FEBS Letters 356 (1994) 339–341

FEBS 14898

Nucleotide sequence and analysis of the mouse SPC3 promoter region

Tadashi Hanabusa[a], Shinya Ohagi[a,**], Joseph LaMendola[b], Shu Jin Chan[a,b], Donald F. Steiner[a,b,*]

[a]*Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL 60637, USA*
[b]*The Howard Hughes Medical Institute, 5841 South Maryland Avenue, Chicago, IL 60637, USA*

Received 9 November 1994

Abstract Insulin is converted from the higher molecular weight proprotein, proinsulin by highly specific proteolytic cleavage at two dibasic amino acid sites. SPC3 and SPC2, two recently identified prohormone convertase that are specifically expressed in β cells and other neuroendocrine cells, appear to be responsible for those cleavages. We have sequenced the 5′-upstream region of the SPC3 gene and examined its promotor/enhancer activity and most of several deletion mutants in several cell lines. This region contains no CAAT box but has several non-functional TATA-like sequences and several putative transcriptional regulatory elements, including AP-1, Sp1 and cAMP response elements. These features are not unlike those of the human SPC2 upstream region. In βTC3 insulinoma cells, the sequence between the *Eco*RI (620 bp) and *Nsi*I (702 bp) sites seems to be important for gene expression, while the sequence between the *Nsi*I and *Dra*I (775 bp) sites may contain strong enhancer element(s).

Key words: Processing enzyme; SPC3 promoter; Luciferase assay

1. Introduction

Many peptide hormones and neuropeptides are synthesized as precursors that require highly specific proteolysis at exposed dibasic sites to release their secreted active forms. Proinsulin and POMC are examples of such precursors. Recently, two important members of a novel family of subtilisin-like endopeptidases, SPC2 and SPC3, have been identified [1,2]. These two proteases appear to be responsible for the cleavage of various prohormones, including proinsulin and POMC [3,4], at dibasic sites in neuroendocrine (NE) tissues, especially brain, pancreas and pituitary [2]. Both SPC2 and SPC3 are expressed only in NE tissues but SPC2 is expressed at a high level in pancreatic β-cells [5] and the intermediate pituitary lobe [3,4], while SPC3 is expressed at relatively high levels in the anterior pituitary corticotrophs and at lower levels in the pancreatic β-cells [3,4]. The mechanisms by which differential expression of these enzymes is controlled are not yet known. The nucleotide sequence of the human SPC2 promoter has no TATA or CAAT box, but has an extremely GC-rich region. Analysis of CAT activity of several deletion mutants shows that the region from −1100 to −539 bp from the translation start site is essential for its promoter activity [6]. But there are no data on SPC3 promoter activity. Herein we investigate the regulation of the SPC3 gene. We report the nucleotide sequence of 2000 bp of 5′-flanking region proximal to the translation start site in the mouse SPC3 gene and the results of expression studies. Using several deletion mutants linked to a luciferase cDNA, we have measured this reporter activity in pancreatic β-cell or pituitary cell lines, in comparison with a non-endocrine kidney cell line, and have defined regions within the SPC3 promoter that are probably responsible for its neuroendocrine cell-specific expression.

*Corresponding author. Fax: (1) (312) 702 4292.

***Present address:* The First Department of Medicine, Wakayama University of Medical Science, 27 Nanabancho, Wakayama 640, Japan.

2. Materials and methods

2.1. Isolation of genomic clones

A mouse leukocyte genomic library constructed in lambda EMBL3 (Cat# ML1040j, Clonetech) was screened by the mouse SPC3 cDNA labeled by nick translation (Amersham). Fifteen positive plaques were found and screened by PCR using primers which are inside of exon 1 and the positive clones were used for further analysis.

2.2. Mapping, subcloning and sequencing

The 6 kb insert of lambda clone was analyzed by restriction mapping and Southern blotting hybridization using a ^{32}P end-labeled 20-base oligonucleotide 5′-AGTGAGTCCTCTAGCTCTAG-3′, corresponding to the immediately upstream region of exon 1 of the mouse SPC3 gene. The appropriate restriction fragment was subcloned into pGEM 3z (Promega) or pBluescript II KS (Stratagene) and sequenced by the dideoxynucleotide method, using either universal or specific primers.

2.3. Construction of luciferase expression plasmids

The luciferase expression plasmid (pGL2-basic) was obtained from Promega. To analyze the promoter activity, various constructs derived from a 6-kb genomic DNA fragment containing the putative promoter sequences immediately upstream from coding region were separated. We prepared constructs using natural sites in the 5′-flanking region of the SPC3 gene as follows: *Sac*I −3.7 kb, *Spe*I −1.8 kb, *Ssp*I −1.2 kb, *Dra*I −775 bp, *Nsi*I −702 bp, *Eco*RI −620 bp, *Bgl*II −284 bp relative to the ATG codon. These fragments were ligated to the luciferase expression plasmid and used in the expression experiments.

2.4. Transfection experiments

βTC-3 mouse insulinoma cells, AtT-20 mouse anterior pituitary cells or COS7 monkey kidney cells were transiently transfected via electroporation. Forty-eight hours before transfection, the cells were replated and grown to 70–80% confluency in 10 cm diameter tissue culture dishes. The cells were harvested and resuspended with 10 μg of a luciferase plasmid and 5 μg of pSV2-β-gal (containing β-galactosidase cDNA and SV40 promoter) in 0.05 ml of DMEM medium without serum and electroporated at 250 V/800 μF (βTC3 cells) or 350 V/800 μF (AtT-20 cells) or 300 V/800 μF (COS7 cells) using a Cell-Porator (Gibco-BRL) and replated in 10 cm diameter cell culture dishes.

2.5. Luciferase and β-galactosidase assay

At 48 h after transfection, the cells were washed twice with PBS and incubated in 300 μl of Reporter Lysis buffer (Promega). The cell lysates were collected in 1.5 ml microcentrifuge tubes and debris was removed by centrifugation at 15,000 × *g* for 2 min at 4°C. The cell lysates were stored at −80°C until assay. β-Galactosidase assays and luciferase assays were performed by kit (Promega) according to manufacturer's protocol. The luciferase reaction mixture was placed in a Monolight

SSDI 0014-5793(94)01285-7

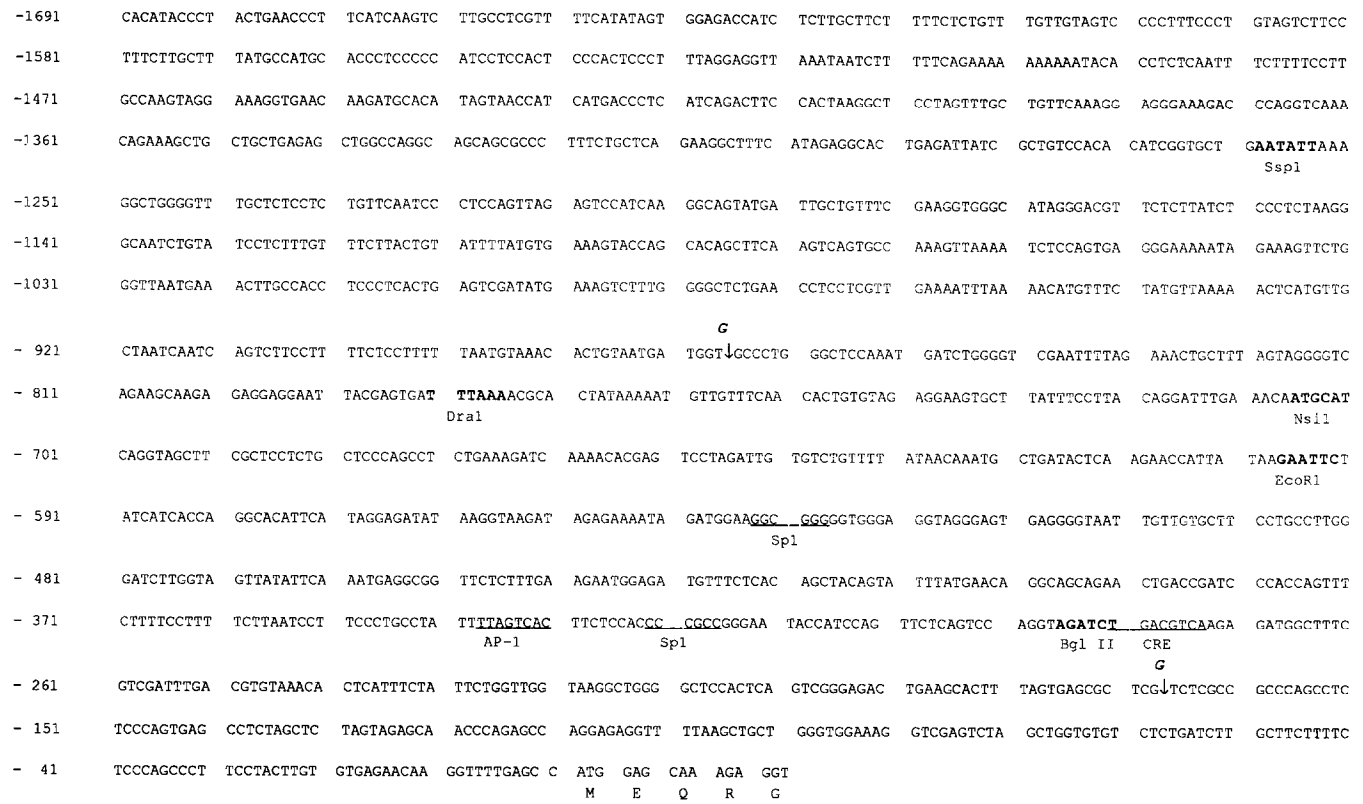

Fig. 1. Nucleotide sequence of the mouse SPC3 gene and 5′-flanking region. The putative regulatory elements are underlined and the restriction enzyme sites used for making the expression constructs are printed in bold. The nucleotides in italics at −868 and −168 are nucleotide insertions reported in the sequence of Ftouhi et al. [10].

2010 luminometer (Analytical Luminescence Laboratories, San Diego, CA), and the light reaction was initiated by injection of 100 μl of 1 mM D-luciferin. Light emission was measured by integration over the initial 10 s of the reaction. Each sample was measured three times and normalized to the β-galactosidase activity.

3. Results and discussion

Proinsulin is converted to insulin by cleavage at the B chain–C peptide junction (R–R↓E) and the C-peptide–A-chain junction (K–R↓G) by SPC3 and SPC2, respectively, members of a recently discovered family of subtilisin and kex-related proteases that includes SPC1 (furin), SPC2, SPC3 and several additional related enzymes [6]. Both SPC3 and SPC2 are specifically expressed in brain and NE cells, but their relative abundance in various NE tissues differs. SPC2 is expressed at high levels in pancreatic α- and β-cells and the intermediate lobe of the pituitary, while SPC3 is expressed at relatively high levels in the anterior pituitary lobe and at somewhat lower levels in pancreatic β-cells. To investigate the regulation of the SPC3 gene, we have sequenced the 5′-upstream region of SPC3 gene and compared it with the corresponding region of SPC2. The 5′ region of the human SPC2 gene lacks TATA or CAAT boxes upstream of the transcription start sites but has a GC rich region [7]. We sequenced 2 kb of the lambda EMBL3 clone which contains 6 kb of 5′ upstream region of mouse SPC3. The nucleotide sequence of mouse SPC3 also contains no CAAT box but has several TATA-like and one GC-box like sequence. However, these TATA-like sequences seem to be non-functional, because they are relatively far from the transcription start sites. We also have noted several putative transcriptional regulatory sequences, including AP-1, Sp1 and cAMP-response elements. These features are not unlike those of the human SPC2 promoter and are consistent with other similarities between these two genes and their presumed origin from a common ancestral processing enzyme. On the other hand, another subtilisin like enzyme, furin (SPC1), is expressed in many tissues throughout the body and has three distinct promoters, only one of which has a TATA box [8].

We performed transient expression experiments using several SPC3 promoter fragments attached to a luciferase reporter gene. The βTC3 cell line is a non clonal tumor cell line derived from insulinomas that developed in transgenic mice expressing the SV40 large T-antigen in the pancreatic islet β-cells [9]. These cells synthesize and secret processed insulin and express SPC2 and SPC3 at medium to high levels. The AtT-20 cell line is also a tumor-derived cell line related to the anterior pituitary corticotrophs. It expresses a high level of SPC3, and very little SPC2. We used the COS7 cell line as a representative non endocrine cell line which does not have neuroendocrine characteristics and expresses neither SPC2 nor SPC3. In βTC3 cells, as shown in Fig. 2, the *Nsi*I construct produced a 16-fold stimulation of luciferase activity and the *Dra*I construct showed the greatest stimulation (over 100-fold); the longer constructs exhibited 21- to 24-fold stimulation above the plasmid without promoter.

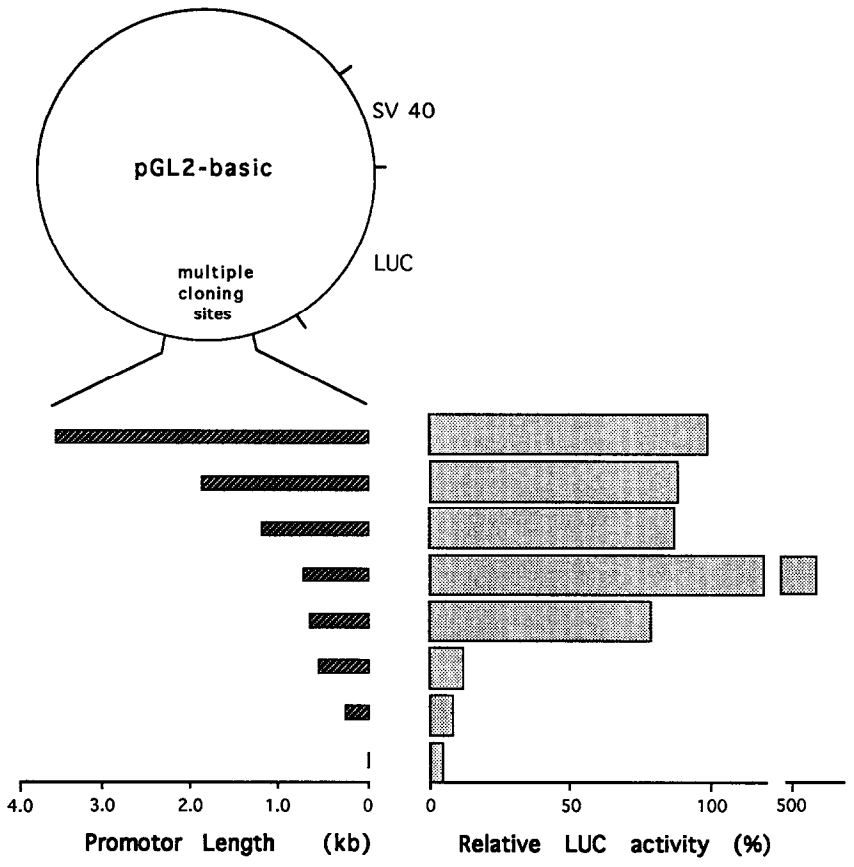

Fig. 2. Schematic constructs containing fragments of 5′-flanking region of SPC3 and luciferase reporter gene, and their luciferase activities in βTC3 insulinoma cells.

Thus, the sequence between the *Eco*RI (620 bp) and *Nsi*I (702 bp) sites (Fig. 1) seems to be important for SPC3 gene expression while the sequence between the *Nsi*I and *Dra*I (775 bp) sites may contain strong enhancer element(s). The transfection results using the AtT20 cell line were similar, while the COS7 cells exhibited no luciferase activity with any of these vectors. We conclude that the 5′-flanking region of the SPC3 gene resembles that of the human SPC2 gene; in the NE cells studied, 702 bp immediately upstream of the initiator ATG of the mouse SPC3 gene is required for tissue-specific gene expression and a strong enhancer element(s) probably exists between 702 to 775 bp from initiator ATG.

Further studies will be necessary to determine the nature of the *trans*-acting factors responsible for the differential regulation of the SPC2 and SPC3 gene in various neuroendocrine cells.

Acknowledgements: We thank Paul Gardner for assistance in preparing oligonucleotide primers and Florence Rozenfeld for assistance with this manuscript. This work was supported by NIH grants DK13914 and DK20595.

References

[1] Smeekens, S.P. and Steiner, D.F. (1990) J. Biol. Chem. 265, 2997–3000.
[2] Smeekens, S.P., Avruch, A.S., LaMendola, J., Chan, S.J. and Steiner, D.F. (1991) Proc. Natl. Acad. Sci. USA 88, 340–344.
[3] Thomas, L., Leduc, R., Thorne, B.A., Smeekens, S.P., Steiner, D.F. and Thomas, G. (1991) Proc. Natl. Acad. Sci. USA 88, 5297–5301.
[4] Benjannet, L., Rondeau, N., Day, R., Chretien, M. and Seidah, N.G. (1991) Proc. Natl. Acad. Sci. USA 88, 3564–3568.
[5] Bennet, D.L., Bailyes, E.M., Nielsen, E., Guest, P.C., Rutherferd, N.G., Arden, S.D. and Hutton, J.C. (1992) J. Biol. Chem. 267, 15229–15263.
[6] Steiner, D.F., Smeekens, S.P., Ohagi, S. and Chan, S.J. (1992) J. Biol. Chem. 267, 23435–23438.
[7] Ohagi, S., LaMendola, J., Lebeau, M.M., Espinosa III, R., Takeda, J., Smeekens, S.P., Chan, S.J. and Steiner, D.F. (1992) Proc. Natl. Acad. Sci. USA 89, 4947–4981.
[8] Ayoubi, T.A.Y., Creemers, J.W.M., Roebroek, A.J.M. and Vau de Ven, W.J.M. (1994) J. Biol. Chem. 269, 9298–9303.
[9] Efrat, S., Linoe, S., Kofod, H., Spector, D., Delannoy, M., Grant, S., Hanahan, D. and Baekkeskov, S. (1988) Proc. Natl. Acad. Sci. USA 85, 9037–9041.
[10] Ftouhi, N., Day, R., Mbikay, M., Chrétien, M. and Seidah, N.G. (1994) DNA Cell Biol. 13, 395–407.