstream of exon 1 and the other located in the single intron of the D_{1A} gene (Lee *et al.*, 1996; 1999; Minowa *et al.*, 1992; 1993). The upstream promoter is active only in neuronal cells, whereas the in-

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Abbreviation: CAT, chloramphenicol acetyltransferase.

tron promoter is active in both neuronal and renal cells. Thus, the kidney expresses only short D_{1A} transcripts lacking exon 1 (Lee *et al.*, 1997). The D_{1A} dopamine receptor is one of five dopamine receptors cloned thus far (Sibley and Monsma, 1992). It is highly expressed in the striatum and to a lesser extent in the prefrontal cortex, and is a key mediator of several important brain functions including movement, behavior and memory (Creese and Fraser, 1987; Sawaguchi and Goldman-Rakic, 1991). In addition, the D_{1A} gene is expressed in the kidney where it regulates electrolyte and water transport (Jose *et al.*, 1992).

Detailed analysis of the transcription control mechanisms of the D_{1A} gene could help us exploit the reason for delivering transgenes into specific neuronal populations in the brain. This strategy would be useful not only in addressing basic scientific questions, but also would help deliver therapeutic genes to the brain. Although strong viral promoters are frequently used for the latter objective, their activity appears to decrease *in vivo* over time, presumably due to the effects of cytokines (Qin *et al.*, 1997). Thus, the use of strong and cell-selective cellular promoters might be advantageous. A number of cellular promoters have been used in experimental animals these include, myelin basic protein (Schiff *et al.*, 2002), prion protein (Lemaire-Vieille *et al.*, 2000), tyrosine hydroxylase (Liu *et al.*, 1997), and brain-derived neurotrophic factor (Timmusk *et al.*, 1995). However, it is difficult to design a compact viral expression vector using these promoters, because many of these promoters are very large. Thus, precise analysis of the D_{1A} promoter could allow us to reduce the size of the vector construct. Previous analysis of the human D_{1A} dopamine receptor gene has shown that the region between nucleotides -1173 and -1136 (ActAR1) might be important for its neural cell-specific expression (Minowa *et al.*, 1993). In addition, the ActAR1 sequence is highly conserved between humans, pig, and rat D_{1A} genes (Lee *et al.*, 1999). Moreover, it was recently reported that human Meis2 and TGIF differentially regulate the transcription of the human D_{1A} dopamine receptor gene by binding to complementary DNA sequences within its Act region (Yang *et al.*, 2000).

In the present investigation, we analyzed the regulatory natures of the human Act and AR1 sequences toward the murine and human D_{1A} dopamine receptor promoters in an effort to explore the utility of these promoter sets in the brain tissue-specific expression of therapeutic transgenes.

Materials and Methods

Plasmid construction The murine D_{1A} dopamine receptor gene-chloramphenicol acetyltransferase (CAT) reporter gene constructs, pCAT-MU-ME, pCAT-MU-MA, pCAT-ActAR1-MU-ME, and pCAT-ActAR1-MU-MA were constructed as described previously (Lee *et al.*, 1999). pCAT-MU-ME possesses the murine D_{1A} upstream promoter region (MU, bases -947 to -767 relative to the first ATG) and the 5′-end of the murine D_{1A} exon 2 (termed ME, -419 to -175) upstream of the CAT gene in pCAT-Basic (Promega, USA). pCAT-MU-MA contained the murine D_{1A} upstream promoter region and the murine D_{1A} intron combined with a ME segment (termed MA, -534 to -175) upstream of the CAT gene in pCAT-Basic. pCAT-ActAR1-MU-ME and pCAT-ActAR1-MU-MA have two tandem copies of the human D_{1A} transactivation region ActAR1 (nucleotides -1174 to -1136) (Minowa *et al.*, 1993) upstream of the murine D_{1A} upstream promoter (MU) in pCAT-MU-ME and pCAT-MU-MA, respectively. Oligonucleotides with two tandem copies of the human D_{1A} transactivation region Act (nucleotides -1174 to -1154) (Minowa *et al.*, 1993) were synthesized. After annealing, the double-stranded oligonucleotide was subcloned 5′ to the murine D_{1A} upstream promoter in pCAT-MU-ME and pCAT-MU-MA to yield pCAT-Act-MU-ME and pCAT-Act-MU-MA, respectively. Oligonucleotides with two tandem copies of the human D_{1A} transactivation region AR1 (nucleotides -1154 to -1136) (Minowa *et al.*, 1993) were synthesized. After annealing, the double-stranded oligonucleotide was subcloned 5′ to the murine D_{1A} upstream promoter in pCAT-MU-ME and pCAT-MU-MA to yield pCAT-AR1-MU-ME and pCAT-AR1-MU-MA, respectively.

The human D_{1A} dopamine receptor gene-CAT reporter gene constructs, pCAT-D1Pro, pCAT-D1Pro-D1Int, pCAT-D1Pro-Ex, and pCAT-D1Pro-All were constructed as described previously (Lee *et al.*, 1996). pCAT-D1Pro possesses the human D_{1A} upstream promoter region (D1Pro, bases -1102 to -963 relative to the first ATG) upstream of the CAT gene. pCAT-D1Pro-D1Int, pCAT-D1Pro-Ex, and pCAT-D1Pro-All have the human D_{1A} intron (termed D1Int, bases -599 to -484), the 5′-end of the human D_{1A} exon 2 (termed Ex, -484 to -239), and the human D_{1A} intron combined with a Ex segment (termed All, -599 to -239) downstream of the human D_{1A} upstream promoter (D1Pro) in pCAT-D1Pro. Two tandem copies of the D_{1A} transactivation region ActAR1 were subcloned 5′ to the human D_{1A} upstream promoter (D1Pro) in pCAT-D1Pro, pCAT-D1Pro-D1Int, pCAT-D1Pro-Ex, and pCAT-D1Pro-All to yield pCAT-ActAR1-D1Pro, pCAT-ActAR1-D1Pro-D1Int, pCAT-ActAR1-D1Pro-Ex, and pCAT-ActAR1-D1Pro-All, respectively. Two tandem copies of the human D_{1A} transactivation regions Act and AR1 were subcloned 5′ to the human D_{1A} upstream promoter in pCAT-D1Pro-Ex to yield pCAT-Act-D1Pro-Ex and pCAT-AR1-D1Pro-Ex, respectively. The integrity of all CAT constructs was verified by restriction analysis and sequencing.

Cell culture and transient expression assays The murine neuroblastoma cell line NS20Y was a kind gift from Dr. Marshall Nirenberg (National Heart, Lung and Blood Institute, USA) and human neuroblastoma cell line SK-N-MC, opossum kidney OK, rat glioma C6, and human hepatoblastoma HepG2 cell lines were obtained from ATCC (Manassas, USA). NS20Y,

C6, and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (BioWhittaker, USA) supplemented with 10% fetal bovine serum (BioWhittaker) at 37°C in a humidified atmosphere containing 10% CO_2. SK-N-MC and OK cells were cultured in Minimum Essential Medium (BioWhittaker, USA) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and 1× MEM non-essential amino acids (all from BioWhittaker, USA). Transfections were carried out using Lipofectamine plus reagent (Invitrogen BV, Netherlands) with serum free medium in 60-mm dishes. Three μg of pCAT plasmid and 1.5 μg of pCMVβ plasmid (Clontech, USA) were used for each transfection. Cells were harvested 48 h later and lysed by freezing and thawing the harvested cells for 4 cycles followed by centrifugation. All plasmids used for the transfections were purified using a Plasmid Midi Kit (Qiagen, USA).

CAT assay was carried out using a CAT enzyme-linked immunosorbent assay (ELISA) kit (Roche Applied Science, USA). All CAT assay results were normalized versus β-galactosidase activity (Sambrook *et al.*, 1989).

Gel mobility shift assay Nuclear extracts from SK-N-MC, NS20Y, C6, OK, and HepG2 cells were prepared using a modification of Dignam's procedure (Dignam *et al.*, 1983; Hennighausen *et al.*, 1987). DNA probes Act (bases -1174 to -1154) and AR1 (bases -1154 to -1136) spanning the human D_{1A} transactivating sequence (Minowa *et al.*, 1993) were generated by annealing ^{32}P-labeled sense oligonucleotide and cold antisense oligonucleotide (Fig. 1). All probes were purified using Sephadex G-25 (Amersham Bioscience, USA) columns. DNA-protein binding reactions were carried out with nuclear extracts from SK-N-MC, NS20Y, C6, OK, and HepG2 cells, and about 5 fmol (> 50,000 cpm) of probe in the presence of 12% glycerol, 12 mM HEPES buffer (pH 7.9), 60 mM KCl, 120 μM EDTA, 50 mM NaCl and 2 μg of poly[dIdC] (Boehringer Mannheim, USA) in 25 μl. In some experiment, a polyclonal antibody to Sp1 (2 μg/μl, Santa Cruz Biotechnology, USA) was added to the reaction mixture before the probe addition. Products of the binding reactions were resolved in a 1× Tris-Glycine buffer, 4% polyacrylamide (30:1) nondenaturing gel (Ausubel *et al.*, 1989).

Results and Discussion

The human D_{1A} transactivation regions Act and AR1 differentially regulate murine D_{1A} promoter(s) We previously reported that the human D_{1A} transactivation region ActAR1 gives tissue-specificity to the murine D_{1A} dopamine receptor promoters (Lee *et al.*, 1999). In the present study, we investigated the regulatory patterns of the human ActAR1 sequence (nucleotides -1174 to -1136) upon murine D_{1A} dopamine receptor promoter(s) by using two distinct protein binding regions of ActAR1, specifically, an Act sequence (nucleotides -1174 to -1154) and an AR1 sequence (nucleotides -1154 to -1136) (Fig. 1 and Minowa *et al.*, 1993). As shown in Fig. 1, the ActAR1 sequence consisted of two distinct protein binding sequences, Act and AR1. Consensus sequences for Meis2 and TGIF binding sites are presented by the Act sequence (Yang *et al.*, 2000), and a deduced consensus sequence for a Sp1 binding site is presented on an AR1 sequence (Fig. 1). Recently, it was confirmed that Meis2 and TGIF bind to the activator sequence Act and that they regulate transcription differentially, and in a cell type-specific manner (Yang *et al.*, 2000). However, the detailed analysis of the regulatory protein, which binds to the activator sequence AR1 has not been performed. To analyze the regulatory patterns of the human Act and AR1 sequences toward the murine D_{1A} dopamine receptor promoters, two tandem copies of the human D_{1A} transactivating regions of ActAR1, Act, and AR1 were subcloned 5′ to murine D_{1A} promoter(s) to generate six hybrid constructs (Fig. 2).

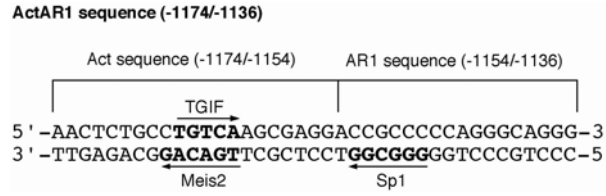

Fig. 1. An ActAR1 sequence of the human D_{1A} dopamine receptor gene. The ActAR1 sequence consists of two distinct protein binding sequences, Act and AR1. Consensus sequences for Meis2 and TGIF binding sites on the Act sequence are shown in bold, and their respective orientations are represented by arrows (Yang *et al.*, 2000). A deduced consensus sequence for the Sp1 binding site on the AR1 sequence is also shown in bold, and its respective orientation is represented by an arrow. Oligonucleotides with two tandem copies of each sequence, ActAR1, Act, and AR1 were used for the plasmid constructions. The Act and AR1 sequences were used in gel shift assays. Nucleotide numberings are relative to the first ATG codon (Minowa *et al.*, 1992).

pCAT-Act-MU-ME showed weak transcriptional activity both in the D_{1A} expressing neuronal cell line NS20Y and in the renal cell line OK, and no significant transcriptional activity in the glial cell line C6 or the hepatic cell line HepG2 (Fig. 2). On the other hand, pCAT-AR1-MU-ME showed strong transcriptional activity up to about 24 and 7 fold that of pCAT-Basic in NS20Y and OK cells, respectively (Fig. 2). Furthermore, pCAT-AR1-MU-ME showed strong transcriptional activity up to about 12 fold in HepG2 cells, but no significant transcriptional activity in C6 cells. pCAT-ActAR1-MU-ME showed strong transcriptional activity of up to about 13 and 7 fold in NS20Y and OK cells, respectively, but weak transcriptional activity in C6 and HepG2 cells (Fig. 2). These results indicate that the human D_{1A} transactivation region Act does not effectively activate the murine D_{1A} upstream promoter, but that the human D_{1A} transactivation region AR1 might stimulate the murine D_{1A} upstream promoter in cell type-

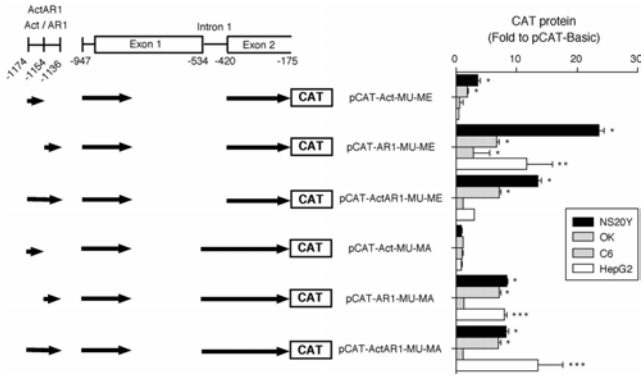

Fig. 2. Transient expression analyses of the human activator region ActAR1 in CAT constructs containing the mouse D_{1A} promoter(s). A schematic structure of CAT constructs is shown. These plasmids were used to transfect; the murine neuroblastoma NS20Y, opossum kidney OK, rat glioma C6, and human hepatoblastoma HepG2 cell lines using Lipofectamine plus. CAT activity was measured using a CAT-ELISA kit and normalized versus β-Galactosidase activity derived from pCMVβ uniformly co-transfected into each plate. Means ± S.E. for three plates are shown as fold changes versus pCAT-Basic. * ANOVA $p < 0.0001$, ** ANOVA $p = 0.0174$, and *** ANOVA $p = 0.0067$ compared with pCAT-Basic. These experiments were repeated twice and reproducible results were obtained.

non-specific manner. Only the human D_{1A} transactivation region ActAR1 activated the murine upstream promoter in a prominent cell type-specific manner.

Analysis of pCAT-Act-MU-MA in the four cell types revealed no significant transcriptional activity (Fig. 2). In contrast, pCAT-AR1-MU-MA revealed relatively strong transcriptional activity up to about 8, 7, and 8 fold in NS20Y, OK, and HepG2 cells, respectively, but no significant transcriptional activity in C6 cells (Fig. 2). pCAT-ActAR1-MU-MA showed strongest transcriptional activity up to about 14 fold in HepG2 cells, and relatively strong transcriptional activity up to about 8 and 7 fold in the D_{1A} expressing NS20Y and OK cells (Fig. 2). On the other hand, pCAT-ActAR1-MU-MA showed no significant transcriptional activity in C6 cells (Fig. 2). These results indicate that the human D_{1A} transactivation region Act cannot activate a combination of murine D_{1A} upstream and intron promoters, but that the human D_{1A} transactivation regions AR1 and ActAR1 can stimulate this promoter combination in a less prominent cell type-specific manner.

Analysis of the CAT constructs containing the murine D_{1A} promoter(s) revealed that pCAT-ActAR1-MU-ME, which contains the murine D_{1A} upstream promoter alone, showed strong transcriptional activity in a prominent tissue-specific manner (Fig. 2). This construct showed no significant transcriptional activity in D_{1A} non-expressing C6 and HepG2 cells. On the other hand, pCAT-ActAR1-MU-MA, which contains the murine D_{1A} upstream and intron promoter, showed strong transcriptional activity (Fig. 2). This construct also showed high transcriptional activity in D_{1A} non-expressing HepG2 cells. Different transcriptional activity profiles of pCAT-ActAR1-MU-ME and pCAT-ActAR1-MU-MA may be due to the presence of the intron promoter in pCAT-ActAR1-MU-MA. In fact, we demonstrated previously that the ActAR1 sequence harbors a potent neural-specific activator element only for the D_{1A} upstream promoter (Lee *et al.*, 1999). Furthermore, although pCAT-AR1-MU-ME showed strongest transcriptional activity in the D_{1A} expressing neuronal cell line NS20Y, it also showed strong transcriptional activity in D_{1A} non-expressing C6 and HepG2 cells (Fig. 2).

The human D_{1A} transactivation regions Act and AR1 differentially regulate human D_{1A} promoter(s) We also tested the regulatory patterns of ActAR1 toward the human D_{1A} dopamine receptor promoter(s) using two distinct protein binding regions of ActAR1, i. e., an Act sequence and an AR1 sequence (Fig. 1). Two tandem copies of the human D_{1A} transactivation regions ActAR1, Act, and AR1 were subcloned 5′ to the human D_{1A} promoters to generate six hybrid constructs (Fig. 3).

Analysis of pCAT-Act-D1Pro-Ex in the five cell types revealed no significant transcriptional activity (Fig. 3). Furthermore, pCAT-AR1-D1Pro-Ex also showed weak transcriptional activity in the D_{1A} expressing neuronal cell lines SK-N-MC and NS20Y, and no significant transcriptional activity in, the renal cell line OK, the glial cell line C6, and the hepatic cell line HepG2 (Fig. 3). On the other hand, pCAT-ActAR1-D1Pro-Ex revealed strong transcriptional activity, reaching about 3, 15, and 3 fold versus pCAT-Basic in SK-N-MC, NS20Y, and OK, respectively, but no significant transcriptional activity in C6 and HepG2 cells (Fig. 3). Furthermore, pCAT-ActAR1-D1Pro also revealed strong transcriptional activity up to about 4, 15, and 12 fold that of pCAT-Basic in SK-N-MC, NS20Y, and OK, respectively, but no significant transcriptional activity in C6 and HepG2 cells (Fig. 3). These results indicate that the human D_{1A} transactivation regions Act and AR1 could not effectively activate the human D_{1A} upstream promoter. Only the human D_{1A} transactivation region ActAR1 activated the human D_{1A} upstream promoter in a prominent cell type-specific manner, and this observation (Fig. 3) is consistent with that of murine D_{1A} upstream promoter (Fig. 2).

To analyze the regulatory patterns of the human D_{1A} transactivation regions ActAR1 of on the human D_{1A} dopamine receptor promoter(s), pCAT-ActAR1-D1Pro-D1Int, pCAT-ActAR1-D1Pro-Ex, and pCAT-ActAR1-D1Pro-All were constructed (Fig. 3). Analysis of pCAT-ActAR1-D1Pro-Ex containing only the human D_{1A} upstream promoter revealed the strongest transcriptional activity,

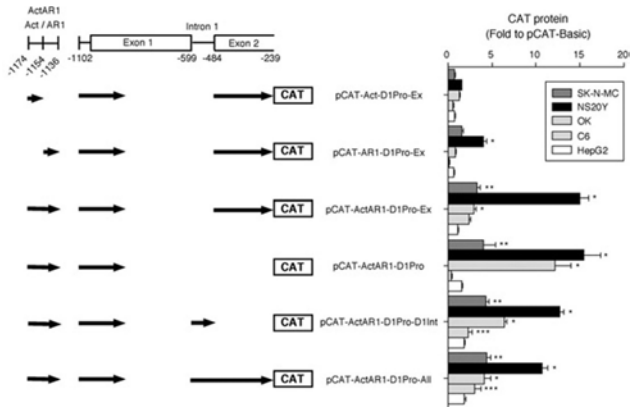

Fig. 3. Transient expression analyses of the human activator region ActAR1 in CAT constructs containing the human D_{1A} promoter(s). A schematic structure of the CAT constructs is shown. These plasmids were used to transfect; the murine neuroblastoma NS20Y, opossum kidney OK, rat glioma C6, and human hepatoblastoma HepG2 cell lines using Lipofectamine plus. CAT activity was measured using a CAT-ELISA kit and normalized with β-galactosidase activity derived from pCMVβ uniformly co-transfected into each plate. Means ± S.E. for three plates are shown as fold changes versus pCAT-Basic. * ANOVA $p < 0.0001$, ** ANOVA $p = 0.0005$, and *** ANOVA $p = 0.0824$ compared with pCAT-Basic. These experiments were repeated twice and reproducible results were obtained.

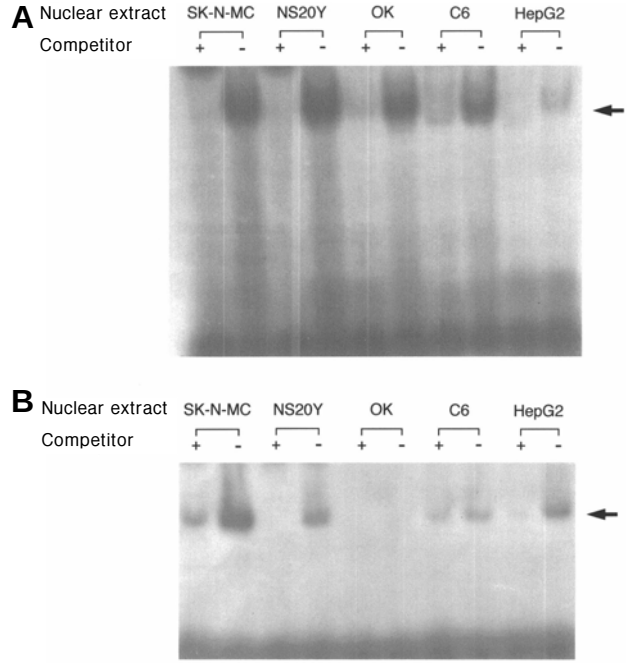

Fig. 4. Gel mobility shift assay using the DNA probes Act (A) and AR1 (B) upon nuclear extracts from 5 different cell lines. DNA probes were generated by annealing a ^{32}P-end-labeled oligonucleotide and its cold complementary oligonucleotide. About 5 fmole (> 50,000 cpm) of probe and nuclear extracts from SK-N-MC, NS20Y, OK, C6, and HepG2 cells were used. Competitors indicate the corresponding unlabeled DNA fragments. The arrow indicates a specific shifted band.

reaching about 15 fold versus pCAT-Basic in the D_{1A} expressing neuronal cell line NS20Y, but the weakest transcriptional activity in OK, C6, and HepG2 cells (Fig. 3). On the other hand, pCAT-ActAR1-D1Pro-D1Int and pCAT-ActAR1-D1Pro-All containing the human D_{1A} upstream and intron promoters revealed significant transcriptional activity in the D_{1A} non-expressing glial cell line C6 (Fig. 3). These results indicate that the human D_{1A} transactivation region ActAR1 can stimulate the human D_{1A} upstream promoter most effectively and in a prominent cell type-specific manner.

pCAT-ActAR1-D1Pro-Ex, which contains the human D_{1A} upstream promoter alone, also showed strong transcriptional activity in a prominent tissue-specific manner (Fig. 3). Although pCAT-ActAR1-D1Pro, which does not contains the Ex, 5′-end of the human D_{1A} exon (-484 to -239), showed almost the same transcriptional activity pattern as pCAT-ActAR1-D1Pro-Ex, it was found to be more active in the D_{1A} non-expressing cell line HepG2 than pCAT-ActAR1-D1Pro-Ex (Fig. 3). We included the 5′-end of the murine and human D_{1A} exons, ME and Ex, respectively in the CAT constructs, on the basis of previous observations. In fact, a previous report revealed that nuclear factor binding can be detected by gel shift assay on the Ex sequence, and that is important for regulating the expression of the D_{1A} gene (Lee *et al.*, 1996).

In conclusion, that transcriptional activity of the D_{1A} promoter(s) induced by an Act sequence might insufficient for the use these promoter combinations in a therapeutic gene delivery system (Figs. 2 and 3). Furthermore, although the AR1 sequence activates the D_{1A} promoter(s) more effective than the Act sequence, it did not give any cell type-specificity to the D_{1A} promoter(s) (Figs. 2 and 3). Only the ActAR1 sequence was found to activate the D_{1A} promoter(s) in a prominent cell-type specific manner.

DNA-protein interactions at the human D_{1A} transactivation regions Act and AR1 We have shown that the human Act and AR1 sequences differentially regulate the murine and human D_{1A} promoters. To evaluate regulatory protein binding at the Act and AR1 sequences of the human D_{1A} dopamine receptor gene, gel mobility shift assays were performed using 5′-end labeled DNA probes with nuclear extracts from; the neuronal cell lines SK-N-MC and NS20Y, renal cell line OK, glial cell line C6, and hepatic cell line HepG2 (Fig. 4). Using an Act probe, a specific retarded band was observed in all cell lines tested, however, this band was very faint in HepG2 cells (Fig. 4A). Using an AR1 probe, a specific retarded band was observed in SK-N-MC, NS20Y, C6, and HepG2 cells, however, no corresponding band was detected in OK cells

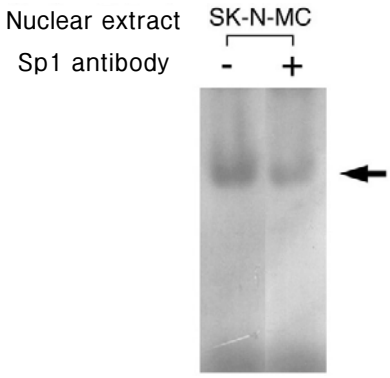

Fig. 5. Gel mobility super-shift assay using the DNA probe AR1 upon nuclear extract from SK-N-MC. The DNA probe was generated by annealing a ^{32}P-end-labeled oligonucleotide and its cold complementary oligonucleotide. About 5 fmoles (> 50,000 cpm) of probe and nuclear extracts from SK-N-MC were used. Four μg of Sp1 antibody was used for the super-shift assay. The arrow indicates a specific shifted band.

(Fig. 4B).

As shown in Fig. 1, we confirmed that Meis2 and TGIF proteins bind to the Act sequence (Yang *et al.*, 2000), however, the transactivation protein, which interacts with the AR1 sequence has not been studied yet (Fig. 1). Recently, we found a deduced consensus sequence for a Sp1 binding site on an AR1 sequence by searching databases (Fig. 1). To investigate whether Sp1 protein can bind to its deduced consensus sequence on the AR1 sequence, a polyclonal antibody to Sp1 was added to the assay mixture along with a nuclear extract from the neuronal cell line SK-N-MC. No mobility change was observed compared with the antibody non-added control (Fig. 5). These results indicate that Sp1 does not interact with the AR1 sequence.

DNA-protein interaction studies indicate that nuclear factors binding to the Act sequence exist in all cell types tested, however, no nuclear factor that binds to the AR1 sequence exist in OK cells (Fig. 4). These observations are consistent with previous reports on Act (Yang *et al.*, 2000) and AR1 (Lee *et al.*, 1997). Nuclear factors that bind to the Act and AR1 sequences are abundantly expressed in neuronal cells but are less expressed in other cells (Fig. 4). Taken together, these observations suggest that the levels of transacting proteins expressed might include the tissue-specific expression of the D_{1A} dopamine receptor gene. Although the AR1 sequence showed a deduced Sp1 consensus sequence (Fig. 1), no further band retardation was caused by the Sp1 antibody (Fig. 5). This observation suggests the existence of a novel unidentified nuclear factor that binds to AR1.

In conclusion, the characterization of the human D_{1A} transactivation region ActAR1 along with the murine and human D_{1A} promoters that are transcriptionally active in a neural cell specific-manner could be useful for targeting transgenes *in vivo*. Because the D_{1A} gene is abundantly expressed in brain, the murine and human promoter combinations, ActAR1-MU-ME and ActAR1-D1Pro-Ex could be useful tools to deliver therapeutic genes into the brain region. However, it should be checked whether these promoter combinations, examined in cultured cells, induce the brain cell-specific expression of transgenes *in vivo*.

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