The Differential Regulation of Gap43 Gene in the Neuronal Differentiation of P19 Cells

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Growth associated protein 43 (Gap43) is a neuron-specific phosphoprotein, which plays critical role in axon growth and synapses functions during neurogenesis. Here we identified two transcription start sites (TSSs) of the mouse Gap43 gene designated as a proximal site at +1, and a distal TSS at –414. RT-qPCR data reveal that the transcripts from +1 increase 10-fold on day-1 post-all-trans retinoic acid (RA) treatment, reached a peak value at day-4 and gradually reduced. By contrast, the distal TSS directs a late, remarkably sharp increase of the transcripts from the day-5 on. An intense signal of Gap43 at the neurites and neural network is determined by the efficient transcription of the distal promoter as shown in Northern blot and RT-qPCR assay. In addition, the targeting of p300 in combination with a differential enrichment of Brm to Brg1 change at the distal promoter region of the gene is induced under RA treatment. The over hundreds of GA rich stretches and the GAGAG elements located between the two TSSs may take parts in the differential transcription of the two TSSs of the Gap43. Our findings provide the first evidence on the identification and differential transcription of the two TSSs of the mouse Gap43 gene, and the preferential distribution of their protein products in the specific stages of RA induced P19 differentiation. These data suggest the efficient transcription of the distal promoter of Gap43 is an important mark for the transition of P19 cells from the progenitor stage into neuronal differentiation.

were constructed with KpnI and HindIII endonuclease sites using pGL3-Gap43 luciferase reporter plasmids. E2F1 expression plasmid was from Dr. YD Gong (Tsinghua University, Beijing). pBabe-E2F1-siRNA plasmid was from Dr. ZQ Yuan (Institute of Biophysics, Chinese Academy of Sciences, Beijing), its targeting sequences are CTGAAGCAACCCTCTGCTGC-3’ and CTTCTTCTCCCTCTCCTTCC-5’. Primers used were shown in Table 1.

Antibodies
Antibody against BRM (ab15597) was from Abcam, Cambridge, UK; antibodies against BRG1 (SC-10768), PCAF (SC-13124), and p300 (sc-32244) were from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Antibody against E2F1 (BS397) and GAP43 (BS6555) were from Bio-world (Shanghai, China).

5’ RACE assay
Total RNA of P19 cells treated with RA for 2 day and mouse liver tissue were isolated using Trizol reagent (Invitrogen). Gap43 TSSs were identified using 5’-Full RACE Kit (Takara, Dalian, China) as follows: converted the uncapped RNA into non-ligated 5’-hydroxyl RNA with calf intestine alkaline phosphatase (CIAP) and removed cap from mature mRNA with tobacco acid pyrophosphatase (TAP). decapped mRNA and 5’ RACE adaptor were ligated with T4 RNA ligase, reverse transcription was carried out using M-MLV and random 9 mers at 42°C. Blots were imaged by autoradiography. DNA probes were generated with the random primer DNA labeling kit ver.2.0 (Takara).

Quantitative real-time RT-PCR
Quantitative real-time RT-PCR assays were carried out as previously described (Li et al., 2007). The relative expression of Gap43 was normalized against GAPDH, using the comparative CT method as the manufacturer’s instructions (Rotor-Gene RG-3000A Real-time PCR System, Corbett Research, Sydney, Australia). Primers were used as in Table 3. Experiments were repeated at least three times with statistical analyses for each individual experimental set. All values in the experiments were shown as mean ± SD.

Chromatin immunoprecipitation (ChIP) and quantitative PCR analysis
ChIP assays were carried out as previously described (Li et al., 2007). For quantitative assays, standard curve and ChIPED DNA samples were analyzed on a Rotor-Gene RG-3000A Real-time PCR System (Corbett Research) with PCR Master Mix for SYBR Green assays (Takara). Primers used as follows: distal primer pairs (d-P) designed for amplifying Gap43 promoter region (−482/−378), forward primer, 5’-TGAGGCTTGCCAGGGAGACT-3’, reverse primer, 5’-GGGATTCTTGCAGATGTTCT-3’, proximal primer pairs (p-P) designed for amplifying Gap43 promoter region (−40/−68), forward primer, 5’-TGAGGCTTGCCAGGGAGACT-3’, reverse primer, 5’-ACGAGCTGCTCAGATGTTCT-3’, the cycle quantity required to reach a threshold in the linear range (Qt) was determined and compared with a standard curve for the primer set generated by 50-fold dilutions of genomic DNA samples of known concentration. The percentage of ChIPED DNA relative to input was calculated and shown as mean ± SD from three independent experiments.

DNA transfection and promoter activity assay
P19 cells were transfected with VigoFect Reagent (Vigorous, Beijing, China) according to the manufacturer’s instructions. Cells were transfected with siRNA, wild-type or mutant constructs and allowed to recover for 48 h prior to promoter activity analysis. To normalize firefly luciferase activity of the reporter construct (pREP4-gap43-luc), 1.50 (mol ratio) of pRL-TK plasmid expressing Renilla luciferase (Promega Bioluminescence System, Madison, WI).

| Table 1. Primers for cloning gap43 promoter |
| Code | Forward primer (5’–3’) | Reverse primer (5’–3’) |
| pGL3 (−706/+41) | GAGCTCCACCTTAGTCTCCTTTGCCG | CTCGAGTTCTTCTTCTGCTTCC |
| pGL3 (−46/+41) | GAGCTGACGGGAAGTAGCAACGCGGAG | CTCGAGTTCTTCTTCTGCTTCC |
| pGL3 (−82/+41) | GAGCTCGAGGAGAGGAGGAGGAGG | CTCGAGTTGCTTCTTCTGCTTCC |
| pGL3 (−359/+41) | GAGCTCGAACCTCTGCTCTTTGCCG | CTCGAGGCTATGCTCAGCGACAGG |
| pGL3 (−464/−327) | GAGCTCGGAGGAGGTGACTGCTTGAG | CTCGAGGCTATGCTCAGCGACAGG |
| pGL3 (−706/−327) | GAGCTCGACCTCTAGTCTCCTTGGC | CTCGAGGCTATGCTCAGCGACAGG |
| pGL4 (−706/−46) | GGAGGGAGGAGGAGGAGGAGGAGGAGG | CTAGCTGCTTCTTCTGCTTCC |
| pREP4 (−706/+41) E2F mutant | AGAGACAGGAGTAGCAACGCGG | |

| Table 2. Primers for 5’ RACE assay |
| Code | Primer sequence (5’–3’) |
| gap44 outer primer (primer1) | ATCGAGCACGCGAGCATCATCG |
| gap44 inner primer2 (primer2) | CAGGCTGACGGGAAGTAGCAACGCGGAG |
| gap44 inner primer3 (primer3) | CTCGAGTTCTTCTTCTGCTTCC |
| gap44 inner primer4 (primer4) | CTTCTTCTCCTCCTGCTTCC |
| 5’ RACE adaptor outer primer (primer5) | CATGCTGACGGGAAGTAGCAACGCGGAG |
| 5’ RACE adaptor inner primer (primer6) | CGGGAGGAGGAGGAGGAGGAGGAGGAGG |
| 5’ RACE control outer primer | AGAGACAGGAGTAGCAACGCGG |
| 5’ RACE control inner primer | TTGGAGTCGCCCTCAGAGAGAG |

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was co-transfected into the cells. Six hours after transfection, the medium was replaced with α-MEM without RA (RA−) or with 0.5 μM RA (RA+). The cells were harvested 48 h after RA treatment and relative luciferase activity was analyzed with GloMax of the Dual-Glo Luciferase Assay System (Promega Bioluminescence System). Experiments were triplicated and all data were shown as means ± SD.

**Immunoblotting**

P19 cells were collected and lysed in RIPA buffer on ice for 1 h. Whole-cell extracts (WCEs) were clarified by centrifugation at 12,000 g for 10 min. Samples were separated in 12% SDS–polyacrylamide gels and analyzed using Western blotting.

**Immunofluorescence**

P19 cells were induced to neuronal differentiation as described previously (Jones-Villeneuve et al., 1982). Briefly, 2 x 10^5 cells were allowed to aggregate in bacterial grade Petri dish with 0.5 μM RA for 4 days. Control cells were incubated in 0.005% DMSO. Aggregated cells or control cells were plated onto cover slips and the cultured for 3 days. Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.25% Triton X-100 for 10 min at room temperature, then were blocked with 1% BSA and 5% goat serum followed by incubated with rabbit anti-GAP43 antibody (1:150), then were washed with PBS and incubated with FITC-conjugated anti-rabbit-secondary antibody. Nuclei were stained with DAPI. Fluorescence images were obtained by confocal microscope (Perkin Elmer UltraView VOX).

**Alignment of the promoter sequences of Gap43 gene**

Promoter regions of human and rat Gap43 gene were downloaded from UCSC genome browser, and were aligned with the promoter of mouse Gap43 gene using DNAMAN 5.2.2.0 software.

**Results**

**Identification of transcriptional start sites of mouse Gap43 gene in P19 cells**

In 5’ RACE analysis, we first synthesized a 3’ primer (P2) started from the 81st G downstream of the first codon to performed PCR with a 5’ primer (P6, per TaKaRa) and obtained four amplified products with 126, 158, 171, and 197 bp in length. Among them, the 171 bp fragment was the most abundant one that comprised of 70% of all the products, indicating an efficient TSS positioned at the 57th bp upstream of the translational initiation site A, and designated as the proximal TSS +1 (Fig. 1A, top part).

As reported earlier that other TSS exists in the far upstream of both human and rat GAP43 genes, we synthesized a second 3’ primer (P4) started from the −290th G relative to +1 to perform a second search and an abundant 158 bp band showed up (Fig. 1A, bottom part). Excluding the length of the 5’ primer (P6), the upstream Gap43 fragment amplified should be 125 bp in length and positioned at −414 relative to the proximal start site of +1. A schematic of the two TSSs identified and the first codon ATG of the Gap43 are shown in Figure 1B.

### Table 3. Primers for real-time RT-PCR

<table>
<thead>
<tr>
<th>Code</th>
<th>Sequence 5’–3’</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer7</td>
<td>CTGGAAAGCTAGCAAAACAATTGT</td>
<td>−409 to −387 bp</td>
</tr>
<tr>
<td>Primer8</td>
<td>CTCTCTCTGTCCCTCTCCTCCT</td>
<td>−311 to −290 bp</td>
</tr>
<tr>
<td>Primer9</td>
<td>CTAACCCAATCTGCTGCTGTT</td>
<td>48–68 bp to ATG on coding sequence</td>
</tr>
<tr>
<td>Primer10</td>
<td>TCAAGAAAGCTAGCCTGAA</td>
<td>169–188 bp to ATG on coding sequence</td>
</tr>
</tbody>
</table>
Promoter activity of Gap43 gene in RA treated P19 cells

We then constructed a pREP4-luciferase based reporter plasmids driven by a series of 5' and/or 3' truncated fragments of the Gap43 gene as indicated in the left part of Figure 2A–C. The reporter constructs were individually transfected into P19 cells with or without RA treatment and their activities were detected by relative luciferase activity and shown in the right parts of the figures.

The activity of the −46/+41 basal construct without RA treatment was set as 1, which was induced by RA to increase twofold over the basal control. For the proximal promoter, the longer the upstream region, the higher the reporter activity is the rule. For example, the highest efficiency of the −359/+41 construct that directed an 11-fold higher activity over the basal construct and was further induced for some 2.5-fold under RA treatment (Fig. 2A).

The basal activity of the distal promoter covering −464/−327 (−50/+87 relative to the distal TSS at −414), did not change much from that of the proximal promoter (Fig. 2B, top row). Extending the upstream sequences to −706 to mimic a construct of −291/+87 relative to −414, the promoter activities were constitutively kept at some 24-fold of the basal level, and further increased to 50-fold in the presence of RA (medium row). In contrast to the above, the extending of the 3' downstream sequences to −47, the −706/−47 construct boosted a 260- and 488-fold higher promoter activity respectively in non-treated and RA induced cells (Fig. 2B, bottom vs. top rows). Summarizing the results from Figure 2A,B, we suggest that the distal promoter is more efficient than the proximal one, in particular, in P19 cells under RA treatment.

To our surprise, the activity of a construct −706/+41 that contained both of the distal and the proximal promoters (Fig. 2C) was only comparable with the −706/−327 (Fig. 2B, medium row) no matter RA was treated or not. Meanwhile, as the distal promoter −706/−47 showed the highest activity over the control (Fig. 2B, bottom row, 260- and 488-fold), which suggested, at least in vitro, the proximal promoter is not only less efficient than its distal counterpart, but also impaired it when both of them coexisted.

p300 and the ATPases of the chromatin remodeling complexes are enriched at the upstream promoter of mouse Gap43 gene under RA treatment

To explore if the above findings can be verified by chromatin remodeling in vivo, chromatin immunoprecipitation (ChIP) assays were performed. Primer pairs for the amplification of the proximal (−40/+68, pP) and the distal (−482/−378, dP) promoter fragments of Gap43 gene were shown in Figure 3A, and the quantification with RT-qPCR assays were performed (Fig. 3B–E).

The two lysine acetyl transferases p300 and PCAF were examined here. In which, p300 was, although transiently, markedly recruited to the distal promoter only on day 3 post-RA treatment but not at all at the proximal region (Fig. 3B). This single day occupancy of p300 extended further upstream to

**Fig. 2.** Activities of the proximal and distal promoters of the Gap43 gene in P19 cells. Relative promoter activity was detected in P19 cells transfected with pREP4-Gap43-luc reporter plasmids driven by (A) Gap43 proximal promoter fragments; (B) distal promoter fragments; and (C) both promoter fragments of Gap43 gene, and normalized with the activity of co-transfected pRL-TK plasmid. Digits represent the first and the last base pair of each promoter fragment relative to proximal TSS of +1. Each bar represents an average value from at least three independent experiments with SD shown on top. RA+ and RA−: with and without RA treatment.
1100 of Gap43 gene (data not shown). However, the targeting of PCAF only slightly increased at the distal but not the proximal promoters (Fig. 3C). By contrast, the ATPase subunits of the SWI/SNF chromatin remodeling complex was first occupied the distal promoter by Brm at day-0 that was replaced by an increased Brg1 at the distal promoter region of the Gap43 gene throughout the fourth day of RA treatment (Fig. 3D,E).

The impact of E2F1 on the promoter activity of Gap43 gene

Since we found an atypical E2F1 consensus of GCGCGAG (Tashiro et al., 2003) existed in the proximal promoter of Gap43 gene, whether it was responsible for the 3.5- to 4-fold higher reporter activity of −82/+41 of Gap43 than the basal control was our next goal to approach. Accompanying the gradually increase of E2F1 during RA treatment (Fig. 4A, top part), E2F1 also occupied the proximal promoter of the Gap43 gene as revealed by ChIP assay (Fig. 4A, bottom part). The reporter activity of Gap43 construct was doubled in wild-type construct of E2F1 transfected over the mock cells (Fig. 4B, left part, open bars), and a ~10-fold higher induction was observed in P19 cells treated with RA (left part, filled bars). Furthermore, co-transfection of the siRNA specific for E2F1 with the reporter plasmid, both of the promoter activities either with or without RA reduced significantly (Fig. 4C). These data indicate the RA induced increase of endogenous E2F1 is positively feedback to synergize with RA in the early activation of the proximal promoter of Gap43 gene.

The preferential expression of the Gap43 transcribed from the proximal and distal TSSs in RA induced P19 cells

RA treated P19 cells aggregated first on the third or fourth day of treatment that were then turned into a neuron-like phenotype in fresh RA-free medium for another 3 days (Wu et al., 2009). Gap43 protein was found early in the cytoplasm, which was significantly enhanced in its outgrowth parts of the neurites in the neural network phenotype, particularly on the 5–7 days of RA treatment during the neuronal differentiation of P19 cells (Fig. 5a).

To examine the differential efficiency of the two TSSs during RA induced differentiation of P19 cells, Northern blot analysis was performed. As P19 cells were continue to grow without RA after day-4, the expression of mRNA initiated from distal promoter increased dramatically and shown as a longer and intense bands around 1.8 kb at day-5 and day-7. On the contrary, the expression of a shorter mRNA at around 1.2 kb
was much less intense in the Northern analysis that showed up on day-2 of RA induction (Fig. 5B).

To validate the results of Northern blot, we quantified the relative level of mRNA with RT-qPCR and normalized with gapdh mRNA as control. In contrast to the immediate early increase that reached a peak value at some 50-fold on day 4, the Gap43 mRNA of the proximal promoter decreased gradually in the following days. By contrast, the mRNA of the distal promoter increased dramatically that ranged between 150 and 300 on day-5 through day-7 (Fig. 5C).

Taken together, the above results indicate that the Gap43 protein expressed and accumulated in the neurites were mostly transcribed from the distal promoter. On the contrary, the early accumulation of Gap43 in the cytoplasm of P19 cells was likely activated via the proximal promoter.

Discussion

The alignment of the upstream sequences of Gap43 gene in mouse with those of the human, and rat genes (Fig. 6) shows that the sequences around the two TSSs identified in mouse −414 and +1 are highly homologous among the three species, in particular, those around the proximal TSS are identical. This finding suggests the Gap43 gene transcribed and regulated in mouse is typical in rat, human and likely in the mammalian world.

In this study, we have identified the distal TSS of Gap43 gene at −441 only with a primer starting from the −290 bp upward in the 5’ RACE approach but not the 3’ primer for the proximal TSS. Further, the distal TSS region (−706/−47) induces a 10-fold higher activity than the dual promoter with 87 bp more in its 3’ end (−706/−41). The findings of two long stretches of GA-motifs (−255/−97) and the sporadic GAGAG elements are both rich between the distal and proximal TSSs of the Gap43 gene, which suggest the DNA sequences between the two TSSs may produce certain structure hindrance to inhibit or impair the proximal promoter of the gene.

The vertebrate homologue of Drosophila GAGA associated factor (dGAF) binds the GAGA element and GA-rich DNA sequences are usually found in clustered over hundreds base-pairs in the upstream of a TSS (Adkins et al., 2006) in the developmental regulated genes (Matharu et al., 2010). It is thus not unusual to have found multiple GAF binding sites in the mouse Gap43 gene.

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possibly involved in the regulation of the accurate timing and differential regulation of the two promoters of Gap43 gene during neuronal differentiation.

In addition, a GCgCGCTaGC sequence at −62/−53 in the upstream of the proximal TSS, which is 80% inversely repeated with a downstream sequence of GCaAGCGaGC at −36/−27. Since these sequences could theoretically form a stem-loop or even cruciform structure that may disrupt the E2F1 binding at −54/−47 and reduce the proximal promoter activity of Gap43 at the later stage of neuronal differentiation.

The P19 embryonic carcinoma cells treated with RA for 4-day become aggregated and change from ES-like pluripotent cells to a progenitor-like stage that is committed to neuronal differentiation. Further cultured in a RA-free medium for another 4-day, the cells are turn into a neuronal differentiation stage as verified by stage specific markers (An et al., 2005). In this study, we have shown that the transcripts from proximal TSS of Gap43 gene increase at the progenitor stage in the presence of RA, which is earlier than, but much less intense than that of its distal counterpart in the differentiating stage of the cells. This implies that Gap43 protein may have two isoforms translated from either the shorter mRNA mainly in the first 1–4 days of RA treatment, or those from the longer mRNA transcribed from the distal promoter of Gap43 in the neuronal differentiation stage of the P19 cells. In addition, the two proposed protein isoforms, designated by the length of their coding mRNAs as S- and L-forms, are distinct in their distribution in the cytoplasm that the S-form is evenly distributed in the cytoplasm, by contrast, the L-form protrudes into the outgrowth neurites and the neural network on days 5–7. However, the major difference, if any, between the two proposed isoforms of Gap43 protein remains to be explored.

The ATPase subunits of the SWI/SNF chromatin-remodeling complexes, Brm and Brg1 are exclusively occupy the distal but not the proximal promoter of Gap43 gene. We show that Brm occupies the distal promoter constitutively, Brg1 substitutes Brm and gradually increases upon RA treatment. An early RA induced Brm to Brg1 switch (Zhang et al., 2010) followed by an efficiently targeting of p300 may provide an open chromatin conformation at the distal TSS to facilitate its fully induction at a later stage of differentiation.

In summary, we have identified two transcriptional start sites in the upstream of the translational initiation site of Gap43 gene. While the proximal promoter responds to the early stage of RA treatment, the distal one is fully activated in the later stage of
neuronal differentiation with Gap43 protein enriched in the neurites and the neuronal network.

Acknowledgments

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Literature Cited


