Transcriptional activity and Sp1/3 Binding to the P1 Promoter Sequences of the Human AβH-J-J Locus, Encoding Aspartyl-β-hydroxylase, Junctin and Junctate

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Running title: Sp regulation of the AβH-J-J locus P1 promoter

ABBREVIATIONS

The abbreviations used are: AβH, aspartyl-β-hydroxylase; $InsP_3R$, inositol 1,4,5 trisphosphate receptor; PCR(s), polymerase chain reaction(s); bp, base pair(s); MEF-2, myocyte enhancer factor 2; Sp1, specific transcription factor 1.

ABSTRACT

Alternative splicing of the locus AβH-J-J generates three functionally distinct proteins: an enzyme (aspartyl-β-hydroxylase) over expressed in various malignant neoplasms, a structural protein of the sarcoplasmic reticulum membrane (junctin) and an integral membrane calcium binding protein (junctate). Junctin and junctate are involved in the regukation of calcium homeostasis in eukaryotic cells. We predictedthat the gene encoding these proteins should contain at least two promoters P1 and P2, and recently characterized sequences contained within P2 controlling tissue specific transcription of junctin, junctate, and aspartyl-β-hydroxylase. Expression of the latter protein from exon 1 occurs by activation by the P1 promoter. In the present report we identified and functionally characterizeds the P1 promoter activity of the AβH-J-J locus. We demonstrate that mRNAs from P1 promoter are actively transcribed in all the human tissues and cell lines analysed and define the transcription start point in RD cells. To investigate the mechanism regulating transcription, we cloned a1.7 kb sequence upstream exon 1 from a human BAC clone and produced progressively deleted reporter constructs. Our results showthat (i) the 1.7 kb fragment isa powerful activator of the reporter gene in HepG2 and RD cell lines, (ii) 544 bp upstream of the transcription start site is essential for maximal promoter activity, (iii) progressive deletions from -544 resuls in gradually decreased reporter expression. The region responsible for maximal transcription contains at least twelve GC-box homologous to Sp1 consensus binding sequence; by electrophoretic mobility shift assay (EMSA) we identified three GC-rich elements which bind Sp family nuclear factors with very high efficiency. Our results suggest that Sp factors positively regulate the core of P1 promoter; by comparing the two promoters present within the AβH-J-J locus our results demonstrate that they are significantly different both in terms of transcriptional efficiency and in their ability to direct tissue specific transcription.

INTRODUCTION

We have previously characterized the human AβH-J-J locus, a genomic sequence which generates three functionally distinct proteins [1]. In addition to the enzyme aspartyl β-hydroxylase (AβH), this locus encodes junctin, a structural protein of sarcoplasmic reticulum, and the membrane-bound calcium binding protein junctate [1]. AβH catalyzes posttranslational hydroxylation of aspartate and asparagine residues in certain epidermal growth factor-like domains present in a number of proteins, including receptors and receptor ligands, involved in cell growth and differentiation, as well as extracellular matrix molecules [2]. AβH is over expressed in infiltrative intrahepatic cholangiocarcinomas, metastasized lung, breast, and colon carcinomas, and malignant neuroectodermal tumors [3-5]. This protein is of interest because it can potentially contribute to the malignant phenotype by increasing motility and enhancing proliferation, survival, and cell cycle progression. Inhibition of AβH expression could represent an attractive approach for gene therapy of infiltrating tumors [2, 6].

Junctate is a novel integral calcium binding protein of sarco(endo)plasmic reticulum membrane, which forms a supramolecular complex with the inositol 1,4,5 trisphosphate receptor ($InsP_3R$) and modulates calcium entry through receptor- and store-activated channels [1, 7]. In addition, junctate induces and/or stabilizes peripheral couplings between the endoplasmic reticulum and the plasma membrane [7, 8].

Our group previously reported two putative promoter sequences present within the human AβH-J-J locus (P1 and P2), which are expected to regulate the transcription of this locus [1, 9]. Theprimary transcripts of the AβH-J-J locus are subjected to alternative splicing resulting in the synthesis of different AβH isoforms, junctin, and junctate [1, 9]. The recently characterized MEF-2 transcription factor dependent P2 promoter sequences drive tissue-specific expression of these proteins, and are responsible for inducing transcription during muscle differentiation [1, 9].

To date knowledge on the sequences regulatingP1 promoter activity is scanty; in the present report we focused our attention on the characterisation on these regulating sequence. To characterize the expression directed by the P1 promoter, we analysed the corresponding mRNAs in different human tissues and cell lines. In addition, we transfectedtwo cell lines () with progressively deleted reporter constructs of the 1.7 kb DNA sequence upstream exon 1 . Computer assisited analysis of the sequences encompassing the region yielding maximal promoter activity revealed the putative presence of several Sp1 binding sites, The interactions of these GC rich elements with factors belonging to the Sp1 family was confirmed experimentally by electrophoretic mobility shift assay (EMSA) [10, 11].

RESULTS

Transcriptional organization of the human AβH-J-J locus and identification of the transcription initiation site proximal to the P1 promoter.

The structural organization of the human AβH-J-J locus is shown in Fig.1. The scheme presented is based both on results previously reported in detail elsewhere [1, 9] and on newly performed studies employing RT-PCR. The combination of data obtained by PCR amplification and sequencing allowed us to define the splicing events (Fig.1) as well as the structure of the 5' region of this locus [1]. The data obtained indicate that the use of different splice donors is involved in the generation of protein diversity by alternative splicing (see lower part of Fig.1) [1, 12]. Furthermore the P1 promoter sequences direct the expression of these proteins in most human tissues [9, 12]. The RT-PCR approach presented in Fig.2A shows that by using an exon 1 specific forward primer and an exon 3 specific reverse primer we were able to amplify all the transcripts starting from the P1 promoter (AβH and is truncated isoforms, including "humbug" (12),). We employed this RT-PCR approach to examine total RNA samples extracted from several human adult tissues and confirmed by DNA sequencing the fidelity of the PCR products. The results obtained from pancreas, brain, adrenal gland, liver, heart and skeletal muscle show that transcription directed from the P1 promoter occurs in all the tissues analyzed (Fig. 2B). On the contrary, we have previously shown that the expression directed by the P2 promoter is tissue specific, with high levels of transcription occurring particularly in skeletal muscle, cardiac muscle, and brain and lower level of transcription in kidney[9, 12]. The P1 promoter directs transcription also in RD, HepG2, MCF7, HeLa and Hek293 tumour cell lines (Fig. 2B).

Since the transcription start site (TSS) of the P1 promoter has not been previously described, we also performed 5' RACE in order to precisely map the origin of transcription. Our experiments were performed on 5'-caped mRNA isolated from HeLa and RD cells. As shown in Fig. 3A, lanes a and b, a prevalent band is evident following electrophoresis of 5'

RACE products. We cloned the PCR- amplified cDNA fragments extracted from the gel , determined their nucleotide sequences (Fig. 3B, arrows) and mappedmultiple TSS endowed with different strengths. The major TSS resulted from the stronger band in the gel, was designated as +1 (Fig. 3B, larger arrow). The nucleotide sequences located upstream from these TSS were considered as potential regulatory regions belonging to the P1 promoter.

Transcriptional activity of the AβH-J-J P1 promoter

To test whether the exon 1 5'-flanking sequences have promoter activity, we cloned a 3.1 kb partially digested *Pst*I fragment (Fig. 1) from the human chromosome 8 BAC 1 clone [1]. The PCR-generated fragment spanning from -1683 to +81 with respect to the principal transcription start site was subsequently inserted into the firefly luciferase reporter vector pGL3-basic, sequenced and used to generate progressively deleted constructs to be analysed for activity after transient transfection in cultured cells [9]. The reporter constructs (a-m), generated as described in the experimental procedures section, are represented on the left side of Fig. 4A. We tested the ability of these constructs to drive luciferase transcription in human hepatoblastoma (HepG2) and embryonic rhabdomyosarcoma (RD) cells. Promoter activity was expressed as fold induction relative to that of cells transfected with pGL3-basic vector (right side of Fig. 4A and Fig. 4B). Our results showe that the largest fragment (-1683/+81) exhibited high reporter gene activity in both cell lines (the fold induction was about 100 in HepG2 cells and 45 in RD cells, respectively). Removal of the -1683/-834 sequence significantly increased promoter activity (compare a and e constructs of Fig. 4A-B). Further removal of the -834/-512 fragment preserved transcriptional activity to comparable levels in the analyzed cell lines (e-h constructs, Fig. 4A-B); however, deletion to nucleotide -389 resulted in a significant decrease of luciferase activity (i construct) and, when the sequence was progressively removed to -240 and -160, reporter expression gradually decreased (l and m constructs). In conclusion our findings indicate that the sequences 512 bp upstream from the transcription start site are essential for maximal promoter activity, and deletions to -389, -240 and -160 result in the progressive reduction of transcription to about 75 %, 40 %, and 7 % respectively.

When we compared the maximal potencies of the P1 and P2 promoter sequences at inducing transcription in RD cells, these appeared dramatically different, the P1 promoter being 16 -fold more active (Fig. 4C). The P1 promoter sequences do not share regions of homology and signals for transcription factors with those found in the P2 promoter, such as MEF-2 binding sites; the latter finding is quite interesting when related to the different tissue specificitiesy of the two promoters [9].

Identification of GC boxes within the -512/+33 AβH-J-J P1 promoter region

Sequence analysis of the region required for maximal expression from the P1 promoter were performed using the *TF search* program. Looking for homology to known signals for transcription factors and imposing an 80% threshold, we identified within -512/+33 at least 12 GC rich boxes similar to Sp1 consensus binding sequences (Fig.5A). The location of these putative Sp1 binding sites within the P1 promoter sequence is shown in figure 5A and B, together with the transcription initiation sites and the ATG signal.

Since the region responsible for maximal P1 promoter transcription contains GC rich elements (h construct, Fig.4), we concentrated our attention on the nuclear extract binding activity to these boxes.

Binding of nuclear factors to GC rich boxes of AβH-J-J P1 promoter.

In order to study protein/DNA interactions and further characterize the transcription factors involved, we performed competitive EMSA [9].

Table 1 and Fig. 5A show the synthetic oligonucleotides used for the band-shift experiments. Figure 6A shows the results obtained using 2 μg of HepG2 cell nuclear extracts and the ^{32}P-labelled Sp1*mer* double-stranded oligonucleotide, which contains the consensus binding site for the Sp1 transcription factor [13, 14]. The probe interacts with nuclear proteins producing the three retarded complex pattern typical of transcription factors belonging to the Sp family. A

fast mobility band and two overlapping low migrating bands (Fig. 6A, lane 1) are generated. As expected, a 100 fold excess of unlabeled Sp1*mer* oligonucleotide completely abolished sequence specific interactions of the nuclear proteins to the probe (Fig. 6A, lane 2). Competitive experiments performed using unlabeled oligonucleotides containing the previously identified GC rich boxes of the P1 promoter region (Table 1) demonstrated that F/G*mer*, H/I*mer* and D*mer* interfere with the formation of the three complexes (Fig. 6A, lanes 3,4 and 7). These results suggest that the last three oligonucleotides contain binding elements recognized by Sp family transcription factors [15]. On the other hand, competitive band-shift assays demonstrate that the three Sp bands were only slightly reduced by K*mer* and L*mer* (Fig. 6A, lanes 5 and 6), while J*mer* did not decrease the abundance of the Sp specific complexes (Fig. 6A, lane 8). To better characterize the binding efficiency of the oligonucleotides under investigation, we performed band-shift with the same probe and different fold molar excess of competitors (Fig. 6A and B). As observed for Sp1*mer* (Fig. 6B, lane 10), the three complexes were completely disrupted by a 6 fold molar excess of unlabeled H/I*mer* (Fig. 6B, lane 15), while the same excess of F/G*mer* decreased the binding to about 5% of the control in the absence of competitor (Fig. 6B, lane 12). Furthermore, the competition with 50 fold molar excess of unlabeled D*mer*, K*mer*, and L*mer* decreased the interactions to 12%, 60%, and 75% of the control respectively (Fig. 6C, lanes 20,22 and 24), while J*mer* competitor was not active even if used at 100 fold molar excess (Fig. 6C, lane 26). To confirm whether the previously identified GC rich P1 sequences are able to bind Sp family transcription factors, we performed band-shift assays using as probe the oligonucleotides under investigation, which generated the same complex migration profile obtained with labelled Sp1*mer*. Figure 6D shows an example of the interactions of nuclear extracts with H/I*mer* probe. As expected from our previous assays, the three complexes were completely disrupted by an excess of the competitors Sp1*mer*, F/G*mer* and H/I*mer* (Fig. 6D, lanes 28-33), but not by J*mer* or the unrelated oligonucleotide MyD*mer* (Fig. 6D, lanes 34 and 35) [9].

DISCUSSION

The aim of the present paper was to investigate in detail one of the two putative promoter sequences regulating the transcription of the AβH-J-J locus [1, 9]. We isolated and identified the 5'-flanking region of the exon 1 of this locus. The cloned nucleotide sequence allowed us to characterize the P1 promoter region, which is involved in the regulation of aspartyl β-hydroxylase and junctate expression. We have been able to identify transcripts relative to P1 promoter activity in all tissues and cell lines analyzed [9, 12]. Similar to many housekeeping gene promoters, the region under investigation lacks a TATA box and an initiator element [16, 17]. In contrast, this sequence is GC-rich and presents homologies with Sp1 consensus binding site, according with previous studies showing that the transcription of other TATA-less promoters frequently involves the action of proximal Sp1 sites [18, 19]. The mapping of the initiation of transcription using 5'-RACE revealed the presence of different transcriptional start sites (TSS) located around position -110 relative to the translation initiation start in HeLa and RD cells. We found, cis-elements with negative and positive effects on transcription upstream from exon 1,. Furthermore, the maximal promoter is located within 512 nucleotides from the principal TSS. Computer analysis indicates the presence of at least 12 sites that match the structural determinants of Sp1-binding specificity and the screening by EMSA assays demonstrated three GC-rich elements binding with high efficiency transcription factors belonging to the Sp family [10, 11]. The migration profiles of the complexes produced by nuclear extracts binding to our GC-rich elements resemble the well known electrophoresis pattern obtained with the consensus binding site for Sp1 transcription factor [13-15]. Sp1 is a ubiquitous DNA-binding protein, that activates the transcription of many cellular and viral genes [20, 21]. Other transcription factors, Sp2-Sp6, have been described that have similar structural properties and DNA binding specificities as Sp1 [10, 11]. Sp1 and Sp3 are the major DNA-binding constituents observed in nuclear extracts with Sp1 consensus element in EMSA [22]. The actions of Sp1 and Sp3 at a given promoter appear to be complex, but, in many cases, expression of Sp3 is thought to antagonize the stimulatory actions of Sp1 on gene transcription [23, 24]. Moreover Sp3 can act as an activator or repressor of Sp1-mediated activation,

depending on the sequence context and the availability of specific co-activators, co-repressor or other transcription factors [21, 22]. The activity of the Sp proteins on transcription can be influenced by multiple factors, including phosphorylation, redox state, and acetylation [25]. The major conclusions of the present manuscript is that the P1 promoter of the AβH-J-J locus contains Sp1 cis-acting motifs putatively involved in the wide transcription directed by this promoter. While our results do not conclusively address the involvement of other factors on the transcription of the AβH-J-J locus, we suggest that some of the Sp1 binding activities are important for transcription directed by the P1 promoter. The impact of Sp proteins in the transcriptional regulation of this locus will be of future interest considering the potential contributione of AβH to the infiltrating growth of neoplasms by increasing cell migration and enhancing proliferation and survival [2-5]. Inhibition of AβH expression could represent an attractive approach for gene therapy of infiltrating tumors [2]. The interest is also extended to transcriptional regulation of junctate, which is involved in calcium homeostasis in eukaryotic cells [1, 7, 8].

When the P1 and P2 promoter sequences are compared, important differences are clearly detectable [9]. The most interesting result emerging from studies focused on the P2 promoter is that the calcium–dependent transcriptional factor MEF-2 activates the transcription of junctin, junctate and AβH in excitable tissues and to lower extent in kidney [1, 9]. No Sp1 binding sites are present in the P2 promoter. On the contrary, P1 promoter directs the expression of humbug and various isoforms of AβH in many tissues and contains several functionally active Sp1 binding sites [1, 9, 12]. The finding that the sequences present in the upstream P1 promoter are significantly different from those of the P2 promoter is,, of great interest because….

In addition, our data do not exclude a concerted regulation of the two promoter sequences based on interactions between different transcription factors. There is strong experimental evidence demonstrating that transcription factors belonging to the Sp1 family interact with other transcription factors, including some proteins binding to the P2 promoter [26, 27, 9]. For instance, physical interactions between Sp1 and MEF-2 have been demonstrated in DNA

binding complexes formed in vitro by nuclear extracts [28]. An intriguing possibility which should be analyzed in the future, is the generation of a looping structures directed by the physical interactions between P1 and P2 promoters driven by transcription factors able to form heterodimers, such as Sp1 and MEF-2.

The data reported here on the functional characterization of the P1 promoter of the AβH-J-J locus demonstrates that this belongs to the class of Sp1-controlled promoters, and it is different from the P2 promoter, both for transcription factors binding signals as well as potency in supporting transcription [9].

The results shown in the present paper together with previously published reports by our and other research groups, suggest that the AβH-J-J locus contains at least two functionally distinct promoters (P1 and P2). The mechanism regulating the transcription of the AβH-J-J locus is likely to be very complex. The P1 and P2 promoters drive the expression of at least three transcripts encoding aspartyl-β-hydroxylase, junctin, and junctate. In addition several isoforms can be generated by multiple alternative splicing events. We do not know either the relative abundance of each alternatively spliced transcript or the mechanism involved in the tissue specific expression of these transcripts. Western blot analysis with Ab specific for the protein sequence encoded by exon two revealed the expression of this exon in heart, skeletal muscle, brain and to lower exten in kidney (1,8). The elucidation of the exact pattern of the expression of the protein products will be important to define the functional role of the alternatively spliced transcripts in calcium homeostasis and tumor progression. [1, 9, 12].

Experimental procedures

Cell Culture

The human hepatoblastoma, HepG2, embryonic rhabdomyosarcoma, RD, breast cancer, MCF7, cervix epithelial carcinoma, HeLa, and embryonic kidney, Hek293, cell lines, obtained from the ATCC (Manassas, VA, USA), were cultured as described previously [].

RT-PCR

Total RNA from human adult normal tissue was purchased from BD Biosciences Clontech (Palo Alto, CA). Total RNA was harvested from cell lines by using the *TRIzol® Reagent* (Invitrogen, CA). cDNA was synthesized from 1 μg of total RNA using ImProm-II™ (Promega, Madison, WI). PCR was performed using the GeneAmp PCR System 9600 (Perkin Elmer), 2 μl/20 μl of cDNA, the e1F forward primer, 5'-CAA GAG CAG CGG CAA CAG-3', and the e3R reverse primer, 5'-TTC CTG AGA GTC CGC CTT TC-3' (designed to amplify a 134 bp sequence present in all the transcripts relative to P1 promoter activity and spanned one intron in order to rule out amplification from genomic DNA), 2 U Taq polymerase and 33 μM dNTPs. The PCR conditions were as follows: 30 cycles of amplification with 15 second denaturation at 95°C, 30 second annealing at 64°C, and 15 second elongation at 72°C. Starting total RNA were normalized performing RT-PCR of beta-2 microglobulin housekeeping gene (data not shown).

5' RACE

RNA ligase-mediated 5' rapid amplification of cDNA ends (5'-RACE) was performed by using the *GeneRacer* kit (Invitrogen, Carlsbad, CA) with 2 μg of total RNA, isolated from HeLa and RD cells. cDNA was synthesized using the e5R primer (5'-AAT AAA ACT TTG GCA TCA TCC ACA TCA AAA TCT CC-3'), complementary to an exon 5 sequence of the AβH-J-J locus (Fig. 1). After ligation to the RNA oligonucleotide, the cDNA was used as template for polymerase-chain reaction (PCR), which was performed with the *GeneRacer* kit anchor primer,

and the gene-specific e3R primer (Fig. 2). A dilution of the original PCR was re-amplified using the nested e1R primer (5'-TTC TCG TCG CCG TTG TCG TCG TC-3') complementary to an exon 1 sequence. PCR products were analysed by electrophoresis, and amplified DNA purified from gel slices was cloned with the pGEM®-T Vector System (Promega, Madison, WI). Recombinant plasmids were sequenced with the ABI PRISM Big Dye terminator cycle sequencing ready reaction kit using the ABI PRISM 377 DNA sequencer (PE Applied Biosystems, Foster City, CA). The stronger transcription initiation site, defined by the most represented 5'-ends, was numbered +1 and all the other sites are relative to it.

Cloning of the AβH-J-J P1 promoter sequences and reporter plasmid construction

The 3.1 kb partially digested *Pst*I fragment of the human chromosome 8 BAC 1 clone encompassing AβH-J-J exon 1 (Fig. 1) [1] was cloned into the pUC18 vector and sequenced. The sequence corresponding to -1715/+49, relative to transcription initiation site, was amplified by using the GeneAmp® High Fidelity PCR System (Applied Biosystems, Foster City, CA), and the *XF* (5'-CCG CTC GAG TGC AGT GTG AAA ACG GAC TAA TAC AGT G-3') forward and *NR* (5'-CAT GCC ATG GTG GCG GAC CTC CTT CAG TGC-3') reverse primers, containing a *Xho*I and a *Nco*I restriction site respectively. The PCR product was cloned into the *Xho*I/*Nco*I restriction sites of pGL3-basic firefly luciferase reporter plasmid (Promega, Madison, WI). Ten serial deletion constructs were generated from this recombinant plasmid using the Exo III/S1 Deletion kit (Fermentas, Lithuania). The sequence of all the constructs was confirmed by DNA sequencing. The AβH-J-J P2 promoter reporter construct with maximal promoter activity (-265/+115) were obtained from pGL3-basic vector as previously described [].

Transient transfection and dual luciferase assay

Cells were seeded at 50-70% confluence in 16-mm wells. Transfection was performed after 24 hours using 1.5-2 μg of *Lipofectamine*™ 2000 (Life Tecnologies, Gaithersburg, MD), 75 ng pRL-TK vector (Promega, madison, WI), which contains the *Renilla* luciferase gene as a

transfection efficiency control and 0.75 μg of *Firefly* luciferase reporter plasmid per well. Lysates were prepared 24 h after transfection by adding 100 μl of passive lysis buffer (Dual Luciferase® Reporter Assay System, Promega). Luciferase activity was determined with an analytical luminometer (model TD-20/20, Turner Designs, Sunnyvale, CA); the light intensity produced by *Firefly* luciferase (test plasmid) was normalized to that produced by *Renilla* luciferase (control plasmid). Promoter activity was expressed as *fold induction* relative to that of cells transfected with pGL3-basic vector. At least three independent experiments were performed using each construct.

Nuclear extract preparation

Nuclear extracts were prepared as described by Andrews and Faller []. Briefly, cells were collected, washed twice with ice-cold phosphate-buffered saline, and resuspended in 0.4 ml/10^7 cells of hypotonic lysis buffer (10 mM Hepes/KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.2 mM phenylmethanesulfonyl fluoride). After incubation on ice for 10 min, the mixture were vortexed for 10 seconds, and nuclei were pelleted by centrifugation at 12000 *g* for 10 seconds, then nuclear proteins were extracted by incubation of the nuclei for 20 min at 4 °C with intermittent gentle vortexing in 20 mM Hepes/KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethanesulfonyl fluoride, 1 $\mu g \cdot mL^{-1}$ aprotinin, 1 $\mu g \cdot mL^{-1}$ leupeptin, 2 mM Na_3VO_4, and 10 mM NaF (Sigma); cell debris was removed by centrifugation at 12000 *g* for 5 min at 4 °C. The Bradford method (DC Protein Assay, Bio-Rad) was used to measure the protein concentration in the extract, which was then stored in aliquots at -80 °C.

Electrophoretic mobility shift assays

The double-stranded oligonucleotides (ODN) used in the EMSA are reported in Table 1. 3 pmol of ODN were ^{32}P-labeled using OptiKinase (Amersham), annealed to an excess of complementary ODN and purified from [γ-^{32}P]ATP (Perkin Elmer). Binding reactions were performed by incubating 2 μg of nuclear extract and 16 fmol of ^{32}P-labeled double-stranded

ODN, with or without competitor in a final volume of 20 μL of binding buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 5 % glycerol, 1 mM dithiothreitol, 0.01 % TritonX100, 0.05 $\mu g \cdot \mu L^{-1}$ of poly dI-dC, 0.05 $\mu g \cdot \mu L^{-1}$ of a single-stranded ODN) []. Competitor [either a 6-100 fold excess of unlabeled ODNs] and nuclear extract mixture were incubated for 15 min and then probe was added to the reaction. After a further incubation of 30 min at room temperature samples were immediately loaded onto a 6 % nondenaturing polyacrylamide gel containing 0.25 x Tris/borate/EDTA (22.5 mM Tris, 22.5 mM boric acid, 0.5 mM EDTA, pH 8) buffer. Electrophoresis was carried out at 200 V. Gels were vacuum heat-dried and subjected to autoradiography.

Statistical analysis

All the data were normally distributed and presented as mean ± S.D. Statistical differences between groups were compared using one-way ANOVA (Analyses of variance between groups) software. Statistical significance was assumed at $p < 0.05$.

ACKNOWLEDGEMENTS

This work was supported in part by grants from FIRB, Ministero Università e Ricerca Scientifica e Tecnologica 60 and 40%, the Department of Anesthesia Basel University hospital, HPRN-CT-2002-00331 from the European Union, Italian Space Agency and Swiss Foundation for research on Muscle. R.G. is supported by grants from AIRC.

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Legends to Figures

FIG. 1. Structure of the 5' end of the human locus for aspartyl β-hydroxylase, junctin, and junctate. Arabic numbers over black boxes indicate exons. Intervening sequences are indicated in Roman numbers. The two putative promoters P1 and P2 are indicated. A schematic representation of aspartyl β-hydroxylase, junctin, and junctate exons splicing is reported at the bottom of the panel. The cytoplasmic, TM, positively charged, calcium-binding, and catalytic domains are indicated. The locations of AUG, stop codons, and poly(A) signals are shown. The Pst I (P) plasmid subclone of BAC 1 [1] covering the first exon of the locus is also shown.

FIG. 2. RT-PCR analysis of the transcripts starting from P1 promoter in adult human tissues or cell lines. Panel A, the exon 1 starting mRNAs of aspartyl-β-hydroxylase, junctate, and junctin, the PCR primers and the 134 bp PCR product are represented; black boxes indicate exons common to the three transcripts, grey boxes indicate exons common only to aspartyl-β-hydroxylase, and junctate. Panel B, electrophoresis analysis of oligo dT RT-PCR products obtained with the e1F/e3R primers in the absence of template (a) or in the presence of cDNA from human adult normal tissues (b, pancreas; c, brain; d, adrenal gland; e, liver; f, heart; g, skeletal muscle) or cell lines (h, RD; i, HepG2; j, MCF7; k, HeLa; l, Hek293) total RNA. M, pUC Mix Marker 8 (Fermentas).

FIG. 3. P1 transcription initiation sites mapping. (A) 5' RACE analysis of AβH-J-J locus exon 1 starting transcripts. cDNA, synthesized from HeLa and RD cells total RNA, were amplified with the gene-specific e3R primer. The nested PCRs were performed with the gene-specific e1R primer, complementary to the exon 1, and one-fifth of the reactions, derived from HeLa (a) and RD (b) cells, was analyzed by gel electrophoresis. M, pUC Mix Marker 8. (B) Nucleotide sequence of 5' RACE products. The most represented nested PCR products were gel-purified, cloned and sequenced. The principal transcription start sites (TSS) are shown by

arrows (the stronger TSS was numbered +1). The gene specific reverse primer used for the last PCR is underlined, and the translation start site (ATG) is indicated.

FIG. 4. AβH-J-J P1 promoter activity in HepG2 and RD cell lines. (A) HepG2 cells were transiently transfected with sequentially deleted reporter constructs of the -1683/+81 P1 nucleotide sequence (represented in the left side of the figure, a-m). Transient transfection and luciferase assays were performed in triplicate, and the data (right side of the figure) were normalized to renilla luciferase activity and are shown as relative activities compared to that for pGL3-basic, a reporter vector with a basal promoter. The values are the means ± SD of at least three independent experiments. (B) The same protocol used to generate the data represented inpanel A was performed with the RD cell line. (C) AβH-J-J P1 and P2 promoter activity in RD cell line. Cell were transiently transfected with the reporter constructs that present the maximal transcriptional activity of each promoter [9].

FIG. 5. DNA sequence of the AβH-J-J P1 promoter region. (A) -512/+112 P1 promoter and 5' UTR sequences. Solid and dashed lines indicate the oligonucleotides used in gel shift assays (Table 1). The sequences homologous to Sp1 transcription factor binding site are boxed; the percent of homology was obtained by *TF SEARCH* ver. 1.3. Arrows indicate the characterized transcription initiation sites. The 5' ends nucleotide positions of the progressively deleted promoter sequence present in the reporter constructs are shown in grey. (B) Schematic representation of the -512/+112 region of the P1 promoter. Elements homologous to Sp1 binding site are indicated by boxes and in grey are shown the nucleotide deletions of reporter constructs.

FIG. 6. AβH-J-J promoter P1 elements homologous to Sp1 box bind HepG2 nuclear factors. Band shift assays were carried out on 2 μg of HepG2 nuclear extracts as described in the Materials and methods using Sp1*mer* (A-C) or H/I*mer* (D) probe (Table 1). (-), probe was incubated with nuclear extracts in the absence of competing oligonucleotides. The fold molar

excess of the added competitor (Table 1) is reported at the bottom of each panel. Arrows and asterisk indicate the specific and non-specific complexes respectively.

Figure 1

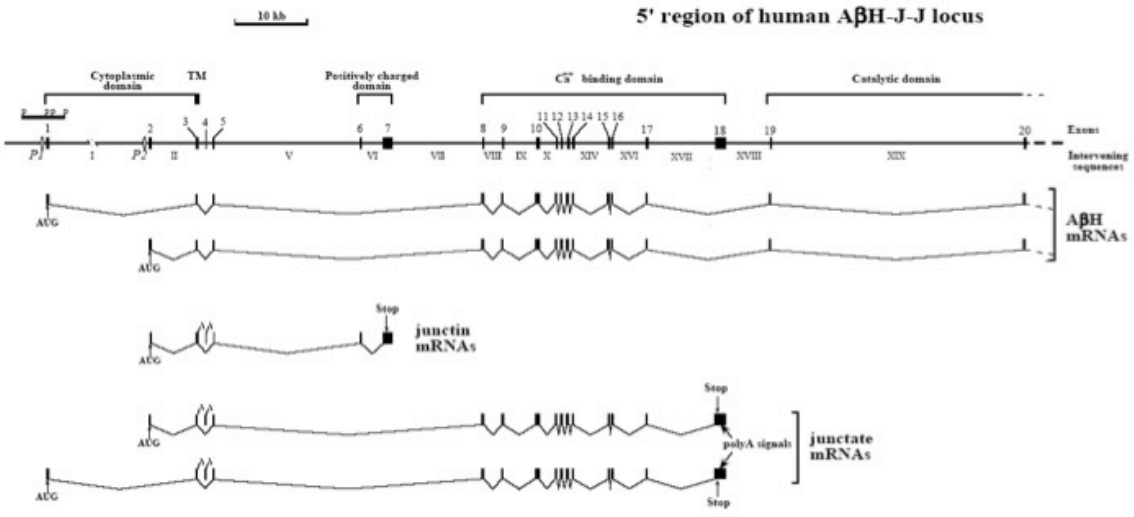

Figure 1

Figure 2

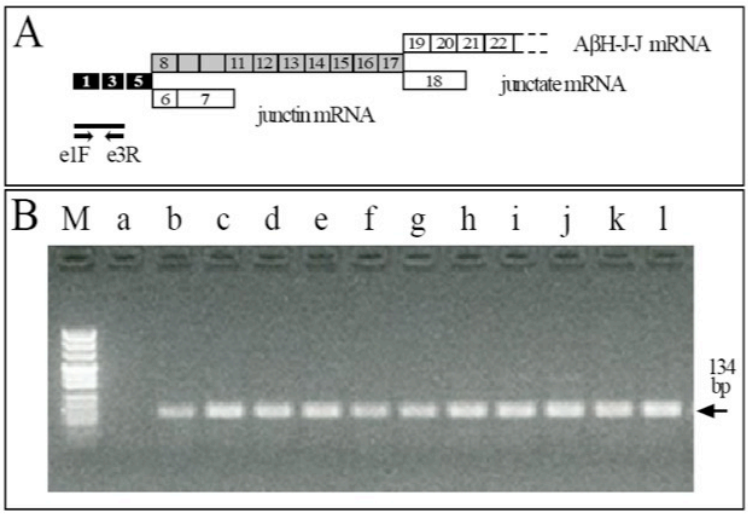

Figure 2

Figure 3

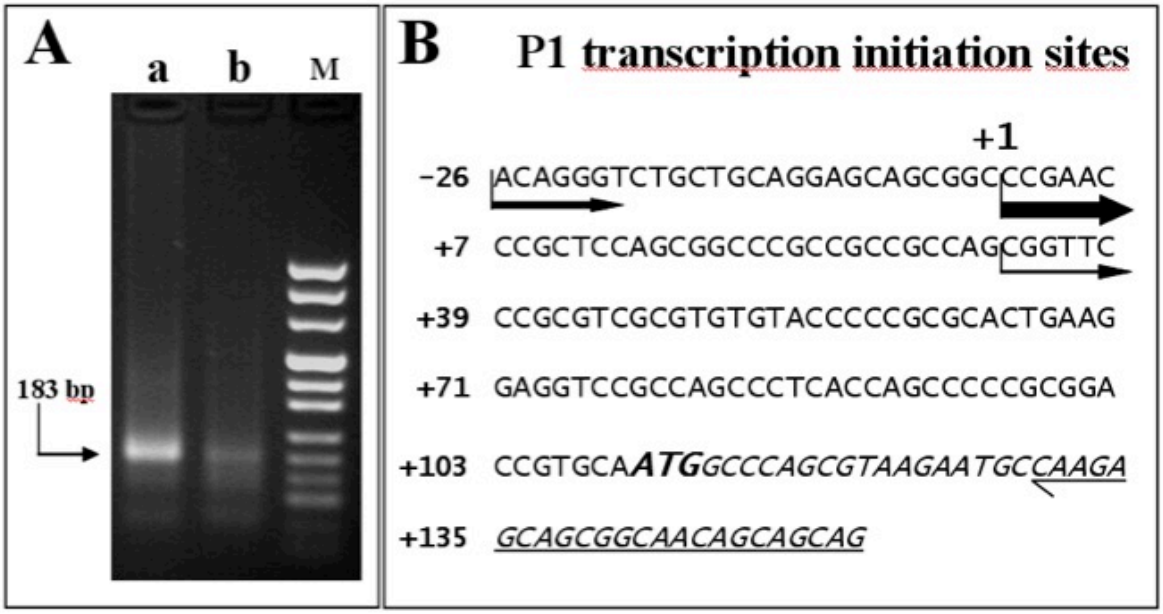

Figure 3

Figure 4

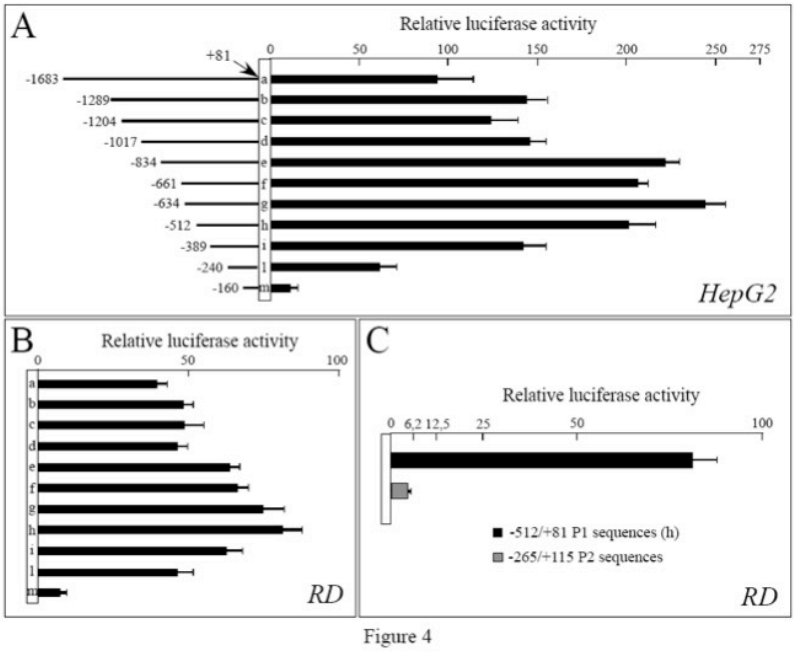

Figure 4

Figure 5

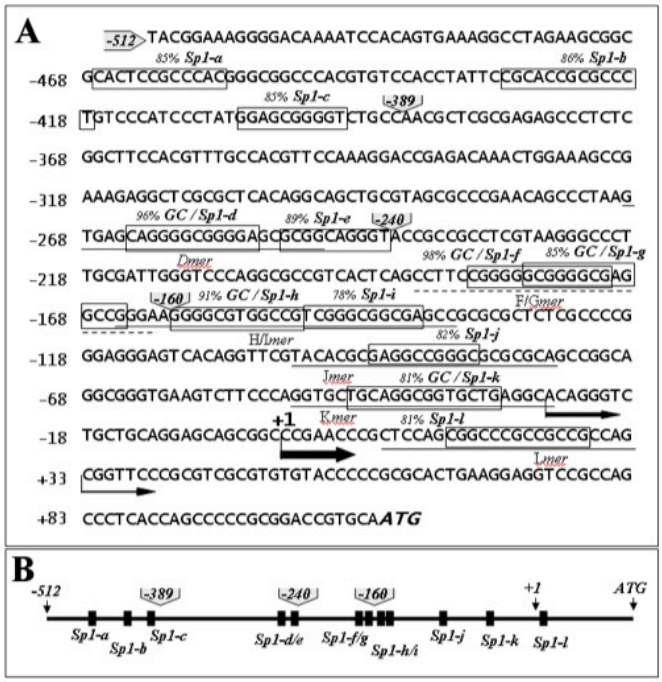

Figure 6

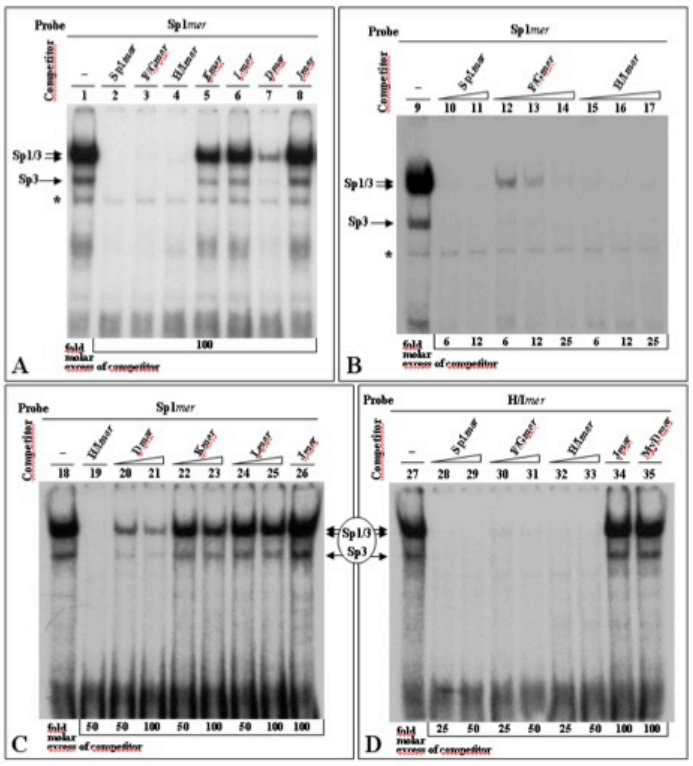

Figure 6

Table 1

Table 1. Double-stranded synthetic oligonucleotides used for gel-shift assays

Sp1mer[a]	5'-CCCTTGGTG***GGGGCGGGGC***CTAAGCTGCG-3'
F/Gmer	5'-CCTTCCGGGGGCGGGGCGAGGCCGGGA-3'
H/Imer	5'-CGGGAAGGGGCGTGGCCGTCGGGCGGCGAGCC-3'
Kmer	5'-GTGCTGCAGGCGGTGCTGAGGCA-3'
Lmer	5'-TCCAGCGGCCCGCCGCCGCCAG-3'
Dmer	5'-GTGAGCAGGGGCGGGGAGCGCGGC-3'
Jmer	5'-TACACGCGAGGCCGGGCGCGCGCA-3'
MyDmer[b]	5'-CCCCCCAACACCTGCTGCCTGA-3'

[a] The oligonucleotide containing Sp1 site was purchased from Geneka (Montreal, Canada); the consensus binding sequence is underlined. [b] [Friday]