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Identification and analysis of the promoter region of the human HAS3 gene

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ABSTRACT

Hyaluronan (HA) is a key component of the vertebrate extracellular matrix that is synthesized at the plasma membrane by the hyaluronan synthases including HAS1, HAS2 and HAS3. The expression and regulation of HAS1-3 are implicated in numerous physiological and pathological processes. The promoters of human HAS1 and HAS2 genes have been identified previously whereas HAS3 promoter remains unclear. In the present study, we have for the first time identified and characterized the human HAS3 gene promoter region. 5′ RACE assay revealed two novel transcriptional variants of HAS3 gene with distinct transcription start sites. Progressive deletion analysis of the 5′-flanking region of HAS3 gene demonstrated that HAS3 proximal promoter is mainly restricted to a 450-bp region (i.e. −761 to −305 bp upstream of the major HAS3 transcription start site), whereas its core promoter is located to a minimal 129-bp region (i.e. −433 to −305 bp upstream of the major HAS3 transcription start site). Transcriptional factor binding analysis indicated that HAS3 gene promoter lacks of canonical TATA box, but contains classical GC box as well as other putative binding sites for transcriptional factors such as C/EBP and NFκB. In addition, site-directed mutagenesis assay demonstrated that the proximal Sp1 binding site is essential for the robust proximal promoter activity of HAS3 gene whereas the core MTE (core promoter motif ten elements) motif is required for the basic core promoter activity of HAS3 gene. Our present study should facilitate further studies on the mechanism regulating the expression of this important gene.

1. Introduction

Hyaluronan (HA), a linear non-sulfated polysaccharide (glycosaminoglycan) with disaccharide repeats of D-glucuronic acid and N-acetyl-D-glucosamine, is a key component of the vertebrate extracellular matrix [1–3]. Despite its simple chemical composition, HA has been shown to be involved in a wide range of physiological and pathological processes including tissue development, inflammation, wound healing, tumor progression and metastasis [1–3].

In mammals, HA is synthesized directly on plasma membrane by the enzymes of hyaluronan synthases (HASs) including HAS1, HAS2 and HAS3 [1]. The three enzymes have distinct enzymatic properties, and they synthesize different sizes of HA molecules with different functions. It has been demonstrated that HAS1 and HAS2 produce large-sized HA whereas HA3 produces HA of a lower molecular mass [4]. The function of HASs also appears to be cell and tissue specific. HAS1 has the lowest enzymatic activity, and is thought to mainly maintain a low and basal level of HA [5]. HAS2 is the most studied and common HAS in mammalian tissue, mainly involved in embryonic and cardiac cushion morphogenesis, and can stimulate cell proliferation and angiogenesis [1,3]. HAS3 is highly expressed in specific conditions such as in tumor cells, and appears to favor the malignant phenotype in many types of malignancies [1,3]. The expression of HAS enzymes is the main determinant of the HA synthesis rate in cells. Conceivably, the various functions of HA are fulfilled by the strict regulation of the three HAS expressions. Accumulating evidence demonstrated that the expression of HAS genes undergoes rapid and dramatic changes during embryo development. In adult tissues, HA synthesis and HAS expression are influenced by injury, inflammation and neoplastic tumors, and activated by a number of cytokines and growth factors [3].

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Dysregulation of HAS genes results in abnormal production of HA and promotion of abnormal biological processes such as transformation and metastasis [1–3].

The expression regulatory mechanisms of HAS1 and HAS2 genes have been well investigated, and the promoters of both two genes have been identified by the same group [6–10]. However, the promoter region of human HAS3 gene has not yet been identified in details till now. In the present study, we have determined the transcription start sites of HAS3 gene by using 5′-RACE technique, and identified its promoter region. The current identification and characterization of the human HAS3 gene promoter provides the basis for further studies on the gene regulation of the HAS3-mediated biological processes.

2. Materials and methods

2.1. Cell culture

Human non-small cell lung carcinoma H1299 cells were grown in a humidified atmosphere containing 5% CO_2 at 37 °C in RPMI 1640 medium supplemented with 50 units/ml penicillin, 50 mg/ml streptomycin, and 10% (vol/vol) FBS.

2.2. Rapid amplification of cDNA ends (RACE) analysis

The 5′-RACE analysis was carried out using the SMART™ RACE cDNA amplification kit (Clontech) following the manufacturer's protocol [24]. Human normal lung total RNAs (Ambion) were reverse-transcribed to obtain first-strand cDNA with the incorporation of a 'smart oligo' onto its 5′ end. Two anti-sense HAS3 gene-specific primers (HAS3-R325: 5′-TTCCGTGTGGATGAACTGG-TAGCCCG-3′, HAS3-R742: 5′-CACCACATCCCGCACACGGTCCATGC-3′), corresponding to positions 325–350 and 742–769 bp of the reference HAS3 mRNA (Accession No. AF234839), were designed and synthesized. Primary PCR amplification was performed using the gene-specific primer (HAS3-R742) and adaptor primer (AP1). An aliquot of the primary PCR products were then used as the template for a nested PCR reaction with the HAS3-R325 and NUP primers. Final reaction products were analyzed by 1.0% agarose gel electrophoresis, purified using the Gel and PCR clean-up system (Promega). The purified 5′ RACE fragments were cloned into the pTA2 Vector (Toyobo) and automatically sequenced by 3730xl DNA analyzer.

2.3. Cloning of the HAS3 gene promoter region

The luciferase reporters of P1953(−318/+1635), P847(+788/+1635), P715(+920/+1635), P2339(−2644/−305), P685(−990/−305), and P456(−761/−305) were generated by cloning the corresponding 5′ flanking genomic region of human HAS3 gene into pGL3-basic promoter-less vector with PCR method. P193(−761/−569) was generated by DNA blunting kit (Takara, Japan) with restriction enzyme digestion and ligation according to the manufacturer's protocols. Three progressive deletion mutants, including P259(−563/−305), P186(−490/−305), and P129(−433/−305) were constructed by Site-Directed Mutagenesis kit (TOYOBO, Japan) following the manufacturer's instructions. The primer sequences and the restriction enzymes used were listed in Supplementary Table 1. Nucleotide sequences of the cloned DNA fragments in all the constructs were confirmed by direct DNA sequencing.

2.4. Site-directed mutagenesis

The luciferase reporter constructs including P456(−761/−305)M2, P456(−761/−305)M1, P186(−490/−305)M2, P186(−490/−305)M1, and P129(−433/−305)M2 were generated by Site-Directed Mutagenesis kit (TOYOBO, Japan) on the basis of the indicated parental constructs according to the manufacturer's instructions. For M1 mutants, the wildtype core MTE (core promoter motif ten elements) sequence of GGAGC at −367 bp was changed into TTCTA. For M2 mutants, the core Sp1 binding site (GC box) sequence CCGG at −467 bp was mutated to AATT. The detailed primer sequences and parental constructs used were listed in Supplementary Table 1. All the mutations were verified by DNA sequencing.

2.5. Transfection and luciferase reporter assay

For luciferase reporter assays, cells were seeded in triplicates into 12-well plates, and transiently co-transfected with 100 ng of the indicated reporter plasmids, 10 ng of pRL-TK plasmid (Promega) encoding Renilla luciferase and 390 ng of the empty plasmid pcDNA3 (Invitrogen) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were lysed with passive lysis buffer and their luciferase activities were measured using the Dual-luciferase assay system (Promega) according to the manufacturer's protocol [24].

2.6. DNA sequence alignment and database analysis

The HAS3 mRNA and genomic sequences were obtained from GeneBank and UCSC database (https://genome.ucsc.edu/). The RACE sequences were aligned with the HAS3 genomic sequences to confirm gene identity, exonal usage, and location of the transcription start sites. Transcription factor binding sites were predicted by online MatInspector professional (http://www.genomatix.de/) and TFSEARCH software. Alignment for the genomic sequences upstream of HAS3 from human, mouse, and rat were carried out using the ClustalW2 algorithm at the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/msa/).

3. Results

3.1. Identification of the transcription start sites of HAS3 gene

To identify the transcription start sites for HAS3 gene, a SMART RACE analysis was performed by using two reverse oligonucleotides (R325 and R824) derived from the second exon of the coding region and two adapter primers provided with the kit for the primary and nested PCR (Fig. 1A). Total RNA prepared from human normal lung tissue (Ambion) was reverse transcribed and the first PCR-based amplification was performed using the outer primer AP1 and reverse primer R824 (Fig. 1A). Then the primary PCR products were used as template for the nested PCR by using NUP and R325 (Fig. 1A). Two distinct PCR products of 250 bp and 170 bp were detected under our experimental conditions, indicating that HAS3 transcript may have at least two transcription start sites (Fig. 1B). The two 5′-RACE products were gel-purified and cloned into the vector pTA2. DNA sequencing of several randomly selected transformants for each band revealed that the two bands are actually 224 bp and 139 bp in length (without the 30 nucleotides of NUP primer), represents two novel HAS3 transcription variants (termed variant I and II). Detailed sequence analysis demonstrated that these two novel variants are distinct from the previously reported HAS3 mRNA cloned from human brain tissue cDNA library (GenBank accession number AF234839, [15]) (Fig. 1C). The three variants have distinct first exons, but with identical partial open reading frames encoding the same amino acid sequences. It is worth noting that, under our experimental conditions, the band intensity of PCR

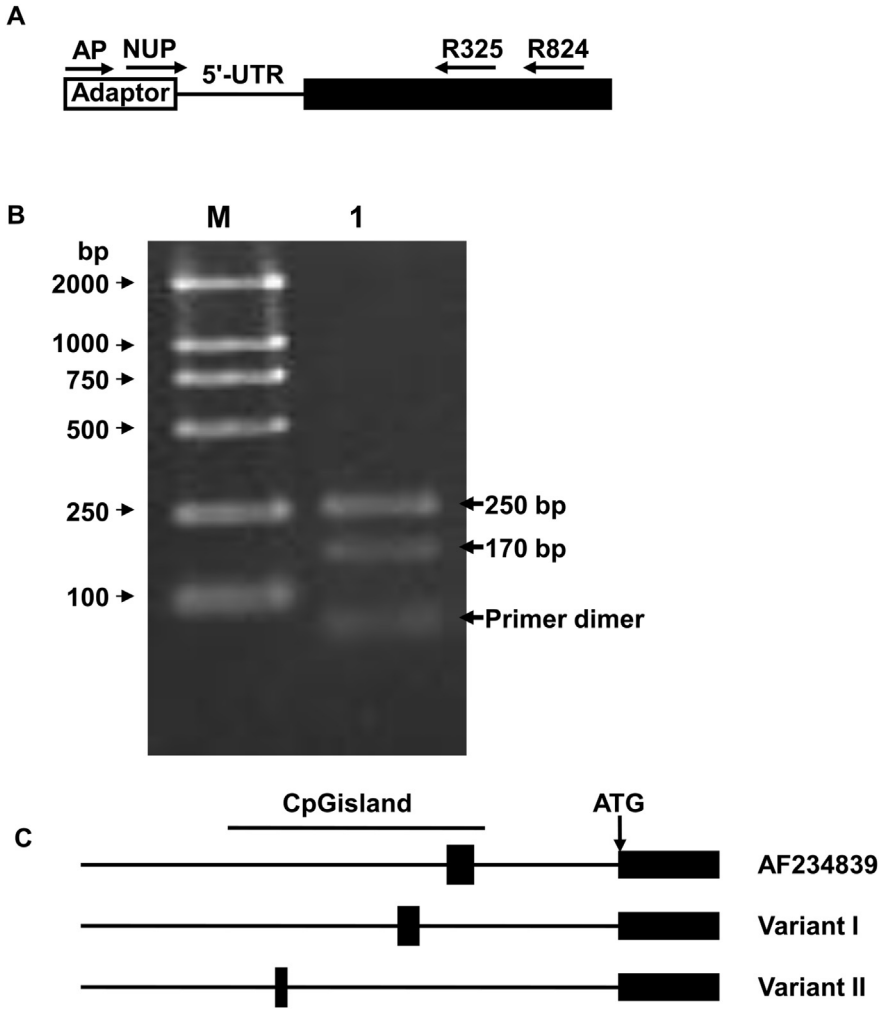

Fig. 1. 5′-RACE analysis of the transcription start site of the HAS3 gene. (A) A schematic representation of the primer design for RACE assays. Only partial of the open reading frame (ORF) of HAS3 is shown. (B) The analysis of RACE products on 1.0% agarose gel. RACE assay was conducted on human lung total RNA. PCR-based amplifications were performed by using AP/GSP2 and NUP/GSP1 for primary and nested PCR, respectively. Lane M: DL2000 molecular markers; Lane 1: Nested PCR products. PCR products was cloned into pTA2 vector and sequenced subsequently. (C) Schematic representation of the two novel variant forms of HAS3 gene. Exons and introns are indicated by filled boxes and thin lines, respectively. Exon 2 contains the translation start codon, ATG. The positions of the putative CpG islands are also shown.

products corresponding to variant I was quite brighter than that of the variant II, whereas HAS3 mRNA AF234839 was undetectable (data not shown). Thus, our present results suggest that the variant I represents the major transcript for HAS3. For further analysis, we defined the 5′-end of variant I as +1 (i.e. the 5′-end of the variant II was located at position −843).

3.2. Identification of the proximal HAS3 promoter region

Sequence analysis revealed that there exist a classic CpG island and putative promoters in the 5′-flanking region of human HAS3 gene. In addition, the existence of more than one transcript variants also suggests the complicity of HAS3 transcription regulation. Therefore, to identify the possible promoter region of HAS3 gene and conduct a systemic functional analysis of the 5′-flanking region of human HAS3 gene, we have generated a variety of luciferase reporter constructs containing the indicated genomic fragments of HAS3 gene (Fig. 2A). These six genomic fragments are overlapped, and covers a 4300-bp region upstream the translation start codon, ATG. H1299 cells were transiently co-transfected with the indicated luciferase reporter constructs together with the Renilla luciferase plasmid (pRL-TK). Forty-eight hours after transfection, cells were lysed and their luciferase activities were measured. As shown in Fig. 2B, luciferase activities were undetectable in cells transfected with P1953(−318/+1635), P847(+788/+1635) or P715(+920/+1635), suggesting that the first intron and exon of HAS3 gene does not harbor promoter activity. Strikingly, a significant increase in luciferase activities was detectable in cells transfected with P2339(−2644/−305), P685(−990/−305) or P456(−761/−305) as compared with that in cells transfected with the empty pGL3-basic plasmid, suggesting that a genomic region from −761 to −305 of HAS3 gene has strong promoter activity (Fig. 2B). Notably, the promoter activities of P685(−990/−305) and P456(−761/−305) are more active than that of P2339(−2644/−305), indicating that the sequence from positions −2644 to −305 contains a silencer negatively regulating the HAS3 transcription.

To gain a preliminary insight into the transcriptional regulatory mechanisms of HAS3 gene, the 5′-flanking region of HAS3 gene from positions −1000 to +130 were extracted to undergo transcription factor binding site analysis. The results showed that the HAS3 gene promoter lacks of classical TATA box, but contains classical GC box as well as other putative binding sites for transcriptional factors such as C/EBP and NFκB (Supplementary Fig. 1).

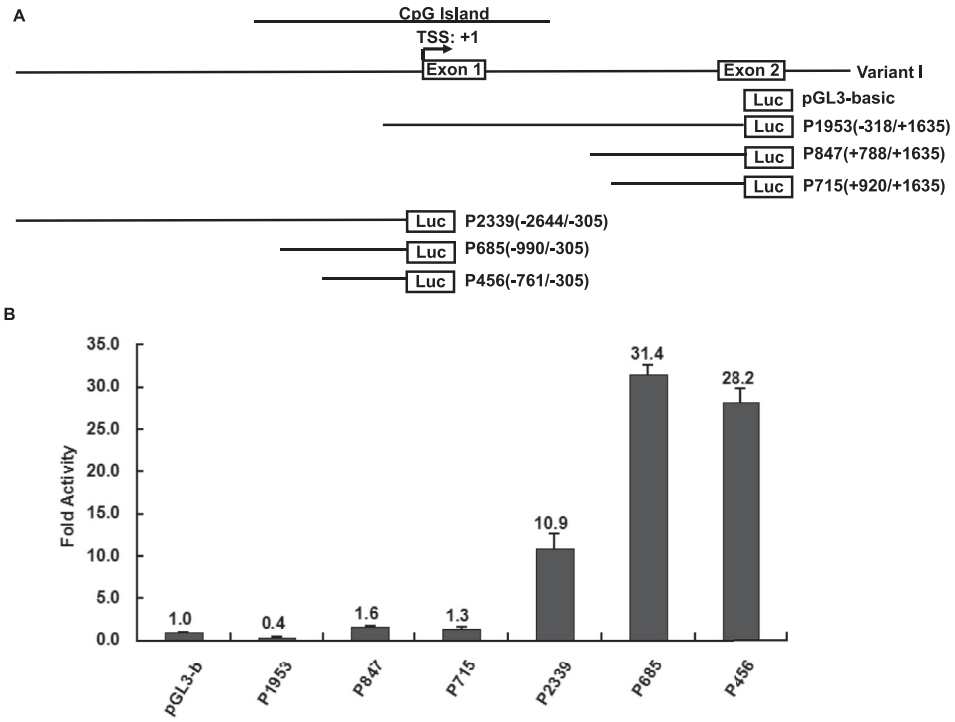

Fig. 2. Identification of the HAS3 proximal promoter region. (A) Schematic diagram of the HAS3 gene promoter reporter constructs. Numbering of the position was relative to the major HAS3 transcription start site, A (adenine, A), which is numbered as +1. Exons and introns are indicated by filled boxes and thin lines, respectively. Exon 1 contains the translation start codon, ATG. The positions of the putative CpG islands are also shown. The constructs were named as 'P-fragment length (start position/end position)'. (B) Luciferase reporter assay. H1299 cells were transiently co-transfected in triplicate in 12-well plates with the indicated luciferase reporter constructs together with the renilla luciferase reporter plasmid (pRL-TK) by using Lipofectamine 2000 transfection reagent. Forty-eight hours after transfection, firefly and renilla luciferase activities were measured by Dual Luciferase Assay System (Promega). Data obtained from a representative of at least three independent experiments were shown as fold induction compared to the activity of cell stransfected with the empty pGL3-basic vector. The results are presented as the mean and SD of triplicates from a representative experiment.

3.3. Identification of the core HAS3 promoter region

To further identify the core promoter region of HAS3 gene, we generated additional four progressive deletion luciferase reporter constructs, including P193(−761/−569), P259(−563/−305), P186(−490/−305) and P129(−433/−305), based on the parental construct P456(−761/−305) (Fig. 3A). Luciferase activities data from these constructs revealed that P193(−761/−569) failed to drive the expression of luciferase reporter gene, whereas a remarkable enhancement of luciferase activities were observed in cells transfected with the remaining luciferase reporter constructs including the parental construct P456(−761/−305) and the shortest construct P129(−433/−305) (Fig. 3B). Thus, we concluded that HAS3 core promoter exists within the genomic region spanning sequences between −433 and −305.

3.4. Site-directed mutagenesis of GC box and MTE in HAS3 core promoter

We noted that a conserved MTE (core promoter motif ten elements) motif predicted to be located at −367 bp and a Sp1 binding site at −467 bp [25,26] (Supplementary Fig. 1). To determine the potential role of MTE and/or Sp1 binding site in the promoter activities of HAS3 gene, mutations were introduced into either MTE site or Sp1 binding site in the indicated luciferase reporter constructs by site-directed mutagenesis (Fig. 3A). H1299 cells were transiently co-transfected with the indicated luciferase reporter constructs along with pRL-TK. Forty-eight hours after transfection, cells were lysed and their luciferase activities were examined. As shown in Fig. 3B, disruption of the Sp1-binding site at −467 bp resulted in a significant reduction of the promoter activity of the long reporter construct P456(−761/−305)M1, but not that of the short construct P186(−490/−305), as compared with the wildtype constructs P456(−761/−305) and P186(−490/−305). Given that P456(−761/−305) shows more significant promoter activity than that of P186(−490/−305), the above intriguing mutagenesis data strongly suggest that Sp1 binding site at −467 bp is critical to maintain the robust promoter activity of HAS3, possibly through interaction of Sp1 with transcription factor(s) binding to the proximal regions from −490 to −761 bp. On the other hand, disruption of the MTE motif led to a remarkable decrease in the promoter activity of the short reporter construct P129(−433/−305)M2, but rather had a negligible effect on the promoter activities of the longer constructs P456(−761/−305)M2 and P186(−490/−305)M2, as compared with the corresponding wildtype parental constructs (Fig. 3B). As P129(−433/−305) contains the core promoter fragment, the results suggest that the MTE motif at −367 bp is required to maintain the basic core promoter activity of HAS3 gene, and the proximal regions −490 to −761 bp harbors alternative MTE motif(s) which could compensate for the disruption of MTE at −367 bp. Consistent with this notion, a further prediction reveal another MTE motif at −545 bp (Supplementary Fig. 1).

3.5. Homology analysis of the HAS3 promoter orthologues

Finally, to determine whether the HAS3 promoter sequences are evolutionally conserved across various species, we conducted homology analysis by using the ClustalW2 algorithm. The results

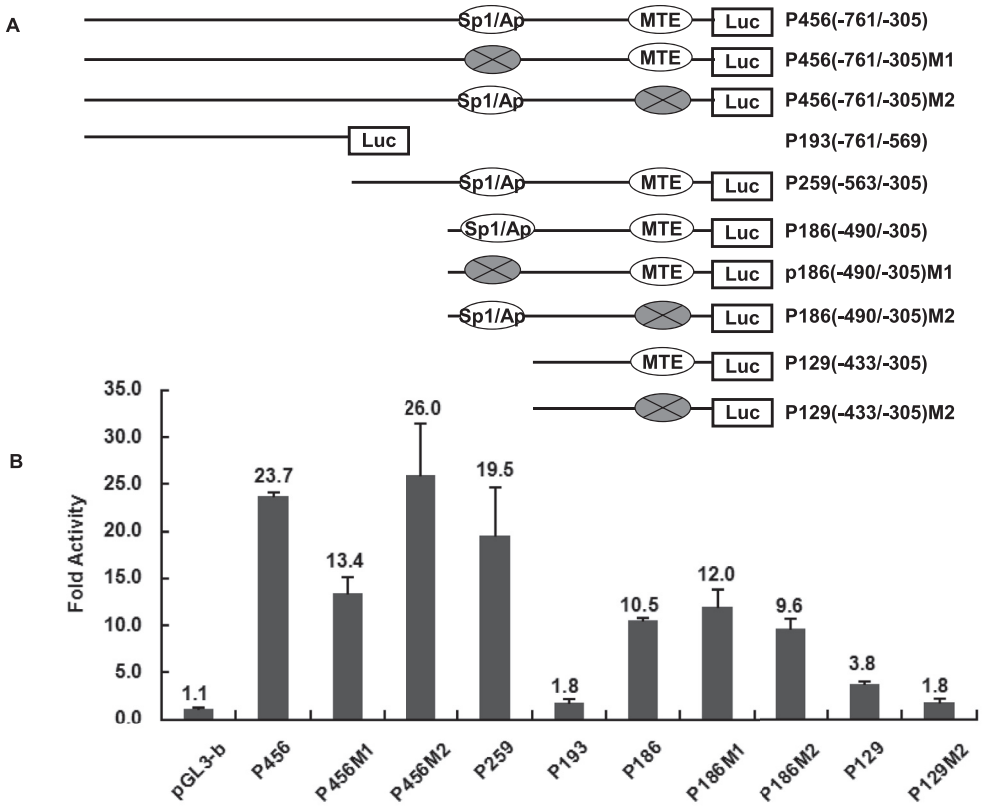

Fig. 3. Identification of the HAS3 core promoter region. (A) Schematic diagram of the HAS3 gene promoter reporter constructs. Serial deletion reporters were constructed as described in Materials and Methods, and site-directed mutations were introduced into the indicated luciferase reporter constructs to disrupt the indicated transcription factor binding sites. (B) Luciferase reporter assay. H1299 cells were transiently transfected with the indicated luciferase reporter constructs and luciferase activities were determined as described in Fig. 2.

revealed that the genomic promoter region shows high evolutionary conservations with high nucleotide identity of the human sequence in comparison to mouse (71%) and rat (73%) species (Fig. 4). The high degree of homology of the promoter region is comparable to that of the coding region of HAS3 (data not shown). With respect to transcription factor binding sites, sequence similarity was seen at the Sp1 binding sites at −467 bp and −342 bp, the core promoter MTE motif at −367 bp, NFκB binding site at −751 bp, and C/EBP binding site at −731 bp.

4. Discussion

HAS3, was the third hyaluronan synthase isolated from mammals in 1997 [11]. It is thought to be more active that HAS1 and HAS2 [4]. Accumulating evidence suggests that HAS3 plays an important role in epidermal development as well as wound healing [17]. During mouse fetal development, HAS3 expression increases when keratinocytes started to stratify on day E15, remains high until birth, with corresponding changes in hyaluronan synthesis. In adult mice, skin injury also stimulate HAS3 expression as well as hyaluronan synthesis [17]. On the other hand, several studies demonstrated that elevated HAS3 expression has been observed in ovarian cancer, breast cancer, and metastatic colon carcinoma cells [12–14], and overexpression of HAS3 can promote tumor growth [14–16]. Several studies also showed that multiple cytokines and growth factors can regulate HAS3 expression, lead to subsequent changed hyaluronan synthesis, and hence participate into the aforementioned various processes [3,17–20]. Taken together, those studies strongly suggest that HAS3 gene expression is finely regulated and contribute to epidermal development and tumorigenesis through stimulation of hyaluronan synthesis.

In the present study, we have for the first time identified and characterized the human HAS3 gene promoters. We found that the HAS3 promoter contains classical GC box as well as other putative binding sites for transcriptional factors such as C/EBP and NFκB. Those binding sites are also well evolutionally conserved among several species. Disruption of the proximal Sp1 binding site resulted in a significant reduction of the promoter activity, strongly suggesting the involvement of Sp1 in the regulation of HAS3 expression. Notably, Sp1 has been shown to regulate a variety of genes involved in epidermal development and tumorigenesis [21–24] as well as the HAS1 and HAS2 genes [3,6,8]. Moreover, NFκB is also well known to regulate a number of downstream target genes in wound healing and tumor development [3,9]. Therefore, further studies are needed to explore whether and how Sp1 as well as NFκB regulates HAS3 in various physiological and pathological circumstances.

In addition, our results revealed that HAS3 promoter contains no canonical TATA box. TATA box is an important cis-acting element found in RNA polymerase II core promoter, which binds to the TATA-binding protein (TBP) subunit of the general transcription factor TFIID, and is responsible for specifying the exact point of transcription initiation. TATA-less promoters usually have multiple transcription start sites to initiate transcription [25,26]. This is consistent with our finding that HAS3 gene promoter has at least two transcription start sites without TATA box. Notably, our data also revealed two novel HAS3 transcription variants which is distinct from the previously reported HAS3 mRNA sequence [15] (Fig. 1C). The existence of different transcription variants implies the regulation of HAS3 expression is even much more complicated than expected and probably involves transcription as well as post-transcriptional levels. Although the N-terminal partial amino acid

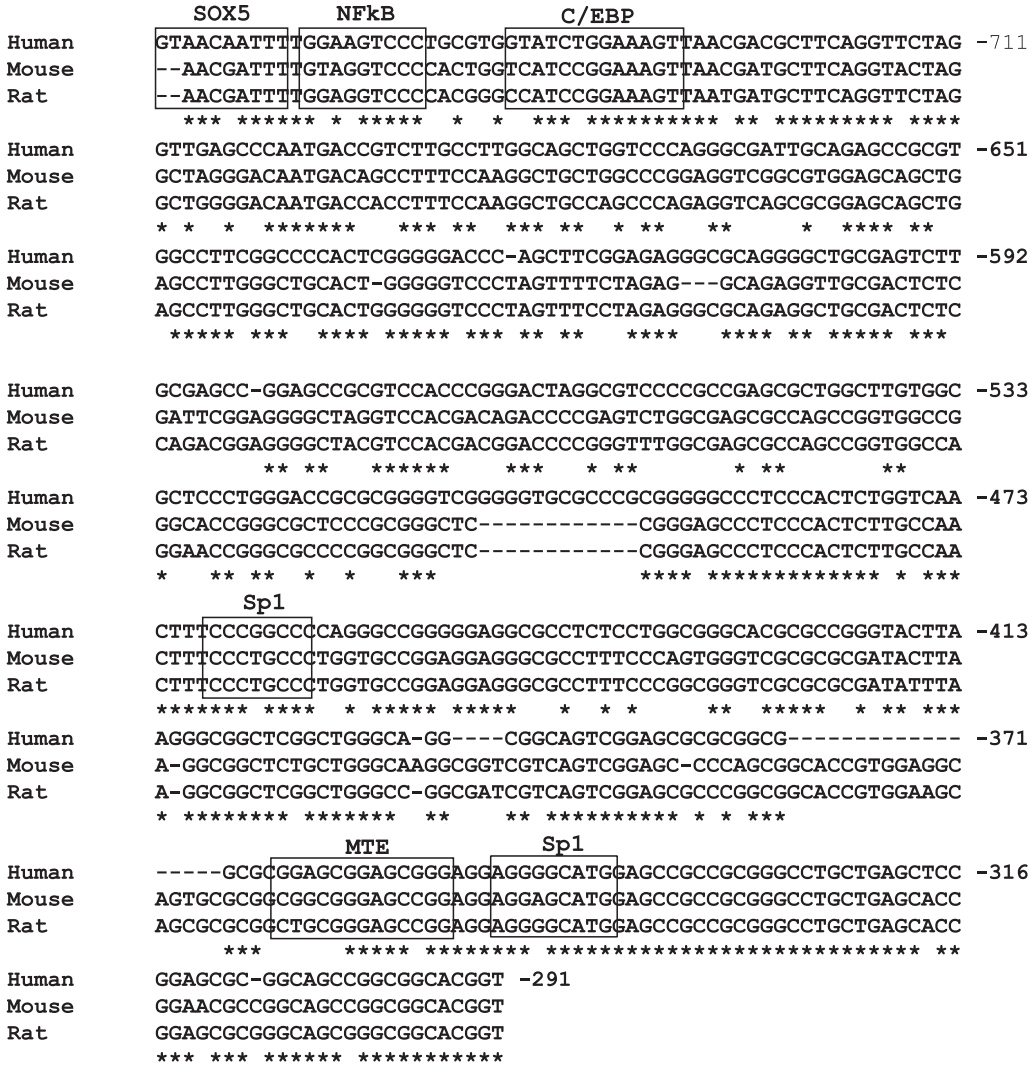

Fig. 4. Sequence alignment of HAS3 gene promoter orthologues. Nucleotides are numbered relative to the major transcription start site of HAS3 gene. Sequence consistent with human HAS3 is labeled by asterisk, and gaps are shown with blank. The conserved putative transcription factor binding sites are boxed.

sequences are identical among the identified three variants, it is unclear whether they have the same or distinct open reading frames. Therefore, it is necessary to identify the full length sequences of all possible HAS3 transcription variants and the corresponding encoding isoforms, and clarify their functional involvement in various biological processes.

In summary, we have for the first time identified and characterized the transcription start sites and promoter region of the human HAS3 gene in the present study, and this study should facilitate further studies on the regulatory mechanisms of HAS3 gene expression and the molecular behaviors of HAS3 in various biological processes.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.142.

Transparency document

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