A Pancreatic β-Cell-specific Enhancer in the Human PDX-1 Gene Is Regulated by Hepatocyte Nuclear Factor 3β (HNF-3β), HNF-1α, and SPs Transcription Factors*

Etti Ben-Shushan, Sonya Marshak, Michal Shoshkes, Erol Cerasi, and Danielle Melloul‡

From the Department of Endocrinology and Metabolism, Hebrew University Hadassah Medical Center, 91120 Jerusalem, Israel

The PDX-1 transcription factor plays a key role in pancreas development. Although expressed in all cells at the early stages, in the adult it is mainly restricted to the β-cell. To characterize the regulatory elements and potential transcription factors necessary for human PDX-1 gene expression in β-cells, we constructed a series of 5′ and 3′ deletion fragments of the 5′-flanking region of the gene, fused to the luciferase reporter gene. In this report, we identify by transient transfections in β- and non-β-cells a novel β-cell-specific distal enhancer element located between −3.7 and −3.45 kilobases. DNase I footprinting analysis revealed two protected regions, one binding the transcription factors SP1 and SP3 and the other hepatocyte nuclear factor 3β (HNF-3β) and HNF-1α. Cotransfection experiments suggest that HNF-3β, HNF-1α, and SP1 are positive regulators of the herein-described human PDX-1 enhancer element. Furthermore, mutations within each motif abolished the binding of the corresponding factor(s) and dramatically impaired the enhancer activity, therefore suggesting cooperativity between these factors.

The mammalian pancreas develops by fusion of dorsal and ventral buds which form as evaginations of the upper duodenal part of the gut. Identification of the homeodomain-containing transcription factor PDX-1 as the first molecular marker temporarily correlates with the pancreatic commitment of the epithelial cells in this region (1–3). Targeted inactivation of this gene in the mouse (4) as well as its mutation in man (5) results in agenesis of the pancreas. The gene is expressed both in endocrine and exocrine cells of the developing pancreas; however, in the adult islet, its expression is predominantly restricted to the β-cell (1–3), where it acts as the mediator of glucose action on insulin gene expression (6–9). In mice, β-cell-selective disruption of pdx-1 leads to diabetes associated with reduced insulin and glucose transporter 2 expression (3).

Development, cell fate, and cell differentiation are complex events that depend on switching on and off the expression of specific sets of genes. Such regulation operates mainly at the transcriptional level by the assembly of multiprotein complexes at the enhancer(s) and the promoter regions of the gene. These complexes are formed and stabilized through multiple protein-DNA and protein-protein interactions. Since PDX-1 plays such a central role in β-cell differentiation and function, the molecular basis of its regulation and, hence, the DNA elements and the interacting proteins involved in this process must be clarified. To this end, a 6.5-kb fragment upstream of the transcription start site of rat pdx-1/lsf-1 (10) and a fragment extending from the −4.5 to +8.2-kb region of mouse pdx-1 (11) were shown to direct the expression of the β-galactosidase reporter gene to pancreatic islet cells in transgenic mice. In transiently transfected β-cells, appropriate expression of the rat pdx-1 gene depended in part on a proximal E box that predominantly binds the ubiquitous transcription factor USF1 (10). Tissue-specific regulation also appeared to require a distal enhancer sequence located between the −6.2- and −5.67-kb region of the rat pdx-1 gene. Analysis of the factors bound to this element indicated that the endodermal factors HNF-3β and Neurod/Beta2 act cooperatively to induce pdx-1 expression in islet cells. Furthermore, it was shown that glucocorticoids reduce pdx-1 gene expression by interfering with HNF-3β activity (12). Studies on the mouse pdx-1 promoter revealed that the region from −2560 to −1880 bp regulates β-cell-specific transcription and directs the appropriate developmental and adult-specific expressions in transgenic animals. It was found that an HNF-3-like element contained within this region is important for the β-cell specificity (11). Additional studies indicate that two highly conserved sequences in the 5′-flanking region of the PDX-1 gene (PH1/area1 and PH2/area2) confer β-cell-specific transcriptional activity (13, 14) on a heterologous promoter. DNase I footprinting and binding analyses revealed that both sequences bind and are transactivated by HNF-3β; this is in accordance with the fact that its absence in mouse embryonic stem cells had a dramatic effect on pdx-1 gene expression (13).

Thus, data from studies with the rat (12), the mouse (11, 13), and the human (13, 14) promoters suggest that HNF-3β is an important regulator of pdx-1 gene transcription. Interestingly, we found that PDX-1 itself binds to the PH1/area1 element and cooperates with HNF-3β to activate transcription (14).

To further identify key components of importance for the expression of the human PDX-1 gene in β-cells, we sequenced about 4.5 kb of the 5′-flanking region of the gene, constructed a series of 5′ deletion fragments fused to the luciferase reporter gene, and tested them in β- and non-β-cells. In this report we provide evidence for a novel β-cell-specific distal enhancer element that appears to be specific to the human PDX-1 gene.

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\[1\] The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) 225952.

‡ To whom correspondence should be addressed: Dept. of Endocrinology and Metabolism, Hadassah University Hospital, P. O. Box 12000, Jerusalem 91120, Israel. Tel.: 972-2-677 83 98; Fax: 972-2-643 79 40; E-mail: Daniele@md2.huji.ac.il.

The abbreviations used are: PDX, pancreatic duodenal homeobox; kb, kilobase(s); bp, base pair(s); Luc, Luciferase; HNF, hepatocyte nuclear factor; CHO, Chinese hamster ovary; TK, thymidine kinase; EMSA, electrophoretic mobility shift assay.
DNase I footprinting analysis revealed two protected regions: one binding the proteins identified as SP1 and SP3 and the second, the transcription factors HNF-3β and HNF-1α. These are thus candidate transcription factors that are involved in regulating selective expression of the human PDX-1 gene in the adult β-cell.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Hamster insulinoma HIT-T15, mouse insulinoma βTC6, and mouse glucagonoma nTC1 cells were cultured in Dulbecco’s modified Eagles medium supplemented with 15% horse serum and 2.5% fetal calf serum (FCS), AR42J, HepG2, CHO, HeLa, and NIH 3T3 cells with 10% FCS. 100 units/ml penicillin and 100 mg/ml streptomycin were added to the media.

**Cell Transfections**—HIT-T15, HepG2, NIH3T3, COS, and CHO cells were transfected using the calcium phosphate coprecipitation method (15), and nTC1, βTC6, and AR42J cells were transfected using the Fugene transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s recommendations with 1.5 μg of human PDX-1 luciferase derivatives and 0.5 μg of the internal control cytomegalovirus-β-galactosidase DNA plasmid (CMV-βGal). In co-transfection experiments 1.5 μg of the reporter plasmid and 0.1–1 μg (as indicated) of the expression plasmids HNF-3α, HNF-3β, HNF-1α, HNF-1β, SP1, and TRβ were used. The cells were harvested 48 h after transfection, and about 10 μg of protein extracts were used to measure luciferase activity with the luciferase assay system (Promega, Madison, WI) and analyzed on a denaturing 6% polyacrylamide gel. Sequencing reactions of each probe were performed using the Maxam and Gilbert procedure (18).

**RESULTS**

**Human PDX-1 Sequences Involved in β-Cell-specific Transcriptional Activity**—To delineate the putative DNA sequences controlling PDX-1 gene expression, we linked a fragment extending from about −7 to +0.11 kb of the 5′-flanking region of human PDX-1 to luciferase reporter gene and constructed a series of 5′ deletions, as depicted in Fig. 1. The chimeric genes were transiently transfected into HIT-T15 β-cells and CHO cells. Expression was strongly β-cell preferential, as shown in Fig. 1. Deletion of sequences between −7 and −3.7 kb led to an approximate 20-fold increase in luciferase activity in HIT-T15 but not in CHO cells, implying the removal of a negative regulatory element(s). When an additional deletion of the distal region located between −3.7 and −2.3 kb was performed, the activity dropped by about 75%, suggesting the presence of a strong positive regulatory element. Further removal of sequences up to −160 bp had no significant effect. In contrast, deletion of the proximal region between −160 and −100 bp abolished the transcriptional activity in both cell lines (Ref. 10 and data not shown). In summary, this data indicate that the 3.7-kb fragment contains a strong positive regulatory region that confers β-cell-specific expression on the reporter gene.

**The Human PDX-1 Sequence −3.7 to −3.45 kb Acts as a β-Cell-specific Enhancer Element**—To localize the sequences responsible for the activity delineated in Fig. 1, the fragment extending from −3.7 to 2.3 kb was subcloned directly upstream of the minimal PDX-1 promoter (−160/+117) fused to the luciferase gene. In transiently transfected HIT-T15 cells, an approximate 6-fold induction in transcriptional activity was observed (Fig. 2A). However, 5′ deletion of sequences between −3.7 and −3.3 kb reduced the transcriptional activity to basal promoter levels. To further delineate the regulatory sequences contained within this 400-bp fragment, 3′ deletions were generated; transient transfections revealed the presence of a positive regulatory element spanning the region from −3.7 to −3.45 kb. This element had the characteristics of an enhancer as it strongly transactivated the PDX-1 promoter when cloned in either orientation upstream of the minimal thymidine kinase promoter (Fig. 2A). Confirming its role as a tissue-specific enhancer, this 250-bp fragment strongly stimulated β-cell-specific expression of the luciferase reporter gene in transected extracted with phenol-chloroform, ethanol-purified, and analyzed on a denaturing 6% polyacrylamide gel. Sequencing reactions of each probe were performed using the Maxam and Gilbert procedure (18).
β-cells, HIT-T15 (80-fold) and βTC6 (34-fold) versus non-β-cells, the exocrine AR42J (no activation), the glucagonoma αTC1 (3-fold), CHO (5-fold), and the hepatoma HepG2 (10-fold) cells (Fig. 2B). From this analysis it emerges that there is a new distal β-cell-specific enhancer located between −3.7 and −3.45 kb. This element appears to be specific to the human PDX-1 gene as no sequence homology was found in the vicinity of this region in the mouse gene (not shown).

**DNase I Footprinting of the Human PDX-1 β-Cell-specific Enhancer Element**—The transcriptional activity driven by the distal enhancer element of human PDX-1 suggested the presence of cis-acting regulatory elements in this region. To assess whether such putative elements interact with specific proteins, we performed DNaseI footprinting analysis using the fragment extending from −3.7 to −3.3 kb as a probe and extracts from HIT-T15 and CHO cells. As shown in Fig. 3A, two protected regions were obtained. The pattern of the first protected sequence E1, −3.473/−3.494 kb, shows a hypersensitive site in the presence of HIT-T15 cell extracts. The second footprinted sequence, between −3.565 and −3.590 kb, E2, occurs within a particularly AT-rich region and shows a slightly different digestion pattern in HIT-T15 and CHO cell extracts. The sequence of the human PDX-1 enhancer element (−3.7/−3.45 kb) is presented in Fig. 3B with the footprinted regions underlined. To further characterize the trans-acting factors binding to the footprinted regions, double-stranded oligonucleotides spanning these sequences were synthesized and used as probes to detect HIT-T15 and CHO proteins by electrophoretic mobility shift assay.

**Members of the SP1 Family of Proteins Interact with the E1 Sequence of the Human PDX-1 Enhancer**—Using the E1 sequence as a probe, two binding complexes (a and b in Fig. 4A) were obtained in HIT cell extracts. Computer analysis for potential binding sequences (19) revealed a GC-box element. To assess whether this motif could interfere with the formation of the E1 complexes, excess unlabeled oligonucleotide containing the SP1 consensus motif GGCGGG was added to the binding reaction. The DNA complexes were competed away by the SP1 oligonucleotide (Fig. 4A, lane 3) but not by a nonspecific one (Fig. 4A, lane 4). To confirm that SP1 family members are involved in E1 complexes, specific SP1 and SP3 antibodies were added separately (Fig. 4B, lanes 6 and 7, respectively) or simultaneously (Fig. 4B, lane 8) to the binding reactions. The results demonstrate that the slower migrating complex a was recognized by anti-SP1 (Fig. 4B, lane 6), whereas complex b reacted with anti SP3 (Fig. 4B, lane 7) antibodies.

**The Transcription Factors HNF-3β and HNF-1α Interact with the E2 Sequence of the Human PDX-1 Enhancer**—In EMSA, using the E2 sequences with HIT-T15 or βTC6 cell extracts, a faint complex labeled a and a strong faster migrating b complex were detected (Fig. 5A). The b complex was observed in all pancreatic cells tested, i.e. the glucagonoma αTC1 and the exocrine AR42J line as well as in the hepatocarcinoma HepG2 cells. In contrast, the a complex was mainly observed in β-cells; a closely migrating complex in AR42J and HepG2 cells runs slightly faster. E2 is contained within an AT-rich region (Fig. 3B), and computer analysis for transcription factors unveiled potential binding sites for several homeodomain-containing proteins (19) including overlapping motifs for HNF-3 and HNF-1. To determine whether HNF-3 or HNF-1 is involved in the observed complexes, electrophoretic mobility shift assays were performed using either the wild type E2 sequence (wt-E2) or the mutant form containing a modified HNF-3/HNF-1 motif (mut-E2). The DNA complexes a and b observed in HIT-T15 cells were competed away by excess of unlabeled wild type oligonucleotide (Fig. 5B, lane 3). In contrast, the unlabeled oligonucleotide containing the mutated HNF-3/HNF-1 motifs (Fig. 5B, lane 4) or a nonspecific (octamer consensus motif) oligonucleotide showed no competition (Fig. 5B, lane 5). When the mutated HNF-3/HNF-1 sequence was used as probe, the a and b complexes were abrogated (Fig. 5B, lane 7).

To verify the presence of HNF-3β and HNF-1α in the E2 complexes, their ability to interact with a series of antibodies was tested. Fig. 5C demonstrates that the b complex is specifically recognized by antibodies against HNF-3β (Fig. 5C, lane 4) but not with anti-HNF-3α (Fig. 5C, lane 3) or antibodies against the homeodomain proteins PDX-1, Oct-4, cdx2, Nkx6.1, or isl-1 (data not shown). Furthermore, the a complex interacted with anti- HNF-1α (lane 5) but not with anti-HNF-1β (lane 6) antibodies. Cell extracts from COS cells transfected with an expression plasmid for HNF-3β or HNF-1α were analyzed for their interaction with the E2 sequence to establish that the protein contained in the b complex corresponds to HNF-3β. Indeed, the HNF-3β and
FIG. 3. DNase I footprinting analysis of the human enhancer element. A, the analysis was performed using the end-labeled fragment spanning the sequences between approximately −3.7 and −3.4 kb and incubated with no extracts (lanes 2, 3, and 9), with extracts from HIT (lanes 4, 5, and 10), or CHO (lanes 6, 7, and 11) cells. G+A and C+T sequencing reactions were run alongside as a marker (lanes 1 and 8, respectively). B, nucleotide sequence of the β-cell-specific enhancer in the human PDX-1 gene. The E1 and E2 footprinted regions are indicated.

FIG. 4. SP1 and SP3 from HIT cells bind the E1 sequence of the human PDX-1 enhancer element. A, EMSA was performed using CHO (lane 1) or HIT (lane 2) cell extracts and a 32P-labeled E1 sequence. Two complexes were formed, labeled a and b, indicated by arrows. Competition for binding of HIT cell extracts to the labeled E1 sequence was performed with a 100-fold excess of an unlabeled oligonucleotide containing an SP1 consensus motif (SP1, lane 3) or with a nonspecific oligonucleotide (NS, lane 4). B, identification of SP1 and SP3 complexes. EMSA was performed with HIT-T15 cell extracts incubated with the labeled E1 sequence (lane 5) in the presence of antisera against SP1 (lane 6) or SP3 (lane 7) or both (lane 8) or preimmune serum (PIS, lane 9).

Human PDX-1-specific Enhancer Element
HNF-1α complexes in COS cells migrated similarly to the α or β complex in HIT cells, respectively, and were also recognized by the corresponding antibodies (data not shown). Taken together, these results demonstrate that the endogenous HNF-1α and HNF-3β in HIT-T15 cells specifically bind the E2 sequence.

Combinatorial Effects of HNF-1α, SP1, SP3, and HNF-3β in the Activation of the Human PDX-1 Enhancer Element—To investigate the effect of the above transcription factors on gene expression driven by the enhancer element, we performed transient transfection experiments in NIH3T3 cells. To this end, the PEn-TK-luciferase construct was cotransfected with increasing amounts of the HNF-1α and HNF-1β plasmids separately or in combination with HNF-3β expression plasmid (Fig. 6A). In parallel, we carried out similar experiments with SP1 and SP3 expression plasmids (Fig. 6B). As presented in Fig. 6A, HNF-3β and HNF-1α but not HNF-1β separately stimulated the PDX-1 enhancer activity in a dose-dependent manner. Furthermore, cotransfection with HNF-3β and increasing amounts of HNF-1α cooperatively activated the expression of the gene. Similarly, HNF-3β and SP1 individually activated the chimeric gene, and cotransfection with a constant amount of HNF-3β and increasing amounts of SP1 significantly stimulated the expression of the gene in a more than additive manner. In contrast, although SP3 lacked any effect on the enhancer activity when acting independently, it dramatically suppressed the HNF-3β-mediated transactivation (Fig. 6B).

Mutant constructs were created to specifically alter the SP1 or HNF-3β/HNF-1 motifs in the context of the human PDX-1 enhancer element (Pen) linked to the luciferase reporter gene and driven by the minimal TK promoter. The mutation that eliminated SP1/SP3 binding (mut-E1) caused more than 90% reduction in enhancer activity in HIT-T15 cells (Fig. 6C). Mutation in the overlapping HNF-3β/HNF-1 motifs (mut-E2) also caused an 80% decrease in enhancer activity. The effect of HNF-3β on enhancer activity was further examined by cotransfection experiments in CHO cells using the wild type reporter construct (PEn) or the E2 mutant form carrying a modified human PDX-1 enhancer element.

DISCUSSION

It is accepted that PDX-1 functions as a master regulator of the exocrine and endocrine pancreatic programs. The pdx-1 gene is expressed early during development in cells of both origins, whereas later it becomes restricted mainly to β-cells. Fragments containing −6.5- and −4.5 kb sequences of the rat (10) and the mouse (11) pdx-1 gene, respectively, were sufficient in targeting its expression to rodent islet cells. Therefore, to further characterize the potential regulatory elements of the human PDX-1 gene, we analyzed about 4.5 kb in the 5′-flanking region of the gene. By transient transfections of β-cell and non-β-cell lines with different 5′ and 3′ deletions, a strong β-cell-specific enhancer element located between −3.7 and −3.45 kb was demonstrated. The 4.5-kb 5′-flanking sequences of the human and mouse pdx-1 genes are markedly different, apart from the conserved proximal promoter region; only three short highly homologous areas located 3′ of the herein-described enhancer element are observed. No homology was observed between this enhancer and the one previously described.
further upstream in the rat gene (12). These observations suggest that several regulatory elements in *pdx-1* gene contribute to its β-cell-specific expression.

To identify the potential transcription factors that bind the distal enhancer element, DNase I footprint analysis was performed, and two protected regions (E1 and E2) were identified. The E1 area was found to bind the transcription factors SP1 and SP3. These factors bind DNA with similar specificity and affinity. SP1 is expressed in most tissues, and targeted inactivation of the gene in the mouse results in retarded growth of the embryos, which die early during gestation (20). Many genes are controlled by SP1, which in some cases acts cooperatively with other transcription factors (21). SP3 has been shown to function either as an inhibitor by competing with SP1 for binding to DNA (22, 23) or as an activator, depending on promoter context and cell type (23, 24). It has also been suggested that the relative amounts of SP1 and SP3 can vary during cellular differentiation, thus modulating the response of target genes (see review, see Ref. 25). High levels of SP1 were found in hemopoietic stem cells, fetal cells, and spermatids.
(20). We also observed about twice as much SP1 protein in pancreatic cells than in fibroblasts but similar levels of SP3 in all tested cells (data not shown). Furthermore, in this report we show that in transfected fibroblasts, whereas excess SP1 has a stimulatory effect on the enhancer transcriptional activity, SP3 shows a rather inhibitory effect on transcription, mainly by inhibiting HNF-3β-mediated transactivation.

The E2 protected area is contained within an AT-rich sequence, and factors binding this region were identified as HNF-3β and HNF-1α. The important role of HNF-3β in transactivating the conserved regulatory elements in the mouse and human pdx-1 5 ’-flanking region was recently shown (13, 14), and its absence in mouse embryonic stem cells dramatically impaired pdx-1 gene expression (13). Moreover, the distal enhancer element identified in the rat pdx-1 (stf-1) gene binds HNF-3β and Neurod/Beta2 factors, which cooperatively induce its expression in islet cells. It was further shown that glucocorticoid-induced reduction of pdx-1 expression was mediated by impaired HNF-3β activity (12). HNF-3β belongs to the forkhead/winged helix family of transcription factors and is essential for endodermal cell lineages (26–28). Since HNF-3β is not restricted to β-cells, selective transcription of pdx-1 must an require additional factor(s). It is believed that HNF-3β, by its structural similarity to histone H5, may alter nucleosomal structure, thus facilitating gene transcription by opening the chromatin structure and thereby providing access to other transcription factors (29, 30). Whereas HNF-3β binds as a monomer, members of HNF1 family of transcription factors bind DNA as homo- or heterodimers (31). HNF-1α and HNF-1β are hepatocyte-enriched transcription factors that are also expressed in other tissues like in the pancreas. The relative abundance of these two proteins differs markedly between the different pancreatic cell lines (Fig. 5A and data not shown). It is conceivable that the relative levels of HNF1 subtypes may also be one of the factors contributing to the tissue specific expression of the PDX-1 gene, as it has been recently shown for the regulation of glucose transporter 2 (Glut2) gene in hepatocytes and β-cells (32). The relative levels of these two proteins have also been reported to affect vitamin D binding protein gene transcription. Although HNF-1α had a stimulatory effect, HNF-1β acted as an inhibitor of HNF-1α-transactivating potency (33).

Our findings that the enhancer element binds HNF-3β/HNF-1α and SP1/SP3 and that mutations in each site dramatically impair its transcriptional activity suggest cooperativity between these factors. Cooperativity between SP1 and members of HNF3 family has also been demonstrated for the surfactant protein B (34) and for the uteroglobin/CC10 (35) genes. The importance of SP1 in the lung epithelium. Hence, SP1 could function as a bridging factor between the hepatocyte nuclear factors and the basal transcriptional machinery, or conversely, HNF-3β may facilitate HNF-1α and SP1 binding by bending the DNA.

Since HNF-3β/HNF-1α and SP1 seem equally important for the human PDX-1 promoter activity, the synergism and possible interactions between these factors need to be analyzed. Nevertheless, HNF-3β, HNF-1α, and SP1 are also present in other pancreatic and hepatic cells, and yet the human PDX-1 enhancer activity was low in these cells, pointing to the possibility that other accessory proteins and/or an additional level of transcriptional control may contribute to the β-specific transcriptional activity of the PDX-1 gene. Transcriptional regulation appears to be a multistep process that may also involve chromatin remodeling, e.g. the methylation status of CpG sequences in a control element (36, 37). Findings showing that methylation of SP1 sites might be a relatively common and physiological mechanism of gene repression have been reported for leukosielin (CD43) (38, 39), cyclin D1 (40), and lung epithelial T1α genes in nonexpressing cells (41). SP1 elements have also been shown to play a key role in protecting a CpG island in the adenine phosphoribosyltransferase (APRT) gene from de novo methylation (37, 42). The effect of methylation on the identified enhancer element transcriptional activity needs to be tested.

We demonstrate that an AT-rich and a GC-box sequences are the major sites of regulation for the herein-described human PDX-1 enhancer element. We suggest that the transcriptional stimulation of pdx-1 gene in β-cells is mediated by a unique combination of protein-protein interactions and that separate modules in its sequence could be active at a given stage by binding a specific set of transcription factors. Indeed, the transcription factors HNF-3β, HNF-1α, HNF-1β, SP1, and PDX-1 itself, which regulate the expression of the PDX-1 gene, have been previously shown, mainly by knockout in mice, to be important developmental regulators.

Recently, mutations in genes coding for three members of the HNF family of proteins have been identified in a subset of type 2 diabetes, MODY (maturation-onset diabetes of the young), HNF-1α (MODY3) (43), HNF-1β (MODY5) (44), and HNF-4α (MODY1) (45). Thus, genes coding for the transcription factors controlling PDX-1 gene expression may be candidates for susceptibility to diabetes.

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