Characterization of the human *STAT5A* and *STAT5B* promoters: evidence of a positive and negative mechanism of transcriptional regulation

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Abstract We recently published the genomic characterization of the *STAT5A* and *STAT5B* paralogous genes that are located head to head in the 17q21 chromosome and share large regions of sequence identity. We here demonstrate by transient in vitro transfection that *STAT5A* and *STAT5B* promoters are able to direct comparable levels of transcription. The expression of basal promoters is enhanced after Sp1 up-regulation in HeLa and SL2 cells while DNA methylation associated to the recruitment of MeCP2 methyl CpG binding protein down-regulates *STAT5A* and *B* promoters by interfering with Sp1-induced transcription. In addition, cross-species sequence comparison identified a bi-directional negative *cis*-acting regulatory element located in the *STAT5* intergenic region.

Key words: *STAT5A*; *STAT5B*; Epigenetic control; Transcriptional regulation; *Cis*-acting element; Cross-species comparison

1. Introduction

The *STAT5A* and *STAT5B* genes belong to the signal transducer and activator of transcription (STAT) family of transcription factors. The family of *STAT* genes is believed to have been produced by successive genome duplication and functional divergence of a single ancestral *Stat* gene early in vertebrate evolution [1,2]. A recent duplication of *STAT5* is believed to have given rise to the closely related *STAT5A* and *STAT5B* homologous genes that encode proteins that in humans are approximately 94% identical, the only consistent difference between the two proteins residing in a five amino acid insertion at the COOH-terminus of STAT5B. The two STAT5 paralogues are able to homo- or heterodimerize with each other and subsequently can translocate to the nucleus. This function is essential for the activity of these transcription factors [3]. Despite the extensive similarity between the two STAT5 proteins, recent reports on *Stat5a/5b* knock-out mice have demonstrated that individual features are associated with the disruption of each individual gene. The double *STAT5a/b*$^{-/-}$ knock-out mouse has an impaired response to a number of cytokines, including erythropoietin, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 2 (IL-2) and growth hormone [4,5] and a profound deficiency in peripheral T-cell proliferation. When only the *Stat5b* gene is disrupted, the mutant phenotype is quite similar to the one observed in growth hormone receptor-deficient mice, including reduced levels of plasma insulin growth factor 1. When *Stat5a* is mutated, the single knock-out has a reduced response to several cytokines affecting lymphoid lineage, in particular GM-CSF and IL-2. The divergent phenotypic effect of the two mutations is also the result of a known tissue-specific difference in the relative abundance of *Stat5a* and *Stat5b* gene expression [5,6].

We have recently isolated and sequenced both *STAT5A* and *STAT5B* human genes finding that they have a very similar genomic organization. A CpG island is present at the 5′ end of each *STAT5* gene covering the 5′ untranslated region (UTR). More surprisingly the two genes share large regions of almost identical sequences that diverge among the different species indicating a species-specific mechanism of preservation. Furthermore, we have identified two alternative 5′ exons in the *STAT5B* gene, the second of which is not embedded in a CpG island [7].

The purpose of the present study is to identify and characterize the promoters of the human *STAT5A* and *B* genes to get insights into the mechanism that regulates the different levels of expression of these two genes. Cross-species sequence comparison of human and mouse sequences has revealed the presence of three conserved regions: two are located in the 5′ flanking region of each *STAT5* gene, spanning the CpG islands and the transcriptional start sites; the third is located in the intergenic region between *STAT5A* and *STAT5B* at a distance of 5200 bp from *STAT5B* and 3700 bp from *STAT5A*, referred to the transcriptional start sites.

Functional analysis of these predicted UTRs in transient transfection in both mammalian and *Drosophila* cells reveals that the two *STAT5* promoters have a quite similar genetic architecture and that the 5′ flanking region of *STAT5* con-

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Abbreviations: STAT, signal transducer and activator of transcription; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-2, interleukin 2; PCR, polymerase chain reaction; CNS, conserved non-coding sequence; UTR, untranslated region

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tains important *cis*-acting elements required for positive and negative regulation of transcription.

2. Materials and methods

2.1. Cell culture

Human HeLa (cervix epithelioid carcinoma) and HepG2 (hepatocellular carcinoma) cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Human Jurkat cells (acute T-cell leukemia) were maintained in RPMI 1640 medium with 10% fetal calf serum. The media were supplemented with penicillin (100 U/ml), and streptomycin (100 μg/ml) and the cultures were propagated at 37°C in a humidified 5% CO_2 atmosphere. *Drosophila* SL2 cells [8], derived from late *D. melanogaster* embryos, were grown in Hyclone serum-free medium (HyQ® CCM3) plus antibiotics at 25°C.

2.2. Expression plasmid construction

The *STAT5A* and *STAT5B* fragments were amplified by polymerase chain reaction (PCR) from human genomic DNA using the primers listed in Table 1. Each primer was designed to contain a tail that includes a specific restriction enzyme sequence (*Bgl*II in the 5′ primer and *HindIII* in the 3′ primer). All the fragments were inserted into the pGL3 Basic Vector (Promega), upstream of the *Photinus pyralis* luciferase coding sequence (pA1Luc, pA2Luc, pA3Luc, pB4Luc, pB5Luc) at the restriction sites reported above and in the orientation indicated in Fig. 3. The generated fragments were sequenced and the orientation was checked after cloning into the pGL3 vector. In addition, the B4 and B5 fragments from *STAT5B* were amplified and cloned also in the pGL3Enhancer vector that contains the SV40 enhancer positioned downstream of the luciferase coding sequence (see Fig. 3B). The Inter 1 and Inter 2 regions were amplified using primers listed in Table 1. The two amplified fragments were inserted upstream of the A2 and B5 fragments in pA2Luc and pB5Luc. Direct and inverted orientations in relation to the transcription start site (see Fig. 5) were obtained in one case using tailed primers containing the recognition sequences *Sac*I in the 5′ primer and *Xho*I in the 3′ primer and in the other case using tailed primer containing the recognition sequences for *Xho*I in the 5′ primer and *Sac*I in the 3′ primer. The Inter 1 and Inter 2 regions, amplified as described above, were also fused in both orientations upstream of the *NEMO/IKK-γ* human promoter in pGL3 Basic vector (pF4-IKK-γLuc; F. Fusco, unpublished results and [13]). All the generated fragments were checked by sequence analysis.

2.3. In vitro DNA methylation

The pA2Luc and pB5Luc constructs were treated with the *Sss*I methylase (New England Biolabs) to obtain methylated DNA; the reaction was carried out in presence of 5 mM *S*-adenosylmethionine in accordance with the manufacturer's protocols. The methylase reaction was assessed by gel electrophoresis after *Hpa*II digestion of DNA. pA2Luc and pB5Luc constructs treated as reported above but without the *Sss*I methylase were used as controls.

2.4. Transient transfections and luciferase assay

Human cell lines were transiently transfected with chimeric luciferase constructs. Unless otherwise reported, 1 μg of DNA was co-transfected with 20 ng of pRL-CMV (Promega) on to approx. 30% confluent cell plate using FuGENE6 reagent (Roche) according to the manufacturer's recommended conditions. The pRL-CMV vector contains the *Renilla* luciferase gene from *Renilla reniformis* transcribed by the CMV promoter. *Drosophila* SL2 cell transfection and pPACSp1 construct were as described [9]. The human MeCP2 expression plasmid was kindly provided by S. Kudo [10].

Each plasmid containing different promoter fragments to be tested was co-transfected with the pRL-CMV vector in the selected cell line. Thus, the dual-luciferase system is based on the measurement of both firefly luciferase and *Renilla* luciferase activities in the same sample. Firefly luciferase activity was assayed in a Turner TD2420 luminometer, as well as *Renilla* luciferase activity. Data are reported as the ratio of firefly/*Renilla* arbitrary units/1000 and indicated as relative luciferase activity in the legends to Figs. 3–5. Each value is the mean and standard deviation of results from three independent experiments. Statistical elaboration of data was performed using Microsoft Excel 2000 software.

2.5. Bioinformatic analysis

Genomic sequences spanning the entire *STAT5A*, *STAT5B* and the intergenic region were analyzed using the alignment software m-Vista: http://www-gsd.lbl.gov/vista/VistaInput that allows you to align two chosen sequences and to determine the percentage of identity between them. Conserved segments can be determined choosing a percentage identity of at least of *x*% and a length of *y* bp. These cut-offs (*x*% over *y* bp) are specified by the user. We chose 80% identity and 70 bp length, as reported in Fig. 1 [11].

CpG islands and relative plot were generated by Microsoft Excel 2000 as previously described [7], the ordinate corresponds to the algorithm used, that is $Y=((140-(9\times(A1-A2+1.5)^2))^{1.5})/160$, where A1 and A2 are the distances of two CpGs along the sequence.

Promoters were predicted with Promoter Scan: http://bimas.dcrt.nih.gov/molbio/proscan/ [12]. Putative regulatory regions were scanned to predict transcription factor binding sites with the Genomatix suite: http://www.genomatix.de/.

3. Results

3.1. Computer analysis and transcriptional activity of the STAT5A and B promoters in HeLa, HepG2 and Jurkat cell lines

In order to identify conserved non-coding sequences (CNSs) in the *STAT5* genes, we performed pairwise alignment of the sequence between the two genes (about 11 kb in human) from human (AC099811: 146 461/160 861) and mouse (AC073918: 100 021/141 301) (about 40 kb in mouse) using the alignment software m-Vista with a range that allows you to identify regions which have at least 80% identity and are 70 bp length (Fig. 1). Three highly conserved regions were identified: *region I* (Figs. 1a and 2a) located between nucleotides −2119 and +1075 in relation to the *STAT5A* major transcriptional start site (*region I*, 38.4% mean identity); *region II* (Figs. 1a and 2a) located at positions −1690/+1739 (*region II*, 46.8% mean identity) from the *STAT5B* transcriptional start site; and *region III* (Figs. 1a and 2b) that contains two fragments

Table 1
PCR primers

Fragment	Forward	Reverse
A1	5′-gccgagatctgcaggtaatgaaatggaagtc-3′	5′-gccgaagcttgatcctgccaactaagacac-3′
A2	5′-gccgagatctgcaggtaagaaatggaagtc-3′	5′-gccgaagcttaggacgcgccaccgggtc-3′
A3	5′-gccgagatctcctcactccccatctaatac-3′	5′-gccgaagcttgcaggtaagaaatggaagtc-3′
B4/B4Enh	5′-gccgagatctgacccagcgcaggcaact-3′	5′-gccgaagcttcaatgaacataacgcaatat-3′
B5/B5Enh	5′-gccgagatctaaatgtcaccgaagggcaga-3′	5′-gccgaagcttcaatgaacataacgcaatataa-3′
Inter 1	5′-gccggagctctctttccctcagcctaatcc-3′	5′-gccgctcgagaagttctgtgctggtttagg-3′
	5′-gccgctcgagtctttccctcagcctaatcc-3′	5′-gccgagctcaagttctgtgctggtttagg-3′
Inter 2	5′-gccgagctctgctgagtctgtgctggctt-3′	5′-gccgctcgagaaggaaggtcaagtcaaggt-3′
	5′-gccgctcgagtgctgagtctgtgctggctt-3′	5′-gccgagctcaaggaaggtcaagtcaaggt-3′

Primers used to amplify the region from *STAT5A* and *STAT5B* and from *region III*, Inter 1 and Inter 2. The underlined sequences correspond to the restriction sites used for the cloning in the pGL3 vectors.

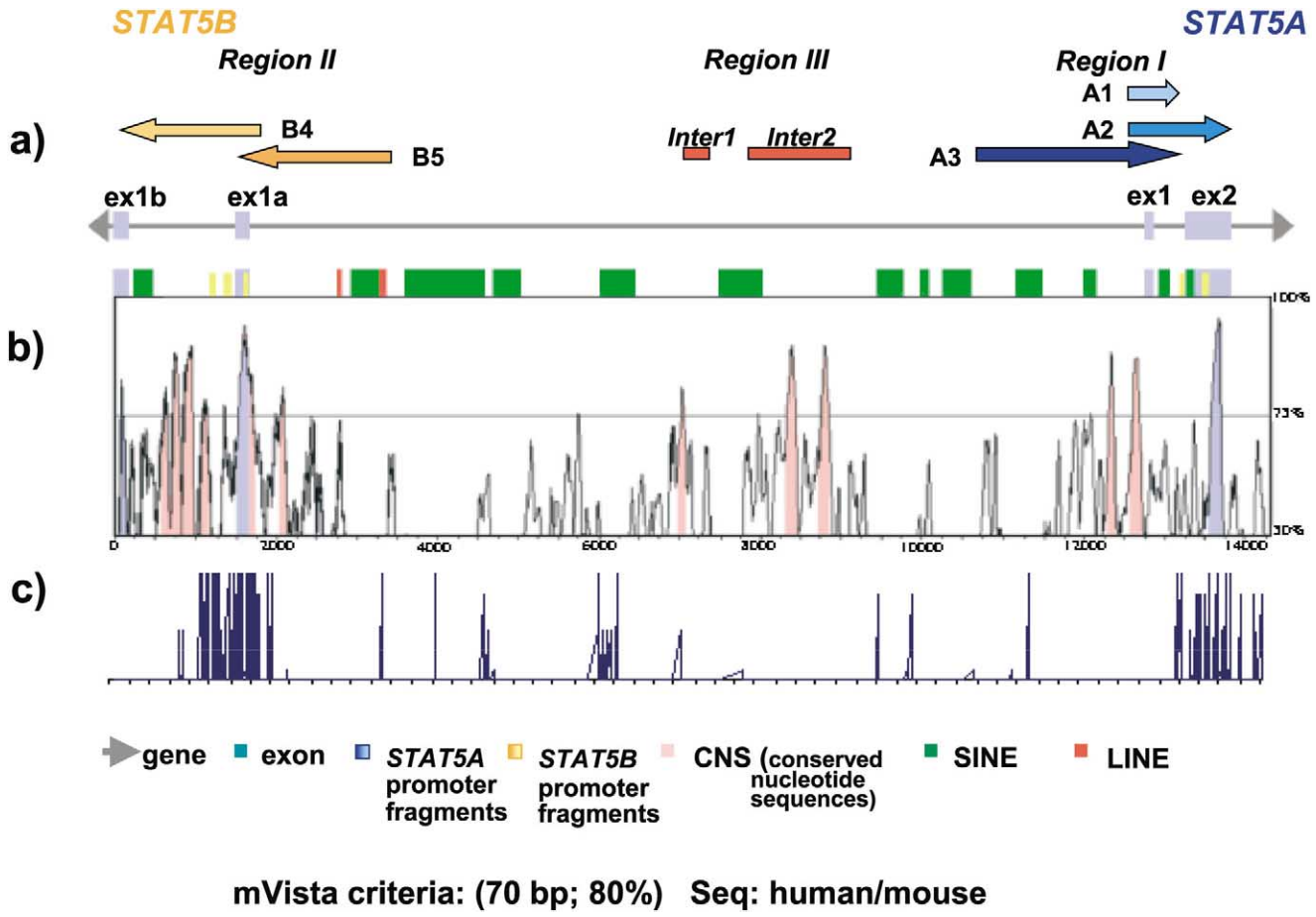

Fig. 1. m-Vista alignment of mouse/human genomic sequences (mouse accession number AC073918, human accession number AC099811) spanning the *STAT5A*, *STAT5B* intergenic region. a: Descriptions of the fragments used in the transient transfection assays: nucleotide positions, referred to human accession number are as follows: *region I*, fragment A1 = 158 858/159 531, fragment A2 = 158 858/160 364, fragment A3 = 157 168/159 531 (total length 3195); *region II*, fragment B4 = 147 961/149 895, fragment B5 = 146 488/148 100 (total length 3429 bp); *region III*, Inter 1 = 153 287/153 876, Inter 2 = 154 266/155 908 (total length 589 bp, 1642 bp, respectively). b: Human and mouse intergenic region alignment: m-Vista criteria used are 80% identity for at least 70 bp length. c: CpG distribution and relative plot were generated using Microsoft Excel 2000 as previously described [7]

with extensive mouse/human identity at −5082 (Inter 1, 50% mean identity) and at −6061 (Inter 2, 50% mean identity) from the *STAT5B* transcriptional start site.

Furthermore, in each of the two 5′ flanking conserved regions (*region I* and *region II*, Fig. 1) a CpG island was identified. A CpG island of 23.2%, extending from the 5′UTR and covering the entire exon 2 of *STAT5A* for a total length of 690 bp is located in *region I*; the other CpG island of 30.2%, 900 bp length covering exon 1a and extending to the putative promoter of *STAT5B* is located in *region II*. Of note, the two start sites are located at position −642 bp from ATG on the *STAT5A* mRNA and at −227 bp from the ATG on the *STAT5B* mRNA [7].

Region I and *region II* contain the *STAT5A* and *STAT5B* major transcription start sites, respectively. Both lack TATA and CAAT positioning elements, and are characterized by putative binding sites for transcription factors known to initiate transcription in TATA-less promoters such as Sp1, CAAT etc. (summarized in Fig. 2). Indeed, Promoter Scan software analysis assigns to *region I* and *region II* the putative promoters.

Region III contains two sequences of 589 bp (Inter 1) and 1642 bp (Inter 2) long, respectively, that share high identity with the intergenic mouse *Stat5* sequence (Fig. 1).

We analyzed the transcriptional activity directed by *region I* and *region II* in mammalian cells. For this purpose we used three *STAT5A* genomic fragments fused to the luciferase reporter gene in the pGL3 Basic vector (A1 fragment from −430 to +244, A2 from −430 to +1075, A3 from −2119 to +244, Fig. 3a). In pGL3 Basic we also subcloned two *STAT5B* genomic fragments (B4 from +1739 to +100 that includes the alternative 1b exon, B5 from −1690 to +236, Fig. 3a). The luciferase diagrams shown in Fig. 3 clearly indicate that the two most active fragments are those containing the CpG islands and that the *STAT5A* promoter is more active than the *STAT5B* one in all cell lines. In contrast, the *STAT5B* alternative promoter (pB4Luc) cannot drive detectable luciferase activity. To determine whether the activity of the *STAT5B* alternative promoter was too low to be measured by standard luciferase assay and/or whether any important regulatory element was lost in our artificial constructs, we fused the B4 and B5 fragments upstream of the SV40 enhancer in the pGL3 Enhancer vector (Fig. 3b). The luciferase diagram clearly shows that while the transcriptional activity of

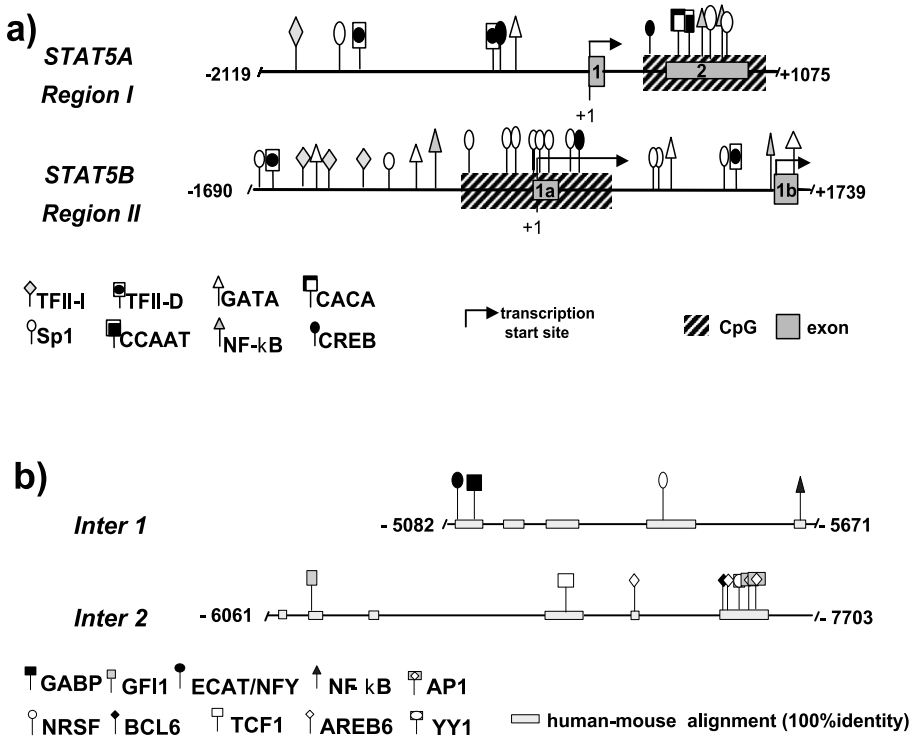

Fig. 2. Analysis of the putative transcriptional binding sites present in the *STAT5A* and *STAT5B* promoters and in Inter 1 and Inter 2. a: *Region I* and *region II* corresponding to the *STAT5A* and *STAT5B* promoters, respectively. The positions are numbered relative to major transcription start sites that are located at position −642 bp from ATG on *STAT5A* mRNA and at −227 bp from the ATG on *STAT5B*. b: Binding sites on the Inter 1 and Inter 2 sequences. The regions of 100% human/mouse identity are boxed.

pB5 Enhancer is strongly enhanced compared to pB5Luc, the luciferase activity driven by pB4 Enhancer remains undetectable.

3.2. *Effect of methylation on* STAT5A *and* STAT5B *promoter activity in constitutive or Sp1-induced transcription in HeLa and* Drosophila *cells*

As reported above, we have found that the regions adjacent to the transcription start site are characterized by a high amount of GC and putative binding sites for Sp1. To confirm the biological effect of Sp1 on the *STAT5A* and *STAT5B* transcription rate, we performed co-transfection experiment in *Drosophila* SL2 cells, which are devoid of Sp1 activity [9]. Some of the *STAT5A* and *STAT5B* luciferase-fused constructs were co-transfected with Sp1-expressing plasmid (pPACSp1) in *Drosophila* cells; the above constructs were not co-transfected when used as a control. The luciferase diagram indicates that Sp1 efficiently transactivates the two chimeric constructs containing the putative Sp1 binding sites (50-fold induction for pA2Luc, 30-fold induction for pB5Luc, Fig. 4a). The basal activity of both *STAT5A* and *STAT5B* promoters in SL2 cells is almost undetectable, thus suggesting that Sp1 is one of the major transcription factors required for the activity of these promoters. In *Drosophila* cells expressing exogenous Sp1, *STAT5A* promoter-driven activity is higher than *STAT5B*.

Sp1 binding sites are well known to play a key role in the maintenance of a methylation-free CpG island in housekeeping genes. Therefore, we tested whether Sp1 sites are still biologically active when promoters are methylated. *STAT5A* and *STAT5B* promoters, contained in the plasmids pA2Luc and pB5Luc respectively, were in vitro methylated by the *Sss*I CpG methylase before co-transfection with Sp1 in SL2 cells. The luciferase diagram shows that methylation only slightly reduces promoter activity for both genes (Fig. 4a). In order to determine whether methyl CpG binding proteins could interfere with the activity of *STAT5A* and *STAT5B* promoters, we co-transfected *Drosophila* cells with an expression vector producing the human methyl binding protein MeCP2. It is well known that CpG methylation down-regulates transcription by preventing the binding of transcription factors to their recognition sequences and that MeCP2 is one such repressor [10]. The luciferase diagram shows that both *STAT5A* and *STAT5B* promoter activity is greatly reduced thus indicating that the presence of methyl CpG binding protein inhibits Sp1 binding and transcriptional up-regulation (Fig. 4a).

Therefore, we tried to test if the down-regulatory effect of methylation on the *STAT5A* and *STAT5B* promoters was reproducible in mammalian cells, which endogenously express both Sp1 and MeCP2. For this purpose the in vitro methylated pB5Luc and pA2Luc constructs were transfected in HeLa cells. The luciferase diagram clearly shows that the transcriptional activity of the *STAT5A* and *STAT5B* promoters is substantially reduced by CpG methylation (Fig. 4b).

3.3. *Functional analysis of conserved CNSs located in* region III

In order to define if the two conserved CNSs located in *region III* (Fig. 1) have any regulatory role in the transcription driven by the two divergent *STAT5* promoters, we fused the Inter 1 and Inter 2 regions upstream of the A2 and B5 fragments in pA2Luc and pB5Luc constructs, respectively, in both orientations (Fig. 5). We found that both Inter 1 and Inter 2 sequences exert a strong orientation-independent negative ef-

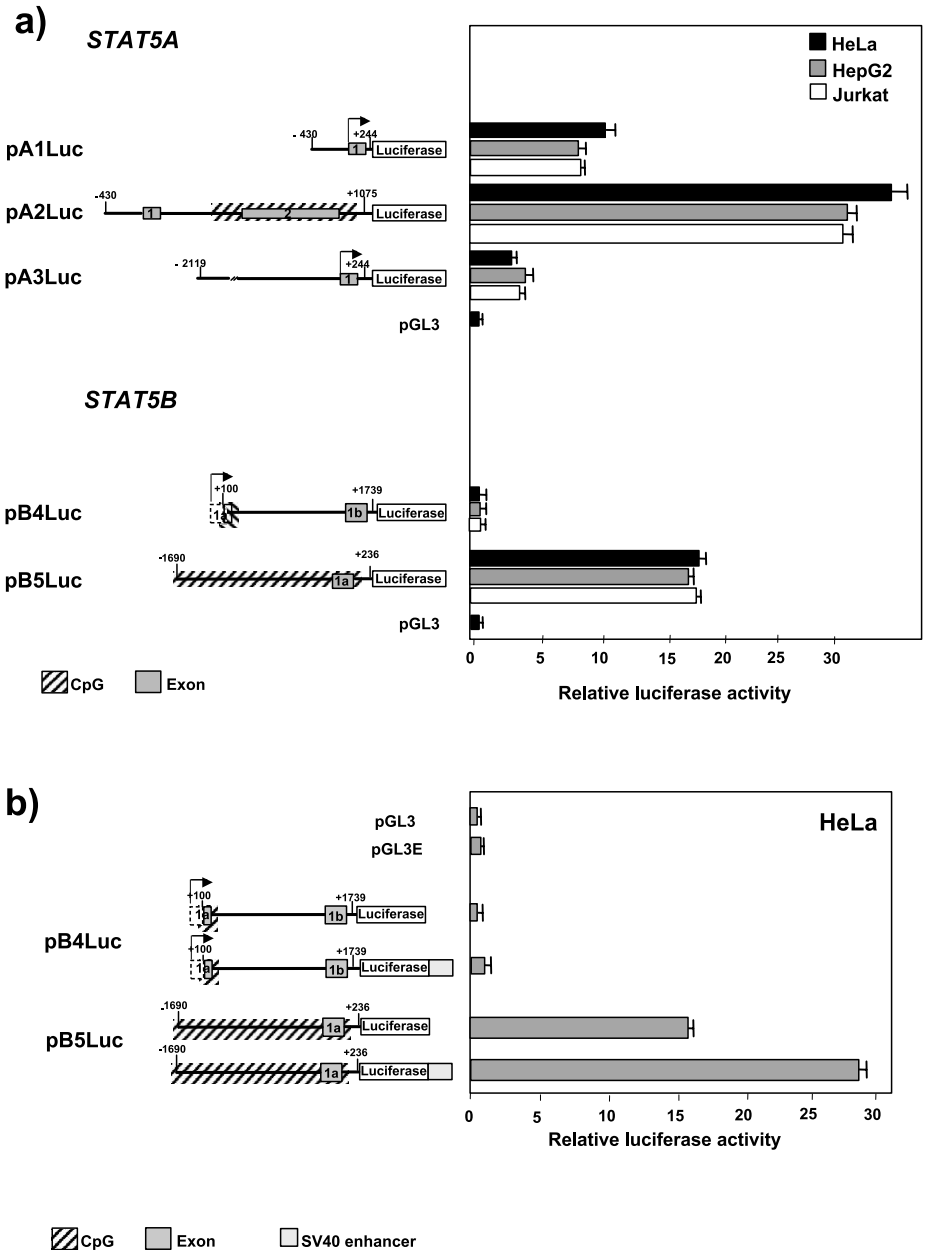

Fig. 3. Transcriptional activity of the *STAT5A* and *STAT5B* promoters in HeLa, HepG2 and Jurkat cells. a: Different constructs spanning the *STAT5A* or *STAT5B* promoters activate luciferase. b: Luciferase activity driven by fragments B4 and B5 inserted in pGL3 Basic vector or pGL3 Enhancer vector in HeLa cells. Empty vectors (pGL3, pGL3E) were used as controls. Relative luciferase activity corresponds to the ratio firefly/*Renilla* activity/1000 for each data point. The results represent the averages ± S.D. of at least three independent experiments. The arrows indicate the +1 transcriptional start site.

fect on the transcription driven by *STAT5* promoters (Fig. 5). This result is not surprising because this region presents a high density of binding sites for negative transcriptional regulators when subjected to in silico analysis with the Genomatix suite (Fig. 2b). Furthermore, we inserted the Inter 1 and Inter 2 sequences in both orientations upstream of the *IKK-γ* human promoter in pGL3 vector. This fragment contains the human *IKK-γ* promoter region spanning from −1089 to +79 (in relation to the transcription start site in exon 1a) (Fig. 5b) [13]. We found that the Inter 1 and Inter 2 regions, in both orientations, can down-regulate this promoter that has a transcriptional activity stronger (six-fold higher) than *STAT5A*.

4. Discussion

In this paper, we report the first functional characterization of the 5′ flanking region of both *STAT5A* and *STAT5B* human genes and their transcriptional activity in mammalian and *Drosophila* SL2 cells. In order to identify sequences that might have a *cis*-acting regulatory role, we searched for conserved regions in the 5′ flanking region of these genes. The two human genes lie head to head at a distance of 11 kb; therefore we screened the entire region comprised between them comparing human and mouse sequences. This analysis revealed three highly conserved upstream CNSs, which are

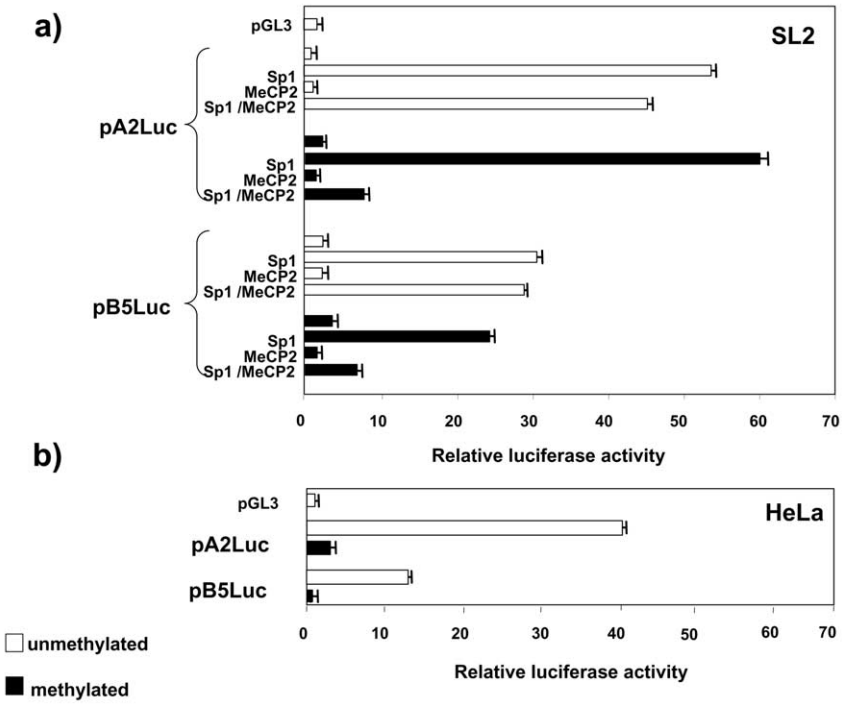

Fig. 4. Transcriptional activity of methylated *STAT5A* and *STAT5B* promoters. a: Methylated pA2Luc and pB5Luc were transiently co-transfected in SL2 cells with indicated expression plasmids (Sp1, MeCP2); unmethylated DNA was used as control. b: Methylation of pA2Luc and pB5Luc fragments eliminates the luciferase activity in HeLa cells. The data represent the averages ± S.D. of at least three independent experiments.

3195 bp (*region I*), 3429 bp (*region II*) and 2400 bp (*region III*) long, respectively. The *STA5A* promoter is predicted in *region I* and the *STAT5B* promoter in *region II*. *Region III* is located 3380 bp from the *STAT5A* transcriptional start site (5082 bp from *STAT5B*) and contains two specific sequences (Inter 1 and Inter 2) that share a high level of identity with a region located between the murine *Stat5* genes (see Fig. 1). Putative recognition sequences for the transcription factors Sp1, GATA, and NF-κB were identified in both mouse and human *STAT5* promoter sequences (*region I* and *region II*), while TATA and CAAT positioning elements were absent (see Fig. 2). A CpG island was identified in both *STAT5A* and *STAT5B* promoters.

Functional analysis of the predicted CNSs by transient transfection in HeLa, Jurkat and HepG2 mammalian cells, as well as in *Drosophila* SL2 cells, using the luciferase reporter system, reveals some interesting features of the *STAT5* promoters.

1. First, we demonstrate that the region just upstream of the transcription start sites of the *STAT5A* and *STAT5B* genes possesses promoter activity when placed 5′ adjacent to the reporter luciferase gene and that the CpG island is required in both promoters for the basal transcriptional activity.
2. *STAT5A* and *B* promoter-directed transcription is comparable in all cells tested. Indeed, prediction of binding sites failed to reveal among them any substantial qualitative and quantitative difference in composition of the putative sites.
3. Sp1, which is the most frequent site observed on both *STAT5* promoters, is a strong activator of *STAT5A* and *STAT5B* transcription in *Drosophila* SL2 cells. Therefore, Sp1 is the major determinant of *STAT5* gene transcription.
4. Both *STAT5A* and *STAT5B* promoters are down-regulated by DNA methylation and recruitment of MeCP2 methyl binding protein. The transcriptional down-regulation is detectable in HeLa cells expressing endogenous MeCP2 as well as in SL2 cells expressing exogenous human MeCP2 and Sp1. Thus we may hypothesize that a cell/stage-specific regulation of *STAT5A* and *STAT5B* expression could be epigenetically determined and maintained through DNA methylation.
5. Finally, the conserved intergenic *region III* that contains several putative negative regulatory binding sites such as GAPB, GFl1, ECAT/NFY, BCL6, AREB6, YY1, does indeed exert a strong orientation-independent *cis*-negative effect on the transcription driven by the *STAT5A* and *B* promoters. This region is also able to exert orientation-independent down-regulation on a heterologous promoter. It is worth noting that several of the predicted repressors such as Ikaros1, POZ/zinc finger (BCL-6), GF1-IB have a role in normal lymphoid development [14,15].

In conclusion, first insights into the regulation of human *STAT5A* and *STAT5B* expression are produced by coupling scanning of their entire intergenic sequence with bioinformatic tools, to in vitro promoter functional assay. These findings should promote a better understanding of the molecular basis for variations of the reciprocal *STAT5A* and *STAT5B* expression in various human tissues as well as a deeper analysis of the methylation status of these two genes in specific developmental stages/tissues. For instance, Buitenhuis et al. [16] have recently demonstrated down-regulation of expression of both

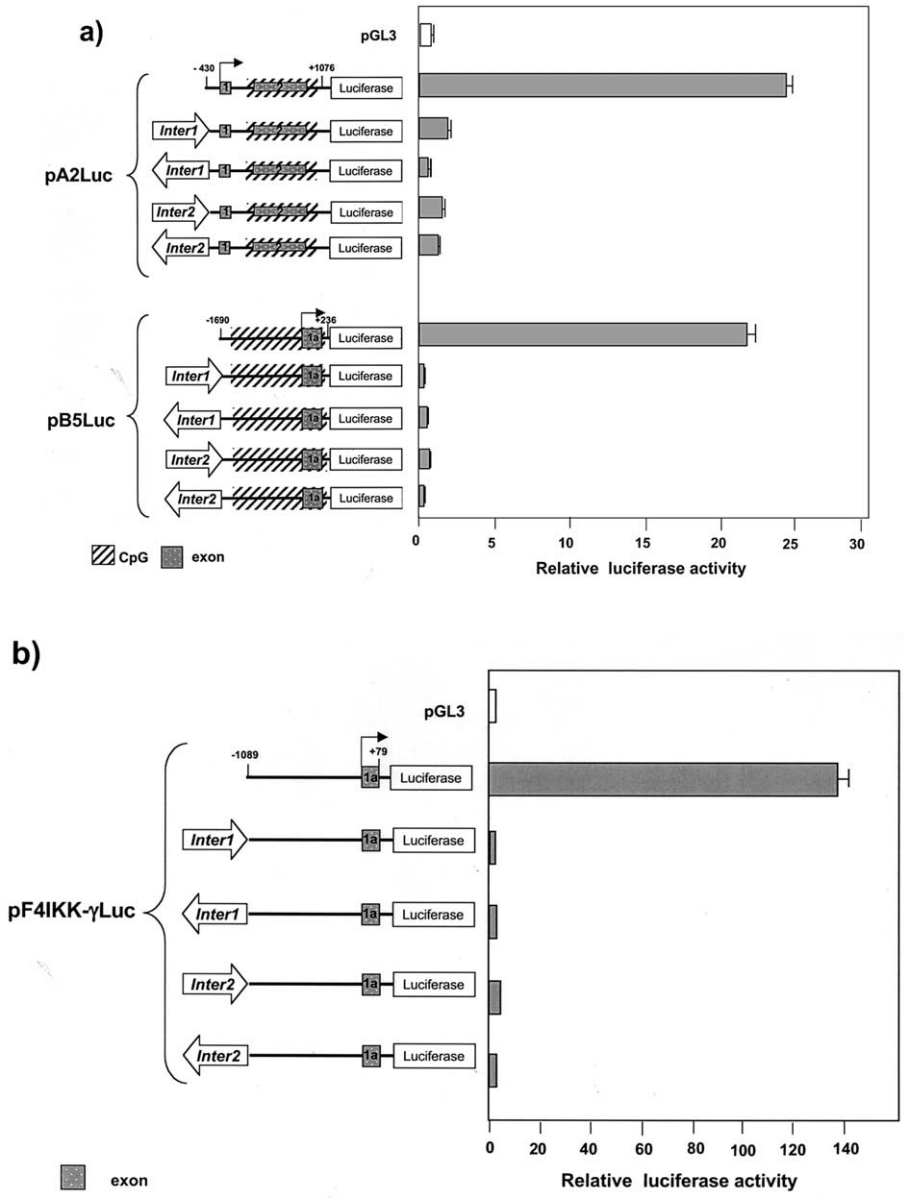

Fig. 5. Down-regulatory effect mediated by *region III*. a: The Inter 1 and Inter 2 fragments were inserted upstream of the A2 and B5 fragments in both orientations. b: The Inter 1 and Inter 2 regions were also fused upstream of the *IKK-γ* promoter in the pF4-IKK-γLuc chimeric construct. The data reported here do not represent a quantitative comparison of the basal promoter activity but only the average down-regulatory effect of Inter 1 and Inter 2 on *STAT5A*, *STAT5B* and *IKK-γ* promoter activity. The data represent the averages ± S.D. of at least three independent experiments. The arrows indicate the +1 transcriptional start site.

*STAT5*s during the final stage of eosinophil differentiation, thus suggesting a critical role for these genes in human primary hematopoietic cell differentiation. However, the molecular mechanism underlying the down-regulation of *STAT5*s remains largely unexplored.

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