Brief Report

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Identification and characterization of the V3 promoter of the *ST3GAL4* gene

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SUMMARY The *ST3GAL4* gene encodes the enzyme Galβ1-4GlcNAc α2,3 sialyltransferase (ST3Gal IV). This enzyme participates in the synthesis of the sialyl Lewis x antigen. In different cancer types altered expression of this antigen has been reported. The transcriptional regulation of this gene is very complex, different mRNA variants (V1-V10) have been reported and are originated by the activity of different promoters and alternative splicing. Only the promoter that gives rise to the V3 variant has not been previously reported. The objective of this work was to identify and characterize the V3 promoter of the *ST3GAL4* gene. For this, the putative V3 promoter of the *ST3GAL4* gene was delimited by *in silico* analysis. The complete promoter and smaller versions were cloned in a reporter plasmid. The constructs were transfected in the HaCaT cells and the promoter activity was evaluated by luciferase reporter assays. The cloned region showed promoter activity, and the basal activity was not dependent on TATA boxes. However, the GC boxes, an initiator element (Inr) and downstream promoter element (DPE) could contribute to basal activity. The promoter contains several binding sites for the nuclear factor of activated T-cells (NFAT) that could participate in inducible activity during the immune response. The minimal promoter corresponds to a fragment of approximately 300 bp, located in the position -347 b to -40 b. The characterization of the V3 promoter of the *ST3GAL4* gene completes the study of the four promoters of this gene, this contributes to the understanding of its complex transcription regulation.

Keywords ST3Gal IV, transcriptional regulation *ST3GAL4*, V3 promoter

1. Introduction

The *ST3GAL4* gene encodes the Galβ1-4GlcNAc α2,3 sialyltransferase (ST3Gal IV), which transfers sialic acid to glycoconjugates containing the terminal structure Galβ1-4GlcNAc or Galβ1-3GalNAc (*1*). This enzyme participates in the synthesis of the sialyl Lewis x antigen, which is a tetra-saccharide highly expressed in different cancer types and during inflammation (*2-4*). Abnormal expression of *ST3GAL4*, either decreased (*5,6*) or increased (*7*), has been reported in several types of cancer.

This gene spans approximately 59 kb (GeneID 6484) in GenBank (*8*) and displays intricate regulation since it contains different promoter regions that participate in the transcription of several mRNA variants. Previously, the presence of mRNA variants named A1, A2, B1, B2, B3, and BX in several human tissues and cancer cell lines has been reported (*9-14*); nevertheless, the current variants described in RefSeq RNA are named V1 to V10 (*15*). Thus, the previously reported variants correspond to those described in RefSeq as follows B1:V1, BX:V2 and B3:V4; the rest of the variants (A1, A2, B2) are no longer listed in GenBank (*6*). The expression of the mRNA variants results from alternative splicing and from the activity of different promoters (named pV1, pV2 and pV4) (*5,9,10,16-18*). Promoter pV1 gives rise to the variants V1, V6 and V8; promoter pV2 gives rise to the variants V2, V5, V7 and V9; pV4 gives rise to the variant V4

and only the pV3 promoter that gives rise to the V3 variant has not been characterized. Molecular studies indicate that the promoter pV4 displays activity in testicular, ovarian, cervical, colon and leukaemia cancer cell lines (*17*). Specifically, the promoter lacks TATA or CAAT boxes but contains regulatory elements as binding sites for MAF, AP2, SP1, LFA1, and HLH (*10*). In a cervical cancer cell line, the binding sites for the transcription factor AP2 are involved in its regulation (*18*). The promoter pV2 contains neither canonical TATA nor CAAT boxes but carries several putative binding sites for SP1, AP-1, and NF-kB transcription factors. Moreover, this promoter showed constitutive activity as well as inducible activity by TNF in a lung cancer cell line, which has been associated with high levels of the transcript isoform V2 (*9*). pV1 has been previously identified but not characterized (*10*). The way that transcription initiation is regulated is important for understanding the cellular processes and diseases in which the transcribed gen is involved.

Our research group recently reported the presence of the variants V1, V2, and V3 (not characterized previously) in keratinocytes and cervical cancer cells. Whereas V1 and V2 were present in all the cell lines (HaCaT, SiHa, C33A and HeLa), V3 was expressed in HaCaT, SiHa, and HeLa cells but it was not detected in C33A, suggesting differential regulation of this variant (*5*). Since the promoter for the V3 variant and its putative regulatory elements have not been described, here we identify and characterize the promotor of the V3 variant of the *ST3GAL4* gene to better understand its complex transcription regulation.

2. Materials and Methods

2.1. Cell culture

The human keratinocyte cell line HaCaT was cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) with 10 mmol/L HEPES, supplemented with 10% foetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich; Merck KGaA) at 37°C with 5% CO_2. Cells were harvested using trypsin (0.025%) and EDTA (0.02%) (Sigma-Aldrich; Merck KGaA) and were washed with phosphate-buffered saline.

2.2. Bioinformatic analysis

Alignment among the V mRNA variants was performed to localize the V3 variant in the genomic map of the human *ST3GAL4* gene and its putative promoter. Then, the putative promoter region was delimited using the promoter prediction program Transcriptional Regulatory Element Database. Additionally, we used the Elements Navigation Tool (ElemeNT) to predict the core promoter elements. The software *PATCH Public 1.0* (Pattern Search for Transcription Factor Binding Sites) was employed to determine possible regulation sites inside the promoter and to design deleted versions of the complete promoter.

2.3. Cloning of the putative pV3 promoter region

Genomic DNA was extracted from HaCaT cell line from keratinocytes of human skin (*Wizard Genomic DNA Purification Kit*, Promega, Madison, WI). Then, the putative promoter of 1735 bp was amplified by PCR using the forward primer 5'-GAGGAAAACCTcgAGGGAATCTTGA-3' and the reverse primer 5'-GGAAAAAGcTTTGGCGTCAGAGG-3'. The underlined nucleotides represent the restriction sites for *XhoI* (forward) and *HindIII* (reverse), and lower-case letters correspond to the nucleotides incorporated into the cloned-promoter sequence. Smaller versions of 689 bp and 484 bp were generated by enzymatic restriction with *KpnI/HindIII* and *SmaI/HindIII*, respectively, of the 1735 bp construction. The promoter fragments were purified and cloned into the reporter vector. The forward primers for the other versions correspond to 5'-GGCTTTGCTCgaGCCCTTTATG-3' for 364 bp, 5'-CTCAGAGCTcGAgCAGCAATGT-3' for 342 bp, 5'-GAAAGGtacCATGTTGTCTTG-3' for 308 bp and 5'-GGCTCGaGAAATTCCTGATTG-3' for 207 bp. The restriction site for the 364 bp, 342 bp and 207 bp versions corresponds to *XhoI*, and that for the 308 bp version corresponds to *Kpn I*. The reverse primer was the same for all versions. The PCR contained 25 µl of PCR Master Mix (Promega, Madison, WI), 0.5 µM of each primer, and 100 ng of DNA template in a final volume of 50 µL, with the following PCR conditions: 95°C for 5 min and 40 cycles at 95°C for 1 min, 58°C for 1 min and 72°C for 2 min, followed by a final extension at 72°C for 7 min. Next, each fragment was electrophoresed in agarose gel, and the band was purified and cloned into the pGL4.12[*luc2CP*] vector (Promega, Madison, WI) using T4 DNA Ligase (Promega). The constructs were verified by digestion with restriction enzymes and subsequent sequencing.

2.4. Luciferase assay

HaCaT cells were seeded in 24-well plates, and when the cells reached 80% confluence, they were transiently transfected with 600 ng of each pGL4.12 construct and 20 ng of the pGL4.74[*hRlucCP*] vector as a reference plasmid (Promega, Madison, WI) using the Lipofectamine 3000 reagent protocol (Invitrogen, Carlsbad, CA). Cells were lysed 24 h after transfection, and firefly and *Renilla* luciferase activities were determined using the commercially available Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luciferase activities were measured in a luminometer

Glomax 20/20 (Promega). The firefly luciferase activity was normalized to the *Renilla* luciferase activity. All transfections were performed in triplicate in three different experiments. Student's *t*-test was used to determine statistical significance between the different constructs; $p < 0.05$ was considered statistically significant.

3. Results and Discussion

The *ST3GAL4* gene encodes the enzyme ST3GAL IV, which participates in the synthesis of the sialyl Lewis X antigen. The expression change of this glycan has been implicated in different pathologies, such as cystic fibrosis and cancer (*2,4,9*). Regulation of *ST3GAL4* gene expression is very complex, giving rise to 10 different mRNA variants (V1-V10), currently reported in the RefSeq RNA database (*15*). These mRNA variants are the result of the activity of different promoters (pV1, pV2, pV3 and pV4) and of alternative splicing. The promoters pV1, pV2 and pV4 have been previously characterized (*10,17*). Recently, we identified the presence of V3 mRNA in cervical cancer cell lines HeLa and SiHa, but not in C33A, nevertheless the promoter of this variant has not been previously reported (*5*). The characterization of promoters that participate in the expression of this gene is of interest, considering the importance of the enzyme ST3Gal IV in cancer and inflammatory disease and the fact that the promoters could respond in a different manner according to the stimulus and cell type (*9*). The promoter of V3 mRNA has not been characterized to date. The objective of this work was to delimitate and characterize the V3 promoter region of the *ST3GAL4* gene and to identify its minimal promoter.

In silico analysis showed that the putative promoter is located from position 126401632 to 126403373 of chromosome 11 and extends up to 1735 bp upstream of the transcription start site of the V3 mRNA variant (GenBank NC_000011.10). The promoter contains five TFIID/TBP (TATA box binding protein) binding sites, four of which are localized far from the transcription start site. Additionally, the promoter contains four initiator elements (Inr), several GC and CCAAT boxes and a downstream promoter element (DPE) (Figure 1). The CCAAT box, GC boxes, and TATA box are promoter elements that have been reported within core promoters (*19*). Other common sequences found close to the transcriptional start site are DPE and Inr, so we identified these elements in the putative V3 promoter (Figure 1). To identify response elements in the putative promoter, we used the software *PATCH Public 1.0* and identified several response elements, including binding sites for AP-1, AP-2, CREB, c-jun, c-Myb, CTCF, ER, GATA-1, NFAT, PAX, E2F, VDR, RAR-α1, RXR-α, and VDR (Figure 1).

We have cloned the complete promoter of 1735 bp, to determine if this region presents activity. The

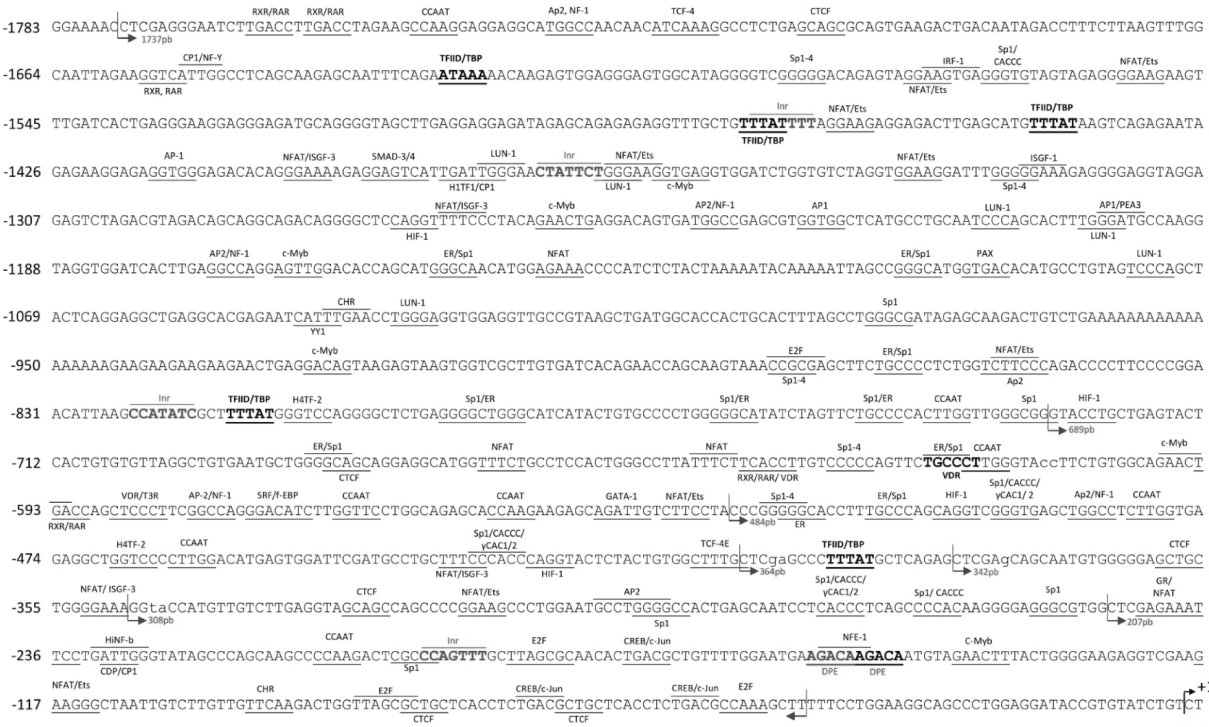

Figure 1. Schematic representation of V3 promoter of *ST3GAL4* gene. It is shown the nucleotide sequence. The reverse arrow represents the 3' extreme of all constructions, and the forwards arrows represent the extreme 5' of the different constructions, the nucleotide size of each promoter fragment is shown. The core promoter elements and the potential regulatory elements are described. Initiator sequences are shown in red. Transcriptional start site is marked with a red arrow. Nucleotides are numbered considering the transcription initiation site as +1.

experimental analysis showed that the region identified and characterized by *in silico* analysis has promoter activity (Figure 2).

To identify the minimal promoter region and the required motifs for basal promoter activity, we generated deleted versions of the complete promoter from the upstream region. Thus, six constructs were generated with the following base pair lengths: 689 bp, 484 bp, 364 bp, 342 bp, 308 bp, and 207 bp (Figure 2). In the first deleted version of 689 bp, four of the five TFIID/TBP sites, three Inr sites and several CCAAT boxes were eliminated, but the same activity of the complete promoter was maintained. The results indicated that the four TATA boxes, the 3 Inr sites, and the several CCAAT boxes eliminated in this construct did not play a role in basal promoter activity. The TATA box is a sequence recognized by the RNA pol II. This element has a position that usually ranges from approximately 40 to 100 nt, but the motifs identified in this region are located far from the transcription start site, which could explain why they do not affect the basal promoter activity (*19*). This could be applied to the Inr sites and the CCAAT boxes deleted in the construct as they are also distant from the transcription start site. In the 484 bp construct, different response elements such as RXR, RAR, cMyb, VDR, NFAT, Ets and two CCAAT boxes were eliminated; nevertheless, the activity was similar with respect to the complete promoter. In the 364 bp construct, a fragment containing GC and CCAAT boxes was eliminated, along with other response elements such as ER, CACCC, HIF1, Ap2, NF-1 and NFAT. The activity of this construct

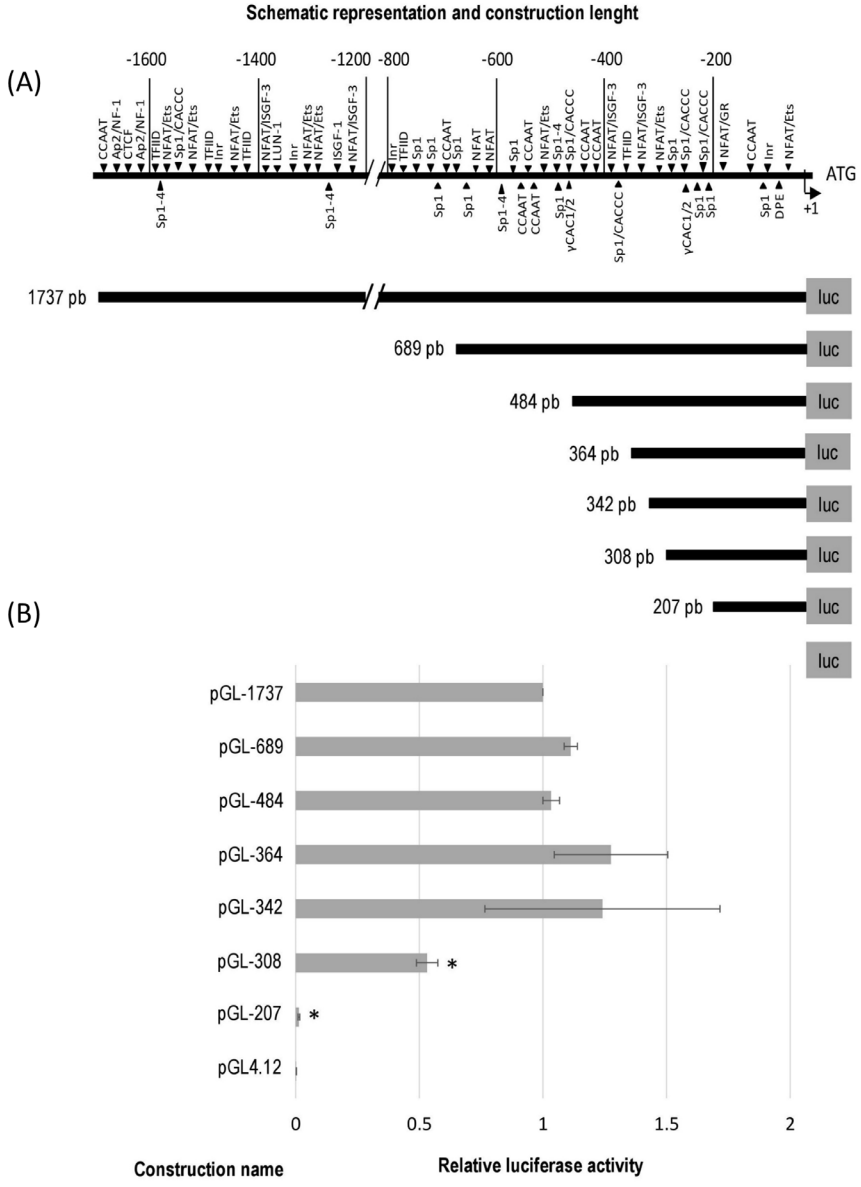

Figure 2. (**A**) Schematic representation of V3 promoter showing different response elements, including the core promoter elements. It is shown the size of the different constructs. (**B**) Relative luciferase activity of the different constructs is shown. All assay transfections were performed by triplicate in three different experiments. Data are expressed as mean ± SD (n = 3). Student's t-test was used and a $p < 0.05$ was considered statistically significant. *Statistical differences between the complete promoter activity and the different constructs.

was similar to that of the complete promoter. Therefore, these elements did not influence basal promoter activity. Next, in the 342 bp construct, the proximal TATA box was deleted, but this deletion did not modify the promoter activity. The constructs of 484 bp, 364 bp, 342 bp maintained the same activity as the complete promoter, none of the sequences eliminated participate in basal promoter activity.

Remarkably, the 308 bp construct had a decreased activity of 47% in comparison with that of the complete promoter. In this fragment, CTCF, NFAT and ISGF3 binding sites were deleted (Figures 1 and 2) ($p < 0.05$). The NFAT and ISGF3 sequences could participate in inducible activity, but not in basal activity. The CTCF binding site is a Zn-finger protein that can regulate gene transcription by activating or repressing promoters. At the promoter level, some evidence indicates that CTCF can activate the promoters of *c-Myc* and *GATA4* genes (*20,21*). In the former case, mutation assays indicate that a binding site for CTCF, alone, is required for the promoter activity of the *c-Myc* gene (*20*). Further molecular analysis, through ChIP assays, indicates that CTCF can bind and recruit RNA pol II at the CTCF binding site (*20*). In the latter case, immunoprecipitation assays show that CTCF can directly bind and recruit TAF3, a TBP-associated core promoter factor, to promoter distal sites of the *GATA4* gene (*21*). Site mutations could confirm the CTCF binding site as an activator of V3 promoter of the *ST3GAL4* gene.

Finally, the 207 bp fragment did not present promoter activity; for this construct, four GC boxes and other response elements, such as CTF, NFAT, Ets, AP2, and CACCC, were eliminated. The 207 bp fragment does not show any activity despite containing three elements: an Inr, a DPE and a CAAT box emphasizing the importance of the Sp1 boxes.

These results indicate that the minimal V3 promoter size of the *ST3GAL4* gene is approximately 308 bp. The region contains GC boxes, a CCAAT box, an Inr and DPE sites that together could maintain promoter activity (Figure 1 and 2). It is important to mention that the minimal construct that maintains the activity does not contain a TATA box, indicating that the V3 promoter is a TATA-less promoter and that other elements must participate in the activity of the promoter. Eukaryotic core promoters can include elements (a TATA box, the TFIIB-recognition element (BRE), an Inr, an MTE, a DPE and a DCE) that are necessary for the transcription preinitiation complex assembly, which includes general transcription factors such as TFIID and the RNA pol II, among others (*22*). Recently, bioinformatic analysis showed that approximately 80% of mammalian protein-coding genes are directed by TATA- less promoters (*23*).

Here, we showed that the version that maintains the promoter activity pGL4-12-308 contains several GC boxes (Sp1 binding sites) and CACCC, Inr and DPE sites. In TATA-less promoters, the presence of Sp1 is necessary for the binding of TFIID to the Inr site (*24*). It has been reported that Sp1 is a transcription factor that recognizes GC boxes and activates transcription through domains rich in glutamine residues (*25*). Therefore, in this core promoter, the binding sites for Sp1 must be essential for the activity. In TATA-less promoters, TFIID can bind to the Inr, primarily if Sp1 is bound to upstream sites (*24,26*). Molecular studies have shown that the DNA-binding domain of Sp1 is in contact with TAF7, and it has been proposed as a co-activator (*27*).

It could be possible that the GC boxes in the 308 bp fragment of the V3 promoter are crucial for the binding of TFIID to the Inr. This was supported by the deletion of the GC boxes in the 207 bp construct, which result in the loss of promoter activity. Additionally, it has been reported that both the Inr and the DPE can participate in basal transcriptional activity when the two elements are separated by 25-32 nt (*19*); however, when the distance between the elements increases, the function disappears (*28*). In the V3 promoter, the distance between these elements is 33 nt, so it could be of interest to determine the role of this binding site in promoter activity. Molecular studies have demonstrated that the Inr, alone or in conjunction with other elements, is capable of directing transcription initiation (*29*). More specifically, the Inr is recognized and binds to TFIID through the proteins TAF1/TAF2 (*24,29*).

It is important to mention that the V3 promoter contains several NFAT sites, and the NFAT (nuclear factor of activated T cells) proteins bind to certain regulatory elements in cytokine genes (*30*). For the pV2 promoter, it has been reported that its expression is induced by TNF, a cytokine that participates in inflammation, showing that the expression of this gene is modulated during inflammation (*3,9*). NFAT is a family of transcription factors that modulate the expression of genes that participate in the immune response, highlighting the importance of this gene in cancer and inflammatory disease (*30*). Nevertheless, further analysis is required to elucidate whether any of these binding sites are necessary for V3 promoter activity.

Finally, we have completed the gene structure including the promoters V1, V2 and V4 (previously reported) and the promoter V3, we also included the genomic structure of the V3 mRNA variant, which contains an extra exon named exon Y. It was named Y, considering that exon X corresponds to the V2 variant. Furthermore, the different exons that constitute the non-coding 5' region of the mRNA variants (V1-V9) are shown. V10 variant was not included because it does not codify for a protein (Figure 3).

In summary, we identified the V3 promoter of the *ST3GAL4* gene, which has a size of 1735 bp, and the core promoter was approximately 300 nt. The basal activity of this promoter is not regulated by a TATA box, and the results suggest that the GC boxes could

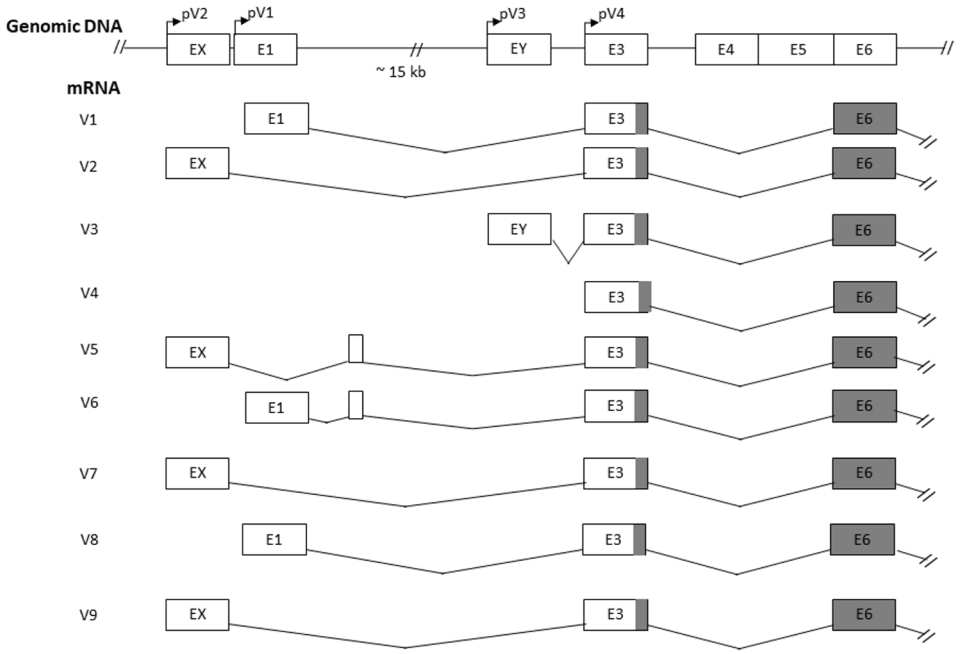

Figure 3. Schematic representation of the *ST3GAL4* gene structure including the promoters V1, V2 and V4 previously reported and the new promoter V3. It is shown in boxes the exons of the mRNA variants V1 to V9. It is also represented the structure of the mRNA variants at the non-coding 5' region, the grey boxes represent the coding region and the white boxes are the exons not translated.

regulate the basal activity in conjunction with the Inr and DPE elements. The promoter contains several NFAT sites that could participate in the transcription regulation of this gene during the immune response.

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