

Transcriptional regulation of human 3'-phosphoadenosine 5'-phosphosulphate synthase 2

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Sulphonation is a fundamental process that is essential for normal growth and development as well as maintenance of the internal milieu. The universal sulphonate donor molecule essential for all sulphoconjugation reactions is adenosine 3'-phosphate 5'-phosphosulphate (PAPS), which is produced from ATP and inorganic sulphate by the action of bifunctional PAPS synthase. There are two isozymes encoded by genes located on chromosome 4 (PAPS synthase 1) and chromosome 10 (PAPS synthase 2). The promoter for PAPS synthase 2 contains neither a TATAAA nor a CCAAT box, although a consensus initiator motif is present. Three human cell lines were used to examine promoter activity after transfection with various lengths of the 5'-flanking region of the PAPS synthase 2 gene fused to a reporter gene. Proximal promoter activity was located between bp -84 and bp -124 upstream of the purported transcription

start site. This region contains two GC/GT boxes that are essential for full promoter activity, as indicated by deletion analysis and supported further by mutagenesis. A nuclear extract of SW13 cells, which highly express PAPS synthase 2, contained proteins that bound to probes possessing promoter-specific GC/GT boxes. Furthermore, the presence of specificity protein (Sp) 1, Sp2 and Sp3 proteins in the nuclear extract was confirmed by supershift analysis. Co-transfection experiments using SL2 cells yielded additional support for the involvement of Sp1 in transcriptional regulation of the PAPS synthase 2 gene; the involvement of Sp2 and/or Sp3 remains to be clarified further.

Key words: adenosine 5'-phosphosulphate kinase, ATP-sulphurylase, sulphoconjugation, sulphonation.

INTRODUCTION

Sulphonation or sulphoconjugation is a major process in the post-translational modification of biomolecules ranging in size from $< 10^3$ to $> 10^6$ kDa, a process that is essential for normal growth and development as well as maintenance of the internal milieu. Sulphonated glycosaminoglycans and proteoglycans are essential components of cell-surface and connective tissue structures. Tyrosine sulphonation is a common post-translational modification of many secretory and membrane proteins. Sulpholipids are concentrated in the brain, peripheral nerves and reproductive tissues. Additionally, the sulphonation of hormones and neurotransmitters as well as drugs and xenobiotics plays a crucial role in biological activity, protein binding and metabolism. Importantly, sulphonation requires the universal sulphonate donor molecule, adenosine 3'-phosphate 5'-phosphosulphate (PAPS), a fact that establishes PAPS as a strategic biomolecule and making its availability of vital importance. The biosynthesis of PAPS from inorganic sulphate and ATP is carried out by the bifunctional enzyme PAPS synthase [1,2]. Human PAPS synthase exists as two isoenzymes that are 77% identical at the amino acid level. Additionally, as a result of alternative splicing, there are two forms of PAPS synthase 2 [3]. The gene for human PAPS synthase 1 is located on chromosome 4 (K. V. Venkatachalam and C. A. Strott, unpublished work), whereas the gene for human PAPS synthase 2 is located on chromosome 10 [4]. Northern-blot analysis indicated that the

human PAPS synthase isoenzymes are differentially expressed [5]. Multiplex reverse transcription (RT)-PCR experiments reveal that the expression of PAPS synthase 1 is ubiquitous, whereas expression of PAPS synthase 2 is variable and tissue specific (H. Fuda, C. Shimizu, Y. C. Lee and C. A. Strott, unpublished work). Particularly striking is the differential tissue-specific expression of the PAPS synthase 2 subtypes. Because these results suggest that the PAPS synthase isoenzymes are uniquely controlled, we set out to investigate their transcriptional regulation and have published recently our initial findings on the transcriptional regulation of the PAPS synthase 1 gene [6]. In this report, we present the results of studies on the regulation of the gene for PAPS synthase 2.

EXPERIMENTAL

Materials

Human RNA, human genomic DNA and Advantage GC-genome PCR kit were purchased from ClonTech (Palo Alto, CA, U.S.A.). The RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) kit was obtained from Ambion (Austin, TX, U.S.A.). Thermoscript RT-PCR system, *Taq* polymerase and reagents for PCR, DNA markers, LIPOFECTAMINE 2000 reagent, cell culture media, fetal-bovine serum (FBS) and antibiotics were obtained from Life Technologies (Grand Island, NY, U.S.A.).

Abbreviations used: CIP, calf intestinal alkaline phosphatase; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; FBS, fetal-bovine serum; PAPS, adenosine 3'-phosphate 5'-phosphosulphate; PCPV, packed-cell-pellet volume; RLM-RACE, RNA ligase-mediated rapid amplification of cDNA ends; RT, reverse transcription; Sp, specificity protein; TAP, tobacco acid pyrophosphatase; UTR, untranslated region.

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Table 1 Oligonucleotide primersUnderlined nucleotides show *Kpn*I or *Xho*I restriction enzyme sites. S, sense primer; AS, antisense primer.

Name	Use	Sense/antisense	Nucleotide sequence
2AS-RT	5'-RACE	AS	5'-CAGGAGAGAACCAGATTTTC
2PE1	5'-RACE	AS	5'-GTCTTTTGTCTTGTATCCCCGACATGCTG
2PE2	5'-RACE	AS	5'-AGCAGAGACCGAAGGACGCAG
TSR3	RT-PCR	S	5'-TGCCATCTGCCGCTCCGCTGCAAGGTC
TSR4	RT-PCR	S	5'-AAGGCTGGGGGCTGCTTGGGAACCGAC
AS2-3	RT-PCR	AS	5'-CAGGAGAGAATCCGAGATTTTC
S2-6	RT-PCR	S	5'-CACTCCCCCTCAAAGGTTTC
AS2-7	RT-PCR	AS	5'-CAGCGTCTCGTAAGATAGC
GMS3	Genomic cloning	S	5'-GGGGTACCTAGTCTGATCCAAAAGAAGTGGGCTCC
GMS4	Genomic cloning	S	5'-GGGGTACCTCAGCTCTGCTCCTAACTAGTGACTACGGTC
GMAS1	Genomic cloning and pGL3S2s	AS	5'-CCGCTCGAGTCCCGGGGAGCAGAGACCGAAGGACGAGGGGAC
LUCS1	pGL3S2-378, D378	S	5'-GGGGTACCTAGGTCCTAGAGGCAGGAAGACTTCTCTTG
LUCS2	pGL3S2-58	S	5'-GGGGTACCAAGGGAAGTGGCAGCTGTCTGCGGAG
LUCS2AS	pGL3S2-d249, d378	AS	5'-CCGCTCGAGTCCGTTCCGCAAGCAAGCAGCCAGCCTTG
LUCS3	pGL3S2-249, D249	AS	5'-GGGGTACCTGCGATCTGCGGCTCCGCTGCAAGGTC
LUCS4	pGL3S2 + 49	S	5'-GGGGTACCGTCCCTGCGTCTTCCGTTCTGCTCCCGGACTCGAGCGG
LUCAS4	pGL3S2 + 49	AS	5'-CCGCTCGAGTCCCGGGGAGCAGAGACCGAAGGACCGAGGGACGGTACCCC
LUCS5	pGL3S2-137	S	5'-GGGGTACCAAGGCTGGGCGCTGCTTGGGAACCGAC
LUCS6	pGL3S2-124	S	5'-GGGGTACCTGCTTGGGAACCGAAGGCGGGGAG
LUCS7	pGL3S2-84	S	5'-GGGGTACCAAGGAGGAGTAGGAGAAGGGGGCTG
ACT-S	RT-PCR	S	5'-CTGGCACCACACCTTCTACAATG
ACT-AS	RT-PCR	AS	5'-AATGTACACGACGATTTCCCGC

Protease-inhibitor-cocktail tablets were purchased from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). Oligonucleotides were obtained from Gene Probe Technology (Gaithersburg, MD, U.S.A.). The pGL3-vector system and luciferase assay kit were purchased from Promega (Madison, WI, U.S.A.). Pfu-turbo polymerase, QuikChange XL site-directed mutagenesis kit and NucTrap probe purification column were obtained from Stratagene (La Jolla, CA, U.S.A.). Plasmid purification kit was purchased from Qiagen (Valencia, CA, U.S.A.). Enzymes were from New England Biolabs (Beverly, MA, U.S.A.), Promega and Sigma (St Louis, MO, U.S.A.). Gel-shift assay kit was purchased from Geneka Biotechnology (Montreal, QC, Canada). Antibodies to human Sp1 (PEP2), Sp2 (K-20), Sp3 (D-20) and Sp4 (V-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The human adrenocortical (SW13), hepatoma (HepG2) and choriocarcinoma (JEG3) cell lines and the Schneider 2 (SL2) cell line derived from *Drosophila melanogaster* were purchased from the A.T.C.C. (Manassas, VA, U.S.A.). Radionucleotides were obtained from NEN Life Science Products (Boston, MA, U.S.A.). T7 Sequenase Version 2.0 DNA sequencing kit was purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.). All oligonucleotide primers used in these studies are listed in Table 1.

RLM-RACE

The recently developed RLM-RACE method (Ambion) was used to determine the transcription start sites of PAPS synthase 2, according to the manufacturer's protocol. Briefly, 10 µg of human brain total RNA was treated with calf intestinal alkaline phosphatase (CIP) for 1 h at 37 °C and then subjected to phenol/chloroform (1:1, v/v) extraction and ethanol precipitation. CIP-treated RNA was incubated with tobacco acid pyrophosphatase (TAP) for 1 h at 37 °C to remove 7-methylguanosine cap structures from mRNA, leaving the 5'-monophosphates to be ligated to an RNA adapter with T4 RNA ligase. RT was performed using thermostable reverse transcriptase (Life

Technologies) and a gene-specific primer (2AS-RT) at 60 °C for 60 min. PCR was performed using primer 2PE1 and an outer RNA adapter primer. PCR conditions were: denaturing at 94 °C for 2 min, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C or 60 °C for 30 s, and extension for 1 min at 72 °C. Nested PCR was performed using primer 2PE2 and an inner RNA adapter primer. PCR conditions were the same as described above, except that the cycle number was 40. PCR products were directly ligated into the pCR2.1 vector (Invitrogen, Carlsbad, CA, U.S.A.), purified and sequenced.

Confirmation of transcription start sites and expression of PAPS synthase 2 by RT-PCR

To confirm transcription start sites, RT-PCR was performed using the gene-specific sense primers TSR3 and TSR4, upstream and downstream of the transcription start sites as determined by the RLM-RACE method. The antisense primer was AS2-3, located on exon 2. In these experiments, 5 µg of human adrenal gland total RNA (ClonTech) was used for the generation of the first strand of cDNA. RT was performed at 50 °C for 50 min using thermostable reverse transcriptase and random hexamer primers. PCR was performed using Advantage GC-genome PCR kit (ClonTech) with 1 M GC-Melt® and the following conditions: denaturing at 95 °C for 1 min, followed by 40 cycles of denaturing at 94 °C for 15 s, and annealing/extension for 90 s at 68 °C. PCR products were analysed by electrophoresis using 2% (w/v) agarose gels.

To analyse the expression of PAPS synthase 2 mRNAs in each cell line, total RNA was extracted using the TRIzol® reagent, according to the manufacturer's protocol (Life Technologies), and 2 µg was reverse-transcribed using random hexamer primers. PCR primers (S2-6 and AS2-7 for PAPS synthase 2, and ACT-S and ACT-AS for β-actin) are depicted in Table 1. PCR conditions were: denaturing at 95 °C for 2 min, followed by either 30 (β-actin) or 40 (PAPS synthase) cycles of denaturing at 95 °C for 15 s, annealing at 55 °C for 15 s and extension for 30 s

at 72 °C. PCR products were analysed by electrophoresis using 2% (w/v) agarose gels.

Construction of luciferase reporter gene vectors

Fragments of DNA located upstream of the transcription start sites were obtained by PCR using human genomic DNA (Clon-Tech) as a template. Primers (GMS3 and GMAS1) were designed on the basis of the genomic sequences described previously for PAPS synthase 2 (GenBank® accession no. AC006191). The Advantage-GC Genomic PCR kit was employed to amplify genomic DNA fragments. PCR was performed with 1 M GC-Melt® and the following conditions: denaturing at 95 °C for 1 min, followed by 40 cycles of denaturing at 94 °C for 15 s, and annealing/extension at 68 °C for 3 min. Nested PCR was performed with primers GMS4 and GMAS1. PCR conditions employed were the same as above, except that the cycle number was 35. The PCR product was digested with *KpnI* and *XhoI* and ligated into *KpnI/XhoI*-digested pGL3-basic vector directly. This fusion plasmid was designated pGL3S2-2255. pGL3S2-1437 and pGL3S2-963 were generated by digestion with restriction enzymes *PvuII* and *StuI* respectively. pGL3S2-2255 was digested with *KpnI* and *PvuII* or *StuI*, blunted and self-ligated. pGL3S2-378, pGL3S2-249, pGL3S2-137, pGL3S2-124, pGL3S2-84, pGL3S2-58, pGL3S2-d378 and pGL3S2-d249 were generated by PCR using the primers shown in Table 1. PCR products were digested with *KpnI* and *XhoI* and ligated into *KpnI/XhoI*-digested pGL3-basic vector directly. pGL3S2-159 was generated by digestion of pGL3S2-378 with *KpnI* and *AvrII*, blunted and self-ligated. pGL3S2+49 was generated using the sequence-matched complementary oligonucleotides LUCS4 and LUCAS4. Following annealing, the resultant double-stranded DNA was digested with *KpnI* and *XhoI* and ligated into *KpnI/XhoI*-digested pGL3-basic vector. Purification of plasmids was carried out using the modified alkaline lysis method (Qiagen), and vector-insert junctions of all constructs were confirmed by sequencing.

Mutational modification of Sp1-binding sites

Mutations of putative Sp1-binding sites were generated using QuikChange XL Site-Directed Mutagenesis kit according to the manufacturer's instructions (Stratagene). Briefly, 10 ng of pGL3S2-378 and pGL3S2-159 were used as templates and the mutated nucleotides as described in Figure 3(A). PCR conditions were: denaturing at 95 °C for 1 min, followed by 18 cycles of denaturing at 95 °C for 50 s, annealing at 60 °C for 50 s, and extension at 68 °C for 12 min. After digestion with *DpnI*, 2 µl of PCR products were used to transform XL10-Gold competent cells provided with the kit. Sequencing identified appropriate clones.

Cell culture

SW13 cells were grown at 37 °C in L15 medium supplemented with 10% (v/v) FBS and antibiotics in the absence of CO₂. HepG2 and JEG3 cells were maintained at 37 °C in 5% CO₂ in Dulbecco's modified Eagle's medium and minimum essential medium respectively, both of which contained 10% (v/v) FBS and antibiotics. Media were changed every other day. SL2 cells were cultivated in Schneider's *Drosophila* medium supplemented with 10% (v/v) FBS at 26 °C in 5% CO₂.

Transfection analysis

All cells were seeded in a 6-well plate (Costar, Cambridge, MA, U.S.A.) at (1–2) × 10⁵ cells/well the day before transfection,

and grown in medium without antibiotics. Transfections were carried out with 2 µg of luciferase fusion plasmids using the lipofection method (LIPOFECTAMINE 2000) with 2 µl of the transfection reagent for SW13 cells and 5 µl for all other cells, according to manufacturer's protocol. pCMV-SPORT β-gal expression vector (100 ng) (Invitrogen) was co-transfected to evaluate transfection efficiency. At 48 h after transfection, cells were harvested and luciferase activity was determined.

In co-transfection experiments, DNA was introduced into SL2 cells essentially as described above. pPac-Sp1 and pPac-Sp3 were generously supplied by Dr R. Tjian, (University of California, Berkeley, CA, U.S.A.) and Dr L. Lania (University of Naples, Naples, Italy) respectively. pRSV-AP2α and pRSV-NN were kindly provided by Dr T. Williams (Yale University, New Haven, CT, U.S.A.), and pRSV-AP2β and pRSV-AP2γ were generously given by Dr H. C. Hurst (Hammersmith Hospital, London, U.K.). SL2 cells were seeded at 1 × 10⁶ cells/well in a 6-well plate. Cells were transfected with 1 µg of luciferase fusion plasmid and either expression vector in the amounts indicated in Figure 5. Total amount of the transfected plasmid was adjusted to 2 µg with the control plasmid (pPac-basic), which contained only the *Drosophila* actin promoter. Cells were grown for 48 h following transfection, harvested and assayed for luciferase activity.

Preparation of nuclear extracts

Nuclear extracts were prepared as described previously [7] with a slight modification. Briefly, cells were scraped from the tissue-culture dishes, washed twice with ice-cold PBS and the packed-cell-pellet volume (PCPV) was measured. Cell pellets were suspended in buffer A [10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol (DTT) and 1 × protease-inhibitor cocktail] to give a 20% (w/v) suspension, with respect to the PCPV. Following incubation on ice for 10 min, the cell suspension was centrifuged at 4560 g for 10 min at 4 °C. Pellets were resuspended in buffer A to give a 50% (w/v) suspension, with respect to the PCPV, and homogenized with 20 strokes of a Dounce homogenizer. After centrifugation at 2560 g for 20 min at 4 °C, pellets were resuspended in buffer B [20 mM Hepes (pH 7.9), 0.42 M NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 25% (v/v) glycerol, 0.5 mM DTT and 1 × protease-inhibitor cocktail] to give a 50% (w/v) suspension, with respect to the PCPV. Suspensions were stirred on ice for 30 min and centrifuged at 12000 g for 20 min at 4 °C. (NH₄)₂SO₄ (0.33 g/ml of supernatant) was gradually added and solutions were stirred on ice for 30 min. After centrifugation at 12000 g for 25 min at 4 °C, pellets were resuspended in one-tenth of the volume of the original supernatant using buffer C [20 mM Hepes (pH 7.9), 20 mM KCl, 1 mM MgCl₂, 17% (v/v) glycerol, 2 mM DTT and 1 × protease-inhibitor cocktail] and dialysed against 300 vol. of buffer C for 5 h. Protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.).

Electrophoretic mobility-shift assay (EMSA)

EMSA was performed using reagents provided in the Nushift kit (Geneka Biotechnology). Nuclear extracts derived from HeLa cells were provided with the kit. Antibodies to human Sp1, Sp2 and Sp3 were purchased from Santa Cruz Biotechnology and used in supershift assays. Oligonucleotide probes, described in Figure 4(A), were end-labelled using [γ-³²P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase, using a standard method [8], and column-purified with a NucTrap probe purification column (Stratagene). After annealing with sequence-matched complementary oligonucleotides, the probes were used for EMSA. The

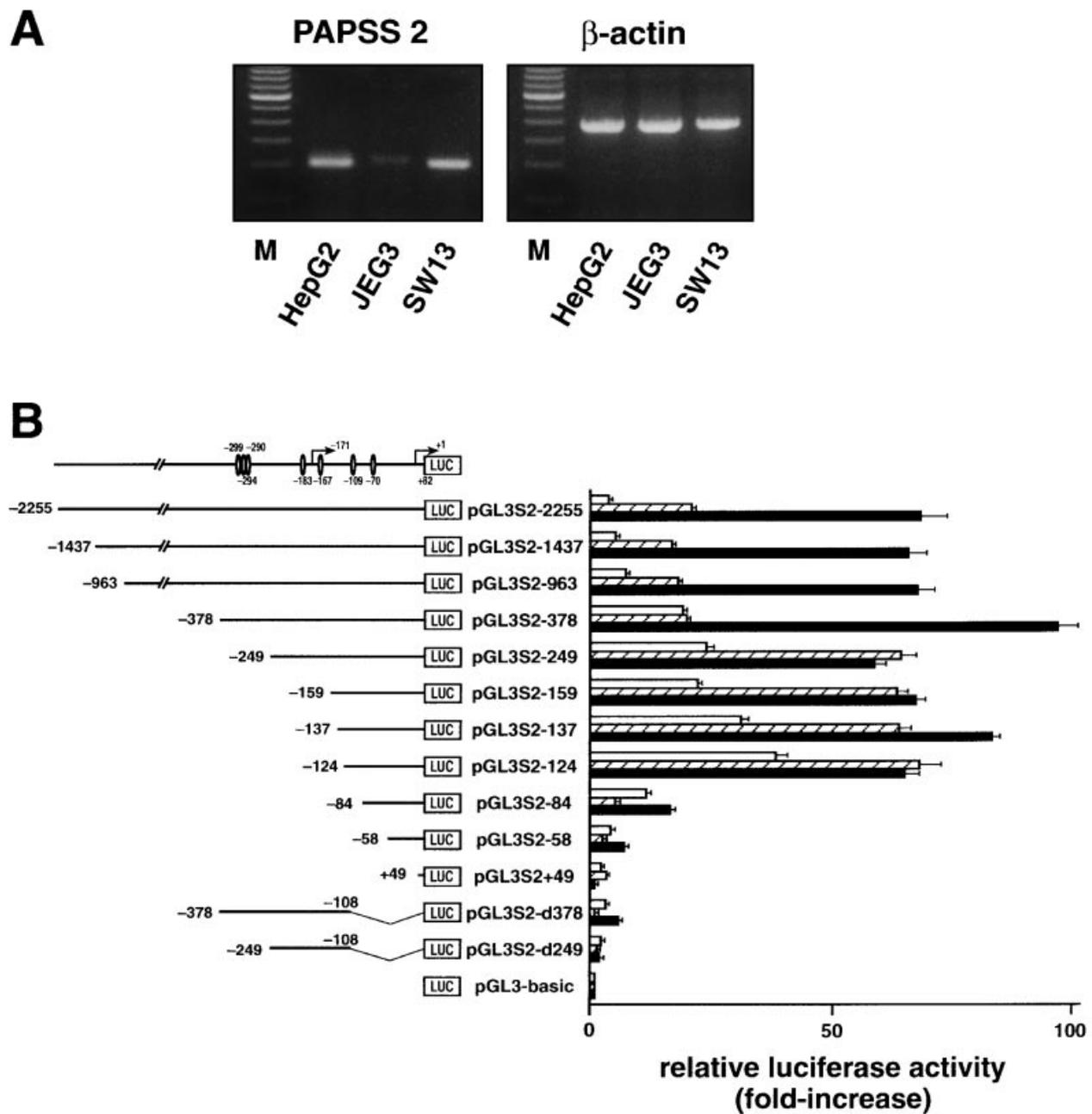


Figure 2 PAPS synthase 2 expression and promoter activity

(A) Gel electrophoresis of RT-PCR products using gene-specific primers for human PAPS synthase 2 (PAPSS 2; left panel) and β -actin (right panel) and RNA extracted from HepG2, JEG3 and SW13 cells. Lane M, 100 bp DNA ladder. All primers are listed in Table 1, and further details are provided in the Experimental section. (B) Reporter gene activity of fused promoter constructs. DNA segments of the 5'-flanking region of human PAPS synthase 2 (solid lines) were fused to a luciferase reporter gene (LUC) and transfected into HepG2 (white bars), JEG3 (hatched bars) and SW13 (black bars) cells. The location of putative Sp1 elements is indicated by vertical ovals and directionally localized by the first bp of each motif. Data (means \pm S.E.M.) are presented as fold-increase in luciferase activity over the pGL3-basic control from at least three experiments, each performed in duplicate.

reaching a maximum at bp -124 , after which, there was a sharp drop in activity. For the JEG3 cells, promoter activity was modest and essentially constant from bp -2255 to bp -249 , at which point there was a sharp increase in activity that remained constant until bp -124 , after which, a sharp fall-off occurred. Promoter activity of the SW13 cells was the highest of the three cell types and remained essentially constant from bp -2255 to bp -124 , after which, it also demonstrated a sharp fall-off. Thus

maximal basal promoter activity for all three cell types was associated with the -124 bp construct (Figure 2B).

Mutational analyses of putative Sp1 elements

Putative Sp1 elements were altered according to the scheme in Figure 3(A). Individual mutations of the fifth (bp -290 to bp -278), sixth (bp -294 to bp -282) and seventh (bp -299 to bp -288) Sp1 elements upstream of the purported start site had

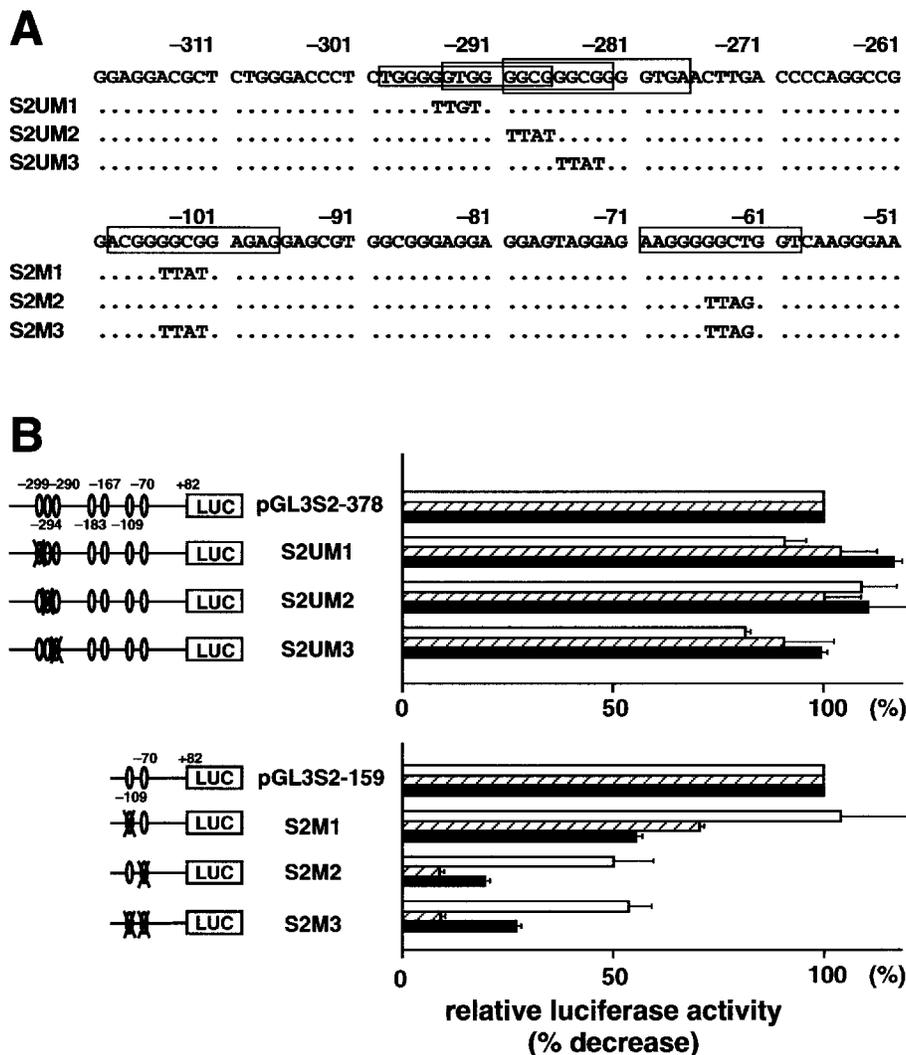


Figure 3 Mutational analysis of putative Sp1 elements

(A) Sequence and location of putative Sp1 motifs (boxed) in the human PAPS synthase 2 promoter as well as substituted nucleotides (S2UM1–3 and S2M1–3) are shown. (B) Mutation of specific Sp1 elements and reporter gene activity for each construct is presented. Further details are provided in the Experimental section. Data (means \pm S.E.M.) are presented as the percentage luciferase activity compared with the non-mutated control from at least three experiments, each performed in duplicate. White bars, HepG2; hatched bars, JEG3; black bars, SW13.

essentially no effect on reporter activity (Figure 3B, upper panel). The third (bp –155 to bp –167) and fourth (bp –183 to bp –171) Sp1 elements upstream of the purported start site were not subjected to mutational analysis since the –159 bp construct, which does not contain these elements, and the –249 bp construct, which does contain them, had similar promoter activities (cf. Figure 2B). To test the first (bp –70 to bp –59) and second (bp –109 to bp –97) Sp1 elements upstream of the purported start site, the –159 bp construct was used. Mutation of the Sp1 element located at bp –109 to bp –97 had no effect on reporter activity in the HepG2 cells, whereas there was an approx. 33% and 50% fall in reporter activity with the JEG3 and SW13 cells respectively (Figure 3B, lower panel). When the Sp1 element located at bp –70 to bp –59 was subject to mutation, however, reporter activity in HepG2 cells fell by approx. 50%, whereas reporter activity with JEG3 and SW13 cells was decreased further to approx. 90% and 80% of the control level respectively (Figure 3B, lower panel). A combined mutation yielded results similar to mutation of only the Sp1 element located at bp –70

to bp –59 (Figure 3B, lower panel). These results indicate that the two Sp1 elements most proximal to the cap site play a significant role in the transcriptional regulation of PAPS synthase 2 gene expression in both JEG3 and SW13 cells. On the other hand, the role of these elements in regulation of the PAPS synthase 2 gene in HepG2 cells appears to be of less importance.

EMSA with probes containing putative Sp1 elements

SW13 nuclear extracts were incubated with radiolabelled PAPS synthase 2 wild-type and mutant probes in the absence or presence of unlabelled competitors (probes are indicated in Figure 4A). Each probe used in these experiments contained a putative Sp1 element that exhibited a significant effect on promoter activity as demonstrated in Figure 3(B, lower panel). The first of the two probes (bp –89 to bp –118) contained the Sp1 element located at bp –109 to bp –97, and the second probe (bp –49 to bp –78) contained the Sp1 element located at bp –70 to bp –59 upstream of the purported start site (cf.

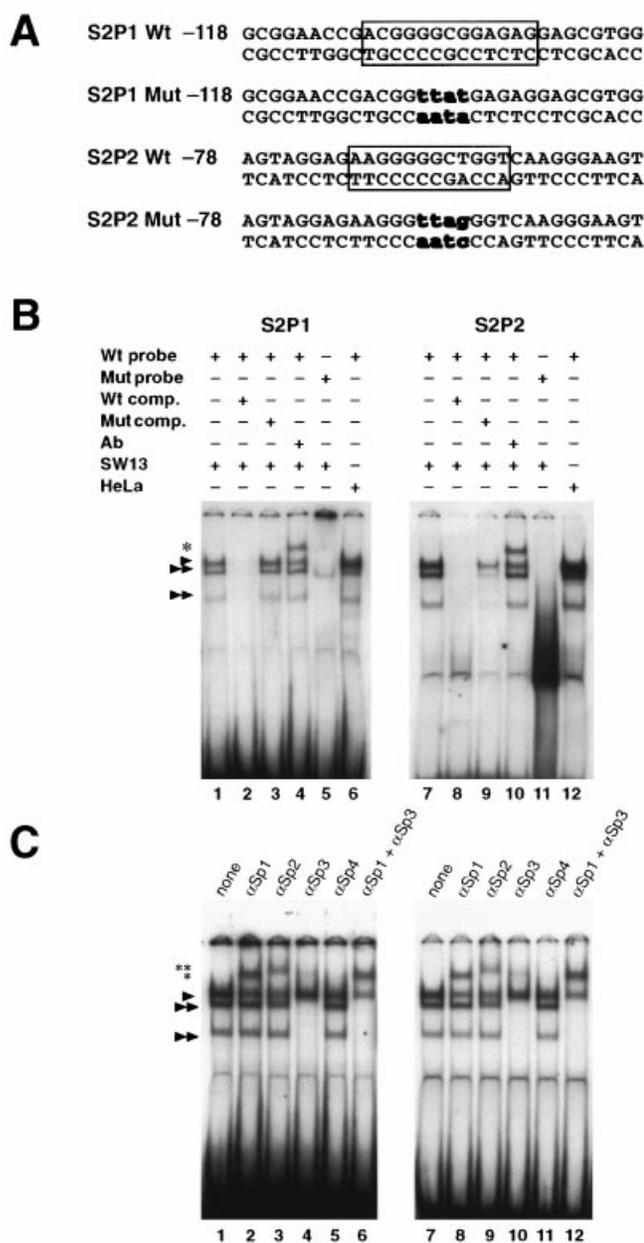


Figure 4 EMSA with SW13 nuclear extract

(A) Wild-type (Wt) and mutant (Mut) sequences, used as radioactive probes and non-radioactive competitors, and their human PAPS synthase 2 promoter locations are shown. The Sp1 elements are boxed and nucleotide substitutions are indicated by lower case and bold lettering. (B) Gel-shift patterns in SW13 nuclear extracts. The ratio of competitor (comp.) to labelled probe was 100:1. HeLa cell nuclear extract was used as a positive control for Sp1 and related proteins. Further details are provided in the Experimental section. A supershifted band with antibody (Ab) to Sp1 is indicated with an asterisk. (C) An expanded EMSA using SW13 nuclear extract and the Wt probe. The use of antibodies (α) to Sp1, Sp2, Sp3 and Sp4 are indicated, and supershifted bands are denoted by single and double asterisks. In (B) and (C), the tentative identification of Sp1 (single arrowhead) and Sp3 (double arrowheads) are indicated.

Figure 3B). Interestingly, similar results were obtained with both probes. That is, with the radiolabelled wild-type probes, the same up-shifted bands were noted (Figure 4B, lanes 1 and 7). Competition experiments with the unlabelled wild-type probe (Figure 4B, lanes 2 and 8) and the mutant probe (Figure 4B, lanes 3 and 9) indicated that the three most up-shifted bands represented

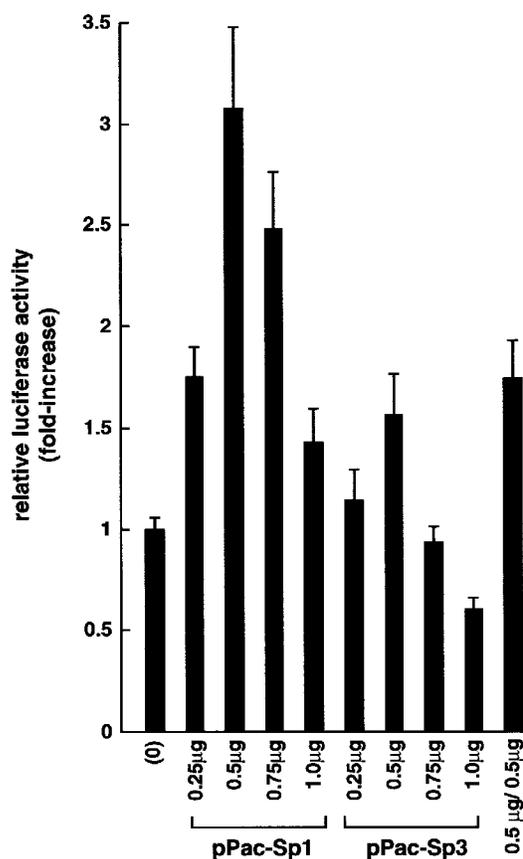


Figure 5 Co-transfection of human PAPS synthase 2 promoter with Sp1 and/or Sp3 expression vectors

Drosophila SL2 cells were co-transfected with the PAPS synthase 2 (pGL3S1-159) promoter fused to a luciferase reporter gene (1 μ g) together with Sp1 (pPac-Sp1) and Sp3 (pPac-Sp3) expression vectors at the indicated doses. Further details are provided in the Experimental section. Data (means \pm S.E.M.) are presented as fold-increase in luciferase activity for co-transfections over that for the promoter construct alone (0) from four experiments, each performed in duplicate.

specific DNA binding, whereas the other bands appeared to be non-specific. This conclusion is supported further by the failure of the labelled mutated probes to produce the same up-shifted bands (Figure 4B, lanes 5 and 11). As a positive control for the Sp1 family of transcription factors, a HeLa cell nuclear extract produced a similar pattern of up-shifted bands (Figure 4B, lanes 6 and 12).

SW13 nuclear extracts were incubated with the same radio-labelled probes used in the previous EMSA experiments in the absence or presence of antibodies to Sp1, Sp2, Sp3 and Sp4. As with the EMSA studies, similar results were obtained with both radioactive probes. That is, antibodies to Sp1 (Figure 4C, lanes 2 and 8), Sp2 (Figure 4C, lanes 3 and 9) and Sp3 (Figure 4C, lanes 4 and 10), but not Sp4 (Figure 4C, lanes 5 and 11), produced supershifted bands. The combination of antibodies to Sp1 and Sp3 produced the most dramatic supershift (Figure 4C, lanes 6 and 12).

Co-transfection of SL2 cells with the PAPS synthase 2 promoter and Sp1 and Sp3 expression vectors

The general transcription machinery is highly conserved between *Drosophila* and mammals and, although a homologue of Sp1 has

been identified in *Drosophila* [11], Sp1 activity is absent in SL2 cells [12]. Thus SL2 cells provide a functionally Sp1-deficient background for the study of Sp1-specific transcriptional regulation [13]. For these experiments, the -159 bp promoter segment of PAPS synthase 2 (cf. Figure 2B) fused to the luciferase reporter gene was used. Sp1 increased reporter gene activity in a dose-dependent manner, whereas Sp3 alone had essentially no effect on reporter activity (Figure 5). When Sp3 was co-transfected together with Sp1, there appeared to be an inhibitory effect on Sp1 induction of reporter activity (Figure 5). Additional studies, however, will be necessary to better understand the interaction between Sp1 and Sp3 in the regulation of the PAPS synthase 2 gene (e.g. dose-dependent experiments involving co-transfection of Sp3 and optimal Sp1-induced promoter activity).

DISCUSSION

The gene for PAPS synthase 2 contains neither a canonical TATAAA nor a CCAAT motif in the proximal promoter region, and our first concern was to establish the transcription start site. Whereas some TATA-less promoters retain the ability to direct transcription initiation from a specific nucleotide, others appear to direct transcription initiation from multiple start sites [14].

Previous reports noting the length of the 5' untranslated region (UTR) of the PAPS synthase 2 cDNA revealed a marked variation from 48 bp to 104 bp [3-5,15]. We originally performed primer-extension analysis and obtained results indicating plausible 5' UTR lengths of 131 bp and 135 bp (results not shown). Furthermore, it was of interest that the 5'-end of one of the extended products matched an adenosine found in an initiator consensus motif $YAA_{+1}NWYY$ (where A_{+1} is the transcription start site) [14,16] (cf. Figure 1B). Standard 5'-RACE was performed next with results suggesting multiple 5' UTR lengths ranging from 107 bp to 231 bp. While this inconsistency might result from the existence of multiple transcription start sites, we could not exclude the possibility of premature termination of RT due to the high GC content in the 5' UTR (calculated 78% GC content between the purported downstream transcription start site and the start of translation). Nevertheless, applying the RLM-RACE procedure, which detects only full-length capped mRNA, consistent reproducible results were obtained revealing two alternate 5' UTR lengths of 107 bp (12 of 12 clones) and 278 bp (11 of 13 clones). Importantly, evidence supporting the location of the purported start sites determined by the RLM-RACE procedure was obtained using RT-PCR. Thus it is believed that the probable transcription start sites and proximal promoter region for the PAPS synthase 2 gene have been established.

The patterns of promoter activity for the three cell types with shortening of the 5'-flanking region are of some interest in that they appear quite distinct. Promoter activity is highest in SW13 cells and is essentially constant from bp -2255 to bp -124. Promoter activity, which is next highest in JEG3 cells, remains constant and relatively low from bp -2255 to bp -249, where it rises sharply to a maximal and constant level until bp -124. Promoter activity is least with HepG2 cells, but begins to rise gradually at bp -378 and reaches a maximum at bp -124. Thus with all three cell types, maximum promoter activity is associated with the -124 bp construct, after which, there is a sharp fall off in activity at bp -84. As discussed below, the region between bp -124 and bp -84 contains important regulatory GC/GT boxes. It was also noted that there were potential binding sites for the family of AP-2 transcription factors between bp -273 and bp -130. However, as a result of a variety of studies and considerations (C. Shimizu and C. A.

Strott, unpublished work), we have concluded that AP-2 does not play an important role in the transcriptional regulation of the PAPS synthase 2 gene.

It is commonly found that many TATA-less promoters are characterized by the presence of multiple GC boxes, which bind the Sp1 transcription activator forming a central role in the assembly of the transcription complex of these promoters [17]. It seems clear that the control of the PAPS synthase 2 gene is at least partly under the influence of the Sp1 family of transcription factors. That is, the 5'-flanking region contains multiple GC/GT boxes and deletion analysis suggested involvement of the GC/GT boxes in the region between bp -84 and bp -124 in transcriptional regulation, a finding further supported by mutational analysis of these elements. Importantly, nuclear extracts from SW13 cells, which highly express PAPS synthase 2, contain proteins that bind to probes containing the same specific GC/GT boxes implicated in gene regulation by the mutational analyses. Furthermore, the pattern of shifted bands during the EMSA experiments was quite similar to the band patterns reported previously in comparable studies with other promoters involving Sp1 and Sp3 [18,19]. Confirmation of the presence of Sp1, Sp2 and Sp3 proteins in the SW13 nuclear extracts was obtained by supershift analyses. Additional support for the involvement of Sp1 in the transcriptional regulation of the gene for PAPS synthase 2 was obtained by co-transfection experiments using SL2 cells. Although direct involvement of Sp3 in transcriptional regulation was not revealed by the SL2 co-transfection experiments, Sp3 is known to exhibit an inhibitory effect on Sp1-induced transcriptional activation, as reported for other promoters [20-23]. The involvement of Sp2 in transcriptional regulation of the PAPS synthase 2 gene is presently unclear and is the subject of further study. These considerations, in association with the finding that maximum promoter activity resides in the region containing key regulatory GC/GT boxes between bp -84 and bp -124, are consistent with the notion that the Sp1 family of transcription factors play a significant role in regulating the gene for PAPS synthase 2.

Sp1, which is expressed ubiquitously in higher eukaryotic cells [12], is a promoter-specific transcription factor first detected in HeLa cells [24]. Sp1 is part of a multigene family which, until now, included three other Sp1-related proteins referred to as Sp2, Sp3 and Sp4 [18]; a fifth member was described recently in the mouse [25]. The four human Sp1 family-related members have similar domain structures: three zinc fingers close to the C-terminus required for DNA binding and a transactivation domain in the N-terminal region that is subdivided into two areas with segments rich in glutamine and serine/threonine residues [18,19]. As with Sp1, Sp2 and Sp3 are also expressed ubiquitously; Sp4 is likewise widely expressed, with high levels in brain [26].

While Sp1 is generally regarded as an activator of transcription, the transcriptional role of Sp3 may involve activation or repression, and is complicated by the presence of three isoforms that are generated by alternative translation start sites and that only the shorter isoforms appear to serve as repressors [22]. Furthermore, Sp1 and Sp3 compete for the same binding site, thus whether Sp3 acts as an activator or as a repressor of Sp1-mediated activation might depend on their relative cellular content [18,19]. Interestingly, the Sp1 protein has been shown to be modified with O-linked oligosaccharides and phosphorylated by a DNA-dependent protein kinase [27,28]; however, the role of such modifications in regulating or modifying Sp1-mediated transcription is not clear [18].

The case for Sp1 involvement in regulating the gene for PAPS synthase 2 is reasonable, but the story is obviously more complicated. At this point in our investigations, Sp1 regulation

would seem to involve primarily proximal promoters, however, more distal involvement is also possible [29]. Activation of a given promoter requires the binding of multiple transcription factors that bind co-operatively to their cognate sites or possibly act synergistically by other mechanisms. Recently, a complex of transcription cofactors has been found to be required for transcriptional activation by Sp1 [30]. Furthermore, it is realized that Sp1 and related family members are not the only proteins to recognize GC/GT boxes. Several other zinc-finger proteins have been found to have a binding specificity similar to Sp1 [19,26].

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