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Transcriptional regulation of the human mitochondrial peptide deformylase (*PDF*)

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ABSTRACT

The last years of research have been particularly dynamic in establishing the importance of peptide deformylase (PDF), a protein of the N-terminal methionine excision (NME) pathway that removes formylmethionine from mitochondrial-encoded proteins. The genomic sequence of the human *PDF* gene is shared with the *COG8* gene, which encodes a component of the oligomeric golgi complex, a very unusual case in Eukaryotic genomes. Since PDF is crucial in maintaining mitochondrial function and given the atypical short distance between the end of *COG8* coding sequence and the *PDF* initiation codon, we investigated whether the regulation of the human *PDF* is affected by the *COG8* overlapping partner. Our data reveals that *PDF* has several transcription start sites, the most important of which only 18 bp from the initiation codon. Furthermore, luciferase-activation assays using differently-sized fragments defined a 97 bp minimal promoter region for human *PDF*, which is capable of very strong transcriptional activity. This fragment contains a potential Sp1 binding site highly conserved in mammalian species. We show that this binding site, whose mutation significantly reduces transcription activation, is a target for the Sp1 transcription factor, and possibly of other members of the Sp family. Importantly, the entire minimal promoter region is located after the end of *COG8*'s coding region, strongly suggesting that the human *PDF* preserves an independent regulation from its overlapping partner.

1. Introduction

Peptide deformylase is an an evolutionary conserved zinc [1,2] and iron [3,4] binding metalloprotease that removes the formyl group of nascent methionines in eukaryotic organelles and bacteria. The critical role of PDF in protein biosynthesis was for long considered unique to prokaryotic organisms and its apparent absence in eukaryotic cells suggested its use as a target for antimicrobial drugs [5]. Giglione and colleagues [6] used *Arabidopsis thaliana* as a model organism and identified PDF homologous proteins in other eukaryotes, including humans. These results substantiated the essential role for PDF in eukaryotic protein processing [7,8] and permitted the design of drugs to target PDF taking into account differences between human and bacterial enzymes. Subsequent studies proved that human PDF is active [8,9] and that its inhibition impairs mitochondrial translation and the assembly of oxidative phosphorylation complexes [9].

Several studies have drawn attention to singular aspects of human PDF. Apart from the well-known unique catalytic features such as an extra N-terminal domain and specific and interdependent amino acid substitutions at critical positions [8], we previously demonstrated [10] that the mRNA sequences of *PDF* and *COG8* overlap at their 3′UTR up to the polyadenylation signal in some mammals. In the case of humans, the genomic distance between *COG8* stop and *PDF* start codons is only 268 bp.

Given the critical role of human PDF in mitochondrial mRNA translation and maintenance of the integrity of oxidative phosphorylation complexes [9], and because no studies have been performed addressing how this gene is regulated, we set out to investigate *PDF* transcriptional regulation. Here we present results that: (a) define *PDF*'s TSSs and minimal promoter region, (b) identify at least one transcription factor critical for gene expression and (c) indicate that *PDF* conserves its own promoter regulation, independently of its *COG8* overlapping partner.

2. Materials and methods

2.1. 5′ Expressed sequence tag (EST) analysis

The *PDF* 5′ EST transcripts used in this study were retrieved from UniGene (http://www.ncbi.nlm.nih.gov/unigene) and aligned using default settings of the MUSCLE 3.6 software implemented in

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Geneious v5.5 (www.geneious.com). The GenBank accession numbers of the 5'ESTs used are: BX405999, CN309857, BF795065, DB504077, AF239156, NM_022341, BI823695 and BM845666.

2.2. 5′-Rapid amplification of cDNA ends (RACE) and transcription start site (TSS) identification

5'-RACE cDNA synthesis was made with the SMARTer™ RACE Amplification Kit (Clontech) using 1 μg of human total placental RNA (Clontech) or 1 μg of total liver RNA (Ambion), according to the manufacturer's protocol. The 5′-RACE PCR was performed as described in [10]. After the 5′-RACE PCR, a hemi-nested PCR was performed using the manufacturer's conditions and 1 μl of the *PDF*-specific primer (Table S1). PCR products were resolved on a 1% agarose gel, gel-extracted and purified. The liver and placenta purified 5′-RACE PCR products were cloned in a TA-cloning plasmid (pUK-TA, in-house developed). Twenty-four clones of each product were selected, extracted and sequenced using TA-F and TA-R primers (Table S1), the Big-Dye Sequencing Kit and an ABI Prism 3130XL Sequencer (Applied Biosystems). To determine the *PDF* TSS and the 5'UTR sequence, clones were aligned against the *PDF* genomic sequence as described above.

2.3. Construction of luciferase reporter plasmids

The fragments of the human *PDF* gene were amplified from 20 ng of genomic DNA of the AGS human cell line using a common reverse primer (immediately upstream of the *PDF* initiation codon) and distinct forward primers (Table S1). These fragments were amplified as previously described [10]. The amplified fragments were gel-extracted, purified and cloned into pTA-Luc, an in house-developed TA-cloning vector with a luciferase gene.

The search for putative transcription factor binding sites in the −168 to −1 *PDF* region was performed in TFSEARCH [11] using a vertebrate matrix and a threshold score of 85.0. Reporter plasmids with mutant putative Sp1 binding sites were generated by PCR-mediated site-directed mutagenesis (primers in Table S1). All plasmids were sequenced using the Luc-F and Luc-R plasmid primers, to assure that they had the predicted sequences. Additional details of plasmid construction are available upon request.

2.4. Cell lines and transient transfections

The HEK293 cell line was maintained in Dulbecco's modified Eagle medium (Invitrogen), supplemented with 10% fetal bovine serum and 1% of a penicillin/streptomycin solution. For transient transfections, 1×10^5 cells per well were plated in 1 ml of medium on a 24-well plate and incubated to 50% confluency. Transfections were done in triplicate using 2.5 μl of Lipofectamine™ 2000 reagent (Invitrogen), 0.5 μg of reporter plasmid and 0.1 μg of pCMV-β-galactosidase (Clontech) per well. Lipofectamine™/DNA mixtures were incubated for 20 min at room temperature prior to addition to the cells. Cells were collected to determine luciferase and β-galactosidase activities 36 h later.

2.5. Luciferase and β-galactosidase assays

Luciferase and β-galactosidase assays were performed according to the protocol recommended by the manufacturer (Promega). Luciferase activities were determined using a 1450 TriLux MicroBeta luminescence counter (PerkinElmer) and β-galactosidase activity was measured at 415 nm in a Microplate Reader (BioRad). Luciferase activities were normalized against the β-galactosidase values.

2.6. Statistical analysis

To determine the statistical significance of differences between average luciferase activity values, we used a two-tailed Student's *t*-test considering a statistically significant difference when *P*-value < 0.05.

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear protein was prepared from the HEK293 cell line using the NE-PER® nuclear and cytoplasmic extraction reagents (Pierce) following the manufacturer's protocol. Oligonucleotide probes for EMSA were designed to include the Sp1 binding site closest to the initiation codon in the *PDF* promoter, at positions −38 to −14 bp from the ATG. Oligonucleotide probes were synthesized with HPLC purification (Thermo Fisher Scientific) with (for biotin end-labeled probes) or without (for unlabeled probes) 3′biotin modification (Table S1). Complementary oligonucleotides were annealed by heat reduction at a 1:1 M ratio. EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's instructions using 1 μg of nuclear protein, 200-fold molar excess (4 pmol) of unlabeled DNA probe for the specific or nonspecific competition assays, 20 fmol of wild-type or mutated biotin end-labeled probes and 2 μg of anti-Sp1 (sc-59x; Santa Cruz Biotechnology), anti-Sp3 (sc-644x) or anti-AP-2α (sc-184x) in the supershift experiments. The reaction products were separated by nondenaturing 6% polyacrylamide gel electrophoresis, transfer to positively charged Biodyne B membrane (Pierce), UV-crosslinked and detected by Chemiluminescent Nucleic Acid Detection Module (Pierce), following manufacturer's instructions.

3. Results

3.1. Identification of PDF transcription start sites and 5′UTR sequence

In order to identify potential human *PDF* TSS, we analysed the 5′ESTs of this gene available at the UniGene database. We verified that all *PDF* sequences are annotated as *COG8* due to the *COG8-PDF* overlap (described in [10]). To guarantee the retrieval of *PDF*-specific sequences, we performed an alignment of all *PDF*-5′ESTs against the *PDF* reference sequence (ENSG00000258429) plus 1 Kb upstream the *PDF* initiation codon. This approach resulted in the recovery of eight small-sized transcripts with multiple TSSs ranging from 5 to 37 bp upstream the *PDF* initiation codon. No obvious dominant TSS could be detected from the analysis of the UniGene data, even though the reference sequence present in the databases has a 5′UTR starting 25 nucleotides upstream the ATG. In order to experimentally assess this apparent TSS variability, we performed 5′RACE in two human tissues (liver and placenta), cloned the resulting PCR products, and determined the sequence of 24 clones for each tissue. As shown in Fig. 1, these experiments revealed a predominant TSS, located 18 bp from the initiation codon (18 clones in placenta and 10 in liver). Interestingly, we observe a wide heterogeneity of transcription start sites (TSSs) in both tissues, leading to differently-sized 5′UTRs, ranging from 3 to 37 bp. Many of these TSSs are not present in the UniGene database.

3.2. A strong promoter immediately upstream of PDF exon 1

The overlap between *PDF* and *COG8* results in transcripts with the same sequence at their 3′ ends [10], though the coding sequences of both genes are separated by 268 bp. Since PDF is essential in post-translational modification of mitochondrial-encoded

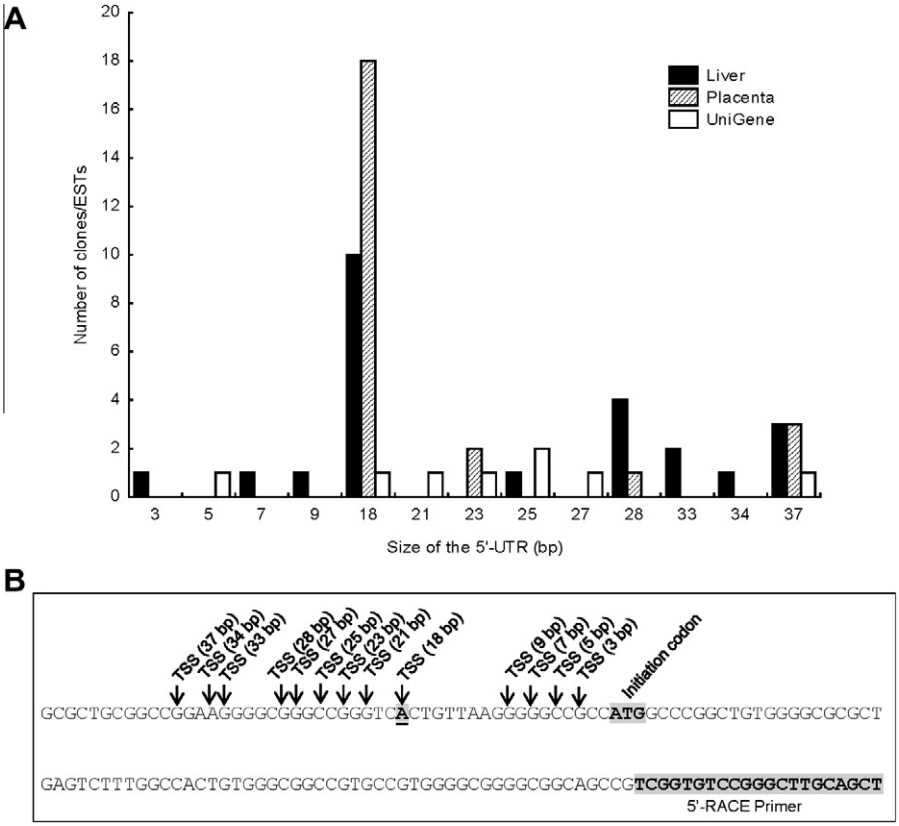

Fig. 1. Heterogeneity of TSSs in the human *PDF*. (A) Number of *PDF* 5′-RACE clones obtained from liver, placenta and 5'ESTs for each distinct 5′-UTR length. (B) TSS mapping in the human *PDF*. The initiation codon, 5′-RACE primer and the most frequent TSS (which gives rise to an 18 bp 5′-UTR) are highlighted in gray.

proteins, and there is no evidence that the *COG8/PDF* mRNA is polycystronic, we reasoned that transcription of *PDF* is directed by its own, hitherto unidentified, promoter. However, given the very close proximity between the two coding sequences, it remained a possibility that, during the complex evolutionary history that ended up in the *COG8/PDF* overlap [10], genomic sequences related to *COG8* could drive *PDF* expression. To address this possibility, we tested four differently sized genomic fragments (168–886 bp), immediately upstream the *PDF* initiation codon (Fig. 2A) that included sequences up to *COG8* intron 4, for their ability to induce expression of a reporter gene in human cells. As shown in Fig. 2B, all four fragments displayed similarly high promoter activity in these assays. Since all of them share the 168 bp sequence (Fig. 2A), a minimal promoter region of *PDF* is likely to be located within this fragment, which does not include any coding sequence from the *COG8* overlapping partner.

An *in silico* analysis of the *PDF* 168 bp putative promoter region using the TFSEARCH software revealed three Sp1, one MZF1 and one ELK1 binding sites in this fragment (Fig. 2C). Given the cellular and pathway specificity of MZF1 [12] and ELK1 [13,14] transcription factors, the high GC content (74%) of the 168 bp fragment, and the fact that it lacks a TATA-box, we focused our analysis on the ubiquitous Sp1 transcription factor.

To evaluate the importance of each of the Sp1 binding sites in promoting transcriptional activation, two types of modifications were made to the reporter plasmid containing the 168 bp fragment: (a) 5′ truncations that remove either one (128 bp fragment) or two (97 bp fragment) of the Sp1 binding sites (Fig. 3A) and (b) two-nucleotide mutations of the Sp1 binding sites singly or combined. The transcriptional activity of these new reporter plasmids was measured in human HEK293 cells (Fig. 3B). For the 168 bp construct, mutating either the *PDF*-ATG adjacent or the distant Sp1 binding site had no significant impact when compared with the wild-type construct. This suggests that, if Sp1 is indeed a *PDF* transcriptional regulator, two binding-sites seem to be sufficient to maintain the basal level of expression. In the 128 bp fragment, a reduction in activity was observed only when the site closer to *PDF*-ATG is inactivated ($P = 0.007$) (Fig. 3). Moreover, a significant reduction in luciferase activity was observed when the wild-type 97 bp fragment, which only harbors the ATG-adjencent Sp1 binding site was compared to its mutated counterpart ($P = 0.003$). Together, these results refine the human *PDF* minimal promoter to a 97 bp fragment and indicate that the Sp1 site closer to the *PDF*-ATG is critical for full transcriptional activity.

Nevertheless, when all Sp1 binding sites were abolished (either in the 128 bp or the 97 bp fragments) the reporters still displayed considerable luciferase activity, similar to that of pGL3-control. These could be due to different, yet not mutually exclusive reasons: (a) Sp1 has an important but not exclusive role in *PDF* expression and (b) the two mutations introduced in the Sp1 binding site are not sufficient to fully abolish functionality. To test the second possibility, an additional experiment was performed in which the effects of two-nucleotide and four-nucleotide replacements (which included the central CG base pair known to bind Sp1 by hydrogen bond contacts [15]) were compared (Fig. S1). Because no significant differences were observed, we conclude that additional transcriptional regulatory elements present within the 97 bp fragment, other than the Sp1 itself, might also contribute to control *PDF* expression.

3.3. Sp family members bind to the PDF minimal promoter region

To demonstrate that Sp1 binds to the minimal promoter region of *PDF*, we carried out EMSA and supershift assays using a probe sequence that includes the Sp1 binding site closest to the *PDF*

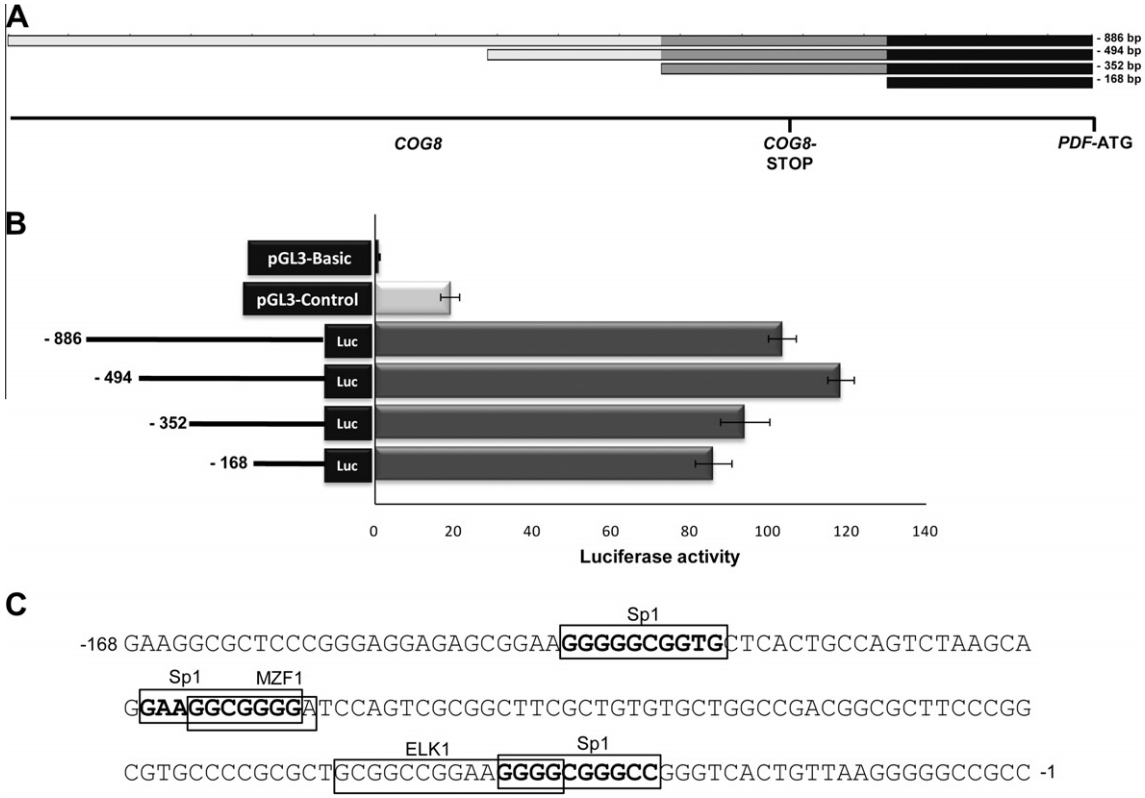

Fig. 2. Functional characterization of the human *PDF* promoter. (A) Illustration of the four fragments used in the luciferase assays. Numbers indicate the total length of the fragments. Fragments start immediately upstream the *PDF* initiation codon and contain different 5′-deletions. All fragments share a 168 bp sequence (black box). The end of the *COG8* coding sequence and beginning of *PDF*'s are represented. (B) Transcription activity in human cells. HEK293 cells were transfected with reporter vectors containing a luciferase gene under control of each of the four different-sized fragments. Luciferase reporter vectors pGL3-Control and pGL3-Basic (Promega) were used as positive and negative controls, respectively. Luciferase values represent the mean ± standard deviation (SD) of triplicate determinations. Luciferase counts were normalized with β-galactosidase activities. The results were confirmed in at least two independent experiments. (C) Location of the putative transcription factor binding sites in the −168/−1 promoter region identified in the TFSEARCH *in silico* analysis. Sp1 binding sites are highlighted in bold.

initiation codon. Two specific bands were detected in the gel shift assay (Fig. 4A, lane 2, higher bands) indicating that at least a protein is binding to the Sp1 probe, forming a DNA–protein complex. The formation of the DNA–protein complexes was fully suppressed by the addition of 200-fold molar excess of unbiotinylated wild-type competitor (Fig. 4A, lane 3), but not by 200-fold excess of a mutant unbiotinylated competitor (lane 4) carrying the replacements used to mutate the Sp1 binding site (Fig. 3). To determine whether Sp1 is the transcription factor binding to the Sp1 DNA probe, a supershift assay was performed using an antibody against Sp1. As illustrated (Fig. 4A, lane 5), a supershifted complex is observed simultaneously with a reduction in the formation of the higher complex-specific band. This demonstrates that Sp1 specifically binds to the Sp1 binding site present in the *PDF* minimal promoter *in vitro*. Because an extra band could not be explained by Sp1 binding and since binding of Sp3 to the same GC-rich sites as Sp1 was previously described [16], we used an anti-Sp3 antibody to assess the presence of Sp3 in the DNA–protein complexes. As observed in Fig. 4A (lane 6), a high molecular weight supershift is observed in combination with the disappearance of the lower complex-specific band (below the Sp1 complex band). On the contrary, addition of an anti-AP2 antibody failed to generate super-shifted complexes (Fig. 4A, lane 7), further demonstrating the specificity of Sp1 and Sp3 bindings. These results represent additional evidence that *PDF* expression is regulated by members of the Sp family of transcription factors, particularly by Sp1, whose interaction with the promoter fragment is stronger than the Sp3. Moreover, a biotinylated probe with the same mutations in the Sp1 binding site that reduced *PDF* promoter activity shown in Fig. 3 formed different complexes from the wild-type sequence probe (Fig. 4A, lane 8). Also, the addition of the anti-Sp1 antibody did not cause any alteration in that band pattern (Fig. 4A, lanes 8 and 9, respectively). Therefore, it is possible that the mutant Sp1 probe binds other transcription factors, which could explain why the activity of the minimal *PDF* promoter is not totally abrogated by mutations in its Sp1 site (Fig. 3 and Fig. S1).

3.4. The PDF core promoter region is highly conserved

To evaluate the conservation pattern of the cis-regulatory elements present in the *PDF* minimal promoter region we compared the homologous sequences from several mammalian species (Fig. 4B). We selected regions that included the Sp1 binding site and the 5′UTR. We found that most of the TSSs and the proximal Sp1 binding site found to be important in humans are conserved in mammals suggesting a role in transcriptional activation of *PDF* in other species as well. Overall, the entire 5′UTR reveals a high degree of conservation, and it is possible to detect invariant elements, such as the CTTCCCGGCGT stretch (Fig. 4B). These may act as cis-regulatory elements, other than the Sp1 binding site, which could explain our observations (Fig. 3) that when the Sp1 binding site is mutated the transcriptional activation is not fully abolished.

4. Discussion

The transcriptional regulation of human *PDF* is currently unknown even though its role in mitochondrial protein deformyla-

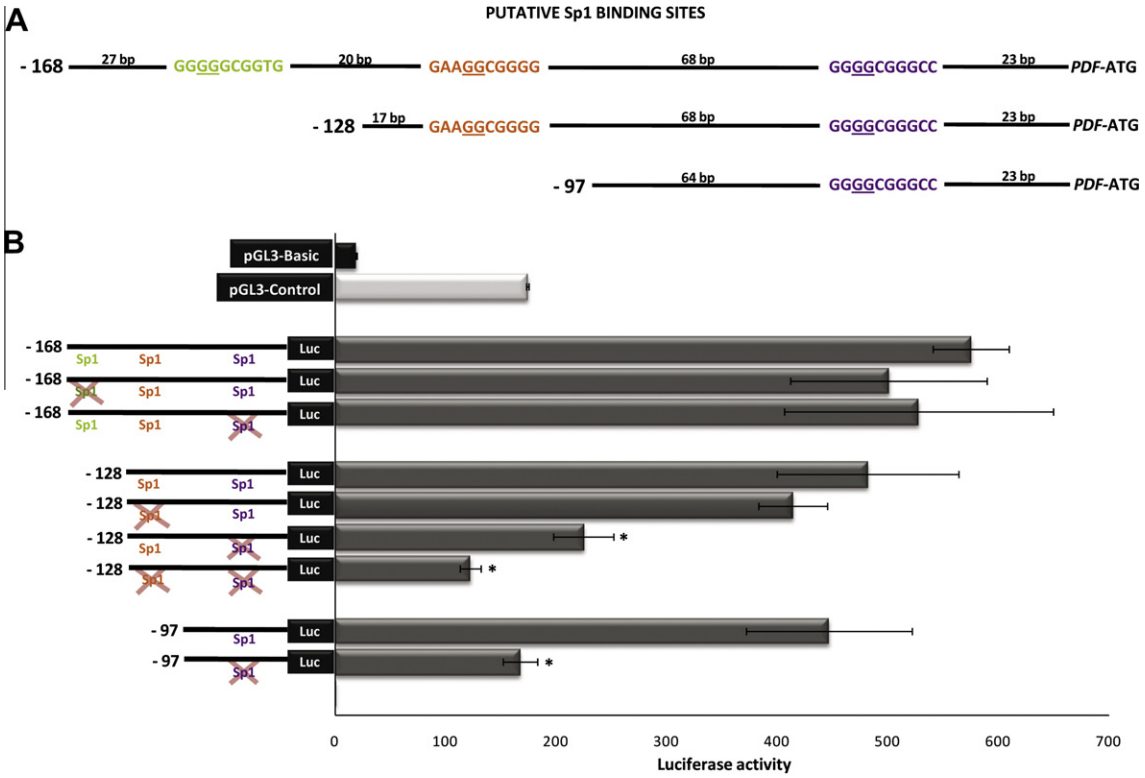

Fig. 3. Sp1 regulates *PDF* transcriptional activity in humans. (A) Position of putative Sp1 binding sites in the 168, 128 and 97 bp fragments. The two conserved guanines altered by PCR site-directed mutagenesis to thymines are underlined. (B) Luciferase activity for wild-type and mutated Sp1 binding site fragments. Mutations in Sp1 binding sites are indicated by an X. Black boxes (Luc) represent the firefly luciferase reporter gene. Asterisks indicate Student's *t*-test significant values (P-value < 0.05) when compared with same-sized wild-type fragments.

tion [8] and in the maintenance of the oxydative phosphorylation complexes integrity [9] has been documented. Apart from the biological importance, *PDF* stands out for its peculiar evolutionary history in primates and rodents [10]. In humans, *PDF* and *COG8* overlap at the mRNA level, that is, the *PDF* transcript shares the second exon with the 3′UTR of *COG8*. At the genomic level, both genes are closely linked as the distance between the *COG8* stop codon and *PDF* initiation codon is only 268 nucleotides.

The close proximity between *PDF* and *COG8* prompted us to test if the transcription regulation of human *PDF* was affected by the *COG8*/*PDF* overlap or, alternatively, if *PDF* still conserves its own promoter sequence. Both *in silico* and experimental analysis showed that human *PDF* has multiple TSSs, the most frequent one located only 18 nucleotides from the initiation codon. The TSSs are located 3–37 bp from the initiation codon and this variability is expected to result in populations of mRNAs inside the cell that differ by very short 5′UTRs. These TSSs could be differentially selected by the RNA polymerase II in different tissues, giving rise to a considerable variability in the transcription initiation of *PDF*, as described for other genes [17]. Consistent with this possibility, we found that the TSSs are more dispersed in liver ($n = 9$) than in placenta ($n = 4$), suggesting that tissue-specific differences might exist in *PDF* transcription initiation.

The human *PDF* gene does not contain a TATA-box, a common cis-regulatory sequence usually located 25–30 nucleotides upstream of the TSSs in the core promoter of many genes [18]. It is known that TATA-less genes usually have GC-enriched promoter regions, frequently include more than one TSS and are associated with ubiquitously expressed genes [19]. All these features are present in the human *PDF* suggesting it does not follow the classical model of transcription regulation, in which a TATA-box guides the transcription of a single TSS.

Using luciferase-reporter assays, a series of 5′-deleted fragments of the region extending from *COG8* intron 4 to *PDF*'s initiation codon showed that the 97 nucleotides upstream the *PDF* initiation codon are sufficient to promote a strong transcriptional activity in human cells. This fragment does not include any coding sequence of the neighboring *COG8* gene. Moreover, we showed that the Sp1 binding site located only 23 bp upstream the *PDF* initiation codon is crucial for the *PDF* transcriptional activation process, since its mutation leads to a statistically significant reduction of reporter gene expression. Furthermore, gel shift and supershift assays demonstrated that the Sp1 transcription factor binds this site, which was also found to be conserved in mammals. Even assuming that other cis-acting elements are also involved in PDF regulation (a likely hypothesis since the removal of all Sp1 binding sites in the promoter region does not completely abolishes transcription activity, as shown in Fig. 3), these results support the notion that Sp1 plays a fundamental role in *PDF* transcription.

It is well established that Sp3 can recognize the same GC-rich binding sites as Sp1 [20]. The Sp3 transcription factor is ubiquitously expressed, binds DNA with high affinity and specificity and can either activate or repress transcription [16,21]. In mammalian cells it can act as an inhibitor of Sp1-mediated activation due to the competition with Sp1 for their binding sites [16]. Because additional bands were detected in the EMSA assay we investigated and confirmed by supershift assay that Sp3 also binds to the ATG-adjacent Sp1 binding site, although less efficiently than Sp1. Hence, it is possible that, depending on the cellular context, Sp3 can also regulate *PDF*. However given the strength of the luciferase activation it seems that Sp1 is the main player of the *PDF* transcriptional regulation.

Overall, our data demonstrate that a 97 bp fragment upstream the *PDF* initiation codon contains the minimal promoter and that

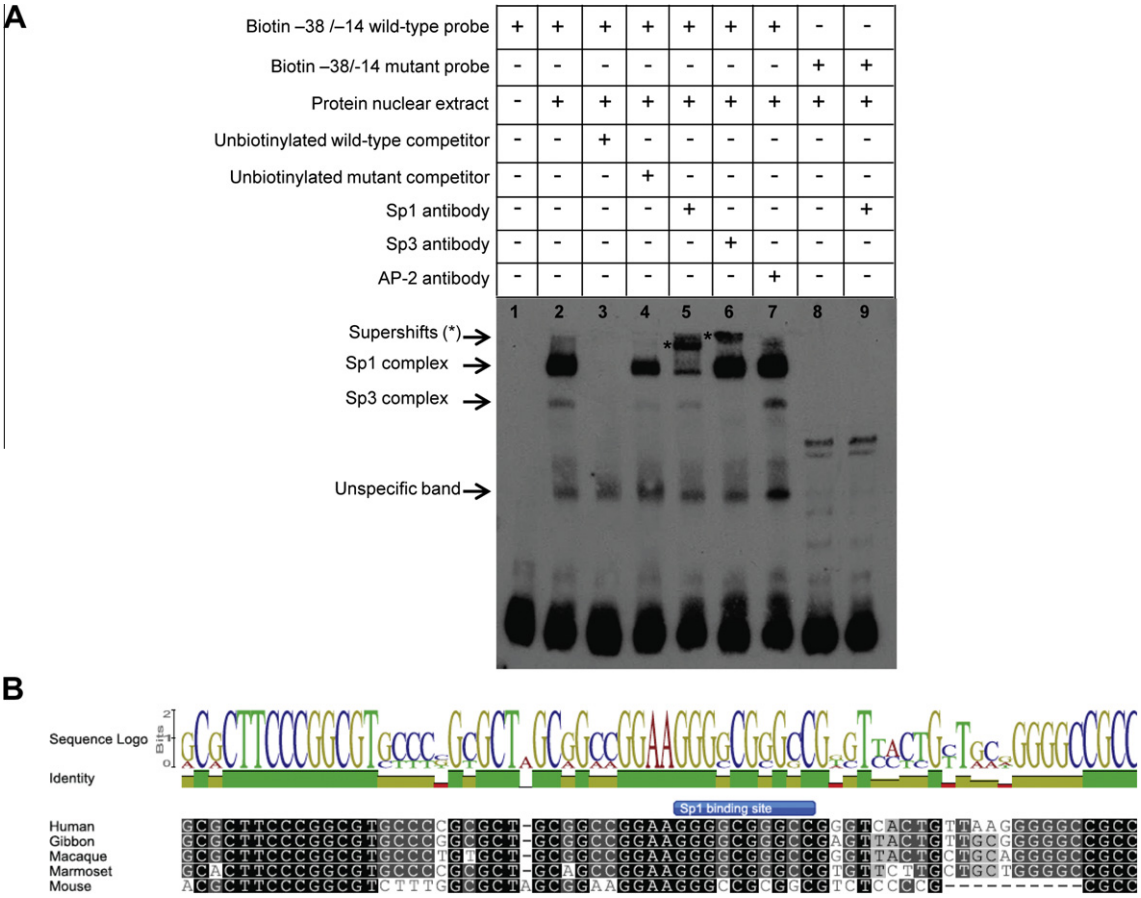

Fig. 4. Sp1 and Sp3 bind the *PDF* promoter *in vitro*. (A) EMSA assay was performed using HEK293 protein nuclear extracts and biotin wild-type or mutant DNA probes from the −38/−14 promoter region that includes the Sp1 binding site adjacent to the *PDF* initiation codon. Specific DNA-Sp1 and DNA-Sp3 complexes are indicated. Sp1 and Sp3 supershifted bands are marked with an asterisk. (B) Conservation of the *PDF* promoter region. Alignment of *PDF* 5′-flanking sequences from different mammals. The human *PDF*-adjacent conserved Sp1 binding site is indicated. Sequence identity and sequence logo are represented.

several TSSs within this fragment are used for transcription initiation. Furthermore, given its strong ability to promote transcription, it is possible that this fragment contains the ancestral *PDF* promoter, rather than a *COG8*-derived sequence, acquired after the overlapping event. In humans, the overlap between *COG8* and *PDF* occurred through the gain of a novel splice donor site between the *COG8* stop and the *PDF* initiation codon and by the loss of the *COG8* ancestral polyadenylation signal as we reported before [10]. These events made *COG8* absolutely dependent on the *PDF* sequence. Conversely, we demonstrate here that *PDF* uses a short promoter that does not include *COG8* coding sequences and, in that sense, the expression of the *PDF* gene is independent of its overlapping partner.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.097.

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