Journal of Genetics, Vol. 97, No. 1, March 2018, pp. 319–322
https://doi.org/10.1007/s12041-018-0883-2

RESEARCH NOTE

Sp1 is an important transcriptional regulation factor for forkhead box N1 in pig

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Received 24 March 2017; revised 25 April 2017; accepted 12 July 2017; published online 8 February 2018

Abstract. The transcription factor forkhead box N1 (*Foxn1*) plays an important role in the development and function of thymic epithelial cells (TECs) in vertebrates. However, the transcriptional regulation of *Foxn1* is still unknown. A series of dual luciferase report vectors were constructed and their relative activities were also detected. The 5′-untranslated regions contains two *cis*-acting elements, *Sp1* and *GATA-1*, as well as *trans*-acting elements between positions −332 to −438. Nevertheless, the relative activities of *Foxn1* promoter were significantly increased in PK15 cells only when the *Sp1* was overexpressed, suggesting that the *Sp1* was the most important sequence for *Foxn1* transcription activation in the pig. Regrettably, the exact *trans*-acting elements were not found.

Keywords. pig; *Foxn1* gene; transcriptional regulation; *Sp1*.

Introduction

The thymus, which is the main site of T lymphocyte development, is one of the most important organs of the immune system. Transcription factor *Foxn1* is an epithelial cell-autonomous gene that predominantly regulates the development of TECs and skin keratinocytes (Palamaro *et al.* 2014). Mutations in the *Foxn1* gene result in the failure of thymus development, hairless nude skin, and short life (Bryson *et al.* 2013; Abitbol *et al.* 2015). On the other hand, *Foxn1* upregulation in the thymus of aged mice could result in the regeneration in thymus function (Bredenkamp *et al.* 2014). *Foxn1* maintains TECs to support T-cell development through mcm2 (Ma *et al.* 2012). miR-18b and miR-518b could upregulate *Foxn1* transcription (Kushwaha *et al.* 2014). The expression pattern of *Foxn1* had obviously temporally and spatially specific. Until date, *Foxn1* was only detected in thymus and skin. Its expression reached the highest level after birth, and then declined (Rode *et al.* 2015).

Materials and methods

Three fragments of 5′-UTR of the pig *Foxn1* gene were obtained by PCR amplification. Primer details are presented in table 1 (A, B, C and R). The restriction enzyme *Nhe*I was introduced to the sense primer, whereas the endonuclease *Hin*dIII was introduced to the reverse primer. The fragments were ligated upstream of the firefly luciferase gene in the plasmid pGL3-Basic. The accuracy of the insert fragment was detected by sequencing. PK-15 cells were transfected with Lipofectamine 2000 Reagent. Cells were cotransfected with the *Foxn1* promoter constructs and a plasmid containing a *Renilla* luciferase (pRL-TK) to assess for transfection efficiency (pRL-TK: *Foxn1* promoter constructs = 1:399). After 24 h transfection, the cells were harvested and firefly and *Renilla* luciferases were assessed by using the Stop and Glo kit (Promega, Madison, USA). Firefly luciferase activity was normalized to that of the *Renilla* luciferase activity, and the results were expressed relative to that of the promoterless

Table 1. Primer pairs used to amplify the *Foxn1* 5′-UTR sequence.

Name	Sequence (5′–3′)	Length (bp)	Location (bp)
A	CTA GCTAGC CACGCCACGGTGGTAACCCAAAC	1001	−973 ~ +28
B	CTA GCTAGC GTTGGGAGTTCTCGTCGTGGCTCA	648	−620 ~ +28
C	CTA GCTAGC AGGCTATTTAACTGCTTCCTTCTGT	252	−224 ~ +28
D	CTA GCTAGC CAGACATTCCACAGTGGGCCAGTGT	360	−332 ~ +28
E	CTA GCTAGC TCCGACTAGACCCCTAGCCTGGGAA	466	−438 ~ +28
F	CTA GCTAGC AAGGATCCGGCGATGCCATGA	558	−530 ~ +28
R	CCC AAGCTT CAGACTGCGGCGGGAGTAGTGACA		
GATA-1F	GAATTC ATGGAGTTCCCTGGCCTC	1239	–
GATA-1R	TCTAGA TCACGAGCTGAGCGG		
Sp1F	GAATTC ATGAGCGACCAAGATCACT	2361	–
Sp1R	TCTAGA TCAGAAGCCATTGCCACT		

The enzyme cut sites are in box; protect base are underlined.

vector. Cotransfections were performed four times, and the luciferase assays were conducted thrice on individual transfection experiments. *Cis*-acting and *trans*-acting elements among 5′-UTR region were all verified using this method.

The complete CDS of *GATA-1* (NC_010461) and *Sp1* (NC_010447) were cloned by RT-PCR. The restriction enzyme *Eco*RI was introduced to the sense primer, whereas the endonuclease *Xba*I was introduced to the reverse primer. The primer sequences are listed in table 1. The PCR reaction mixture (total of 25.0 μL) contained 1.0 μL of genomic DNA (100 ng), 2.5 μL of 10× PCR buffer, 1.0 μL of each primer (10.0 p mol/L), 0.5 μL rTaq polymerase (1.00), 2.0 μL dNTPs and 17.0 μL double distilled H_2O. The cycling profile was 94°C for 4 min; 30 cycles: 94°C for 1 min, 58°C for 2 min, 72°C for 2 min; 72°C for 10 min; 4°C for 5 min. The fragments were ligated in the plasmid pcDNA3.1(+). The accuracy of the insert fragment was detected by sequencing. pcDNA-*GATA-1* and pcDNA-*Sp1* were cotransfected with the pGL3-Basic-*Foxn1*, respectively. Firefly luciferase activity was measured as above.

All data were expressed as the mean ± SEM. Statistical analysis was performed using the student's *t*-test, using the SAS 9.2 statistical software. The threshold of significance was defined as $P<0.05$ or $P<0.01$.

Results and discussion

The 5′-UTR sequence of the pig *Foxn1* of 1001 bp was cloned, the indicated fragment length is the length of the total fragment and does not refer to the nucleotides before the initiation codon. A series of 5′-UTRs of the *Foxn1* promoter at nucleotides positions −973, −620, and −224 bp and with a common 3′-terminus at position +28 bp were cloned into luciferase reporter vector pGL3-basic. In the present study, the first nucleotides of the initiation codon was designated as +1. The accuracy of the constructs was confirmed by sequencing.

The *Foxn1* promoter activities of the 5′-deletions mutants were assessed by using a dual-luciferase reporter assay after transient transfection into PK15 cells. Figure 1a shows that the most remarkable changes in promoter activity were observed between pGL3-basic (control) and (−224/+28 bp; $P<0.01$), (−224/+28 bp) and (−620/+28 bp) ($P<0.01$), (−620/+28 bp) and (−973/+28 bp) ($P<0.01$). It is possible that *cis*-acting elements were present within the −224 to +28 bp region and −620 to −973 bp region, as well as *trans*-acting element within the −224 to −620 bp region, *cis*-acting should be promoter elements and *trans*-acting should be repressor elements.

The online database (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi) was used to

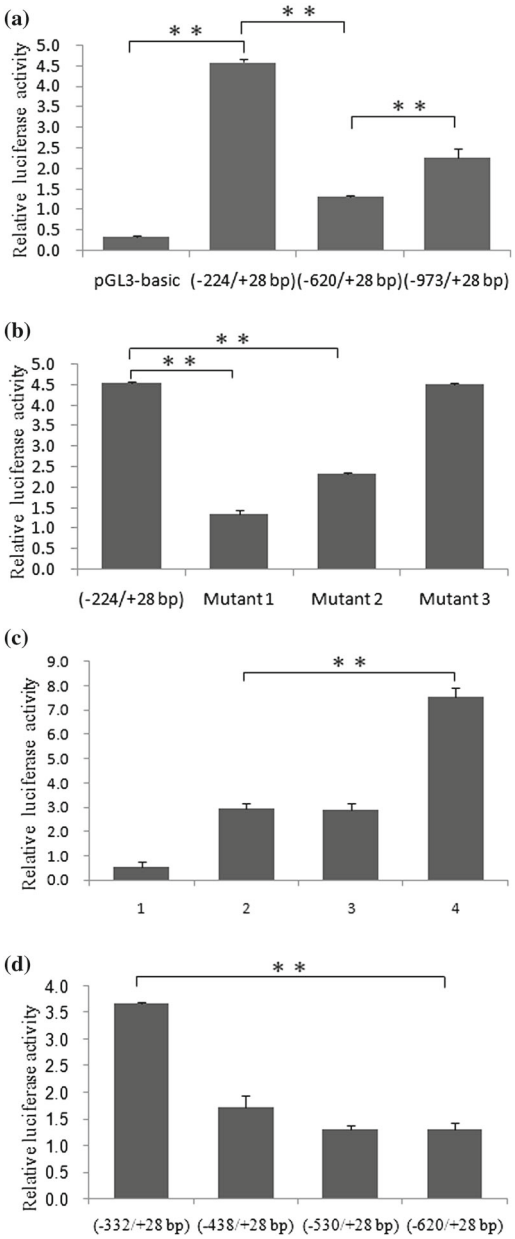

Figure 1. The relative luciferase activity of Foxn1 promoter in PK15 cells. The luciferase activity of Foxn1 reporter constructs in PK15 cell lines are: (a) The relative luciferase activities of each fragment are all significant different to pGL3-basic. (b) The relative luciferase activity of mutants 1 and 2 are all significant different to (−224/+28 bp) fragment, but mutant 3 was not. It is mainly for detecting the *cis*-acting element of Foxn1 promoter. (c) The relative luciferase activity of Foxn1 after cotransfected with *GATA-1* and *Sp1*. The first column means only pGL3-basic plasmid. The second column means only pGL3-basic-*Foxn1* plasmid. The third column means pGL3-basic-*Foxn1* and pcDNA3.1-*GATA-1* cotransfection. The forth column means pGL3-basic-*Foxn1* and pcDNA3.1-*Sp1* cotransfection. The relative luciferase activity of pGL3-basic-*Foxn1*+pcDNA-*Sp1* is significantly higher than others. (d) The relative luciferase activity of (−438/+28 bp) fragment is significantly different to (−332/+28 bp) fragment. It is mainly for detecting the *trans*-acting element of *Foxn1* promoter. **Significant difference at $P < 0.01$.

analyse the potential transcription factor-binding sites between −224 to +28 bp. Several transcription factors were detected such as *GATA-1*, *GATA-2*, *GATA-3*, *SRY*, *E2F*, *v-Myb*, *Sp1* and *MZF1*, and all these can be bounded to this region. To identify the most important *cis*-acting elements within the *Foxn1* promoter, overlap-extension PCR was performed for site-specific mutagenesis within the − 224 to + 28 bp regions. Three mutants were constructed. Mutant 1 showed a deletion of the sequence GGGTGATGGTGTCACTACTCCCGC CGCAGTCTG, which included the transcription factor binding site (TFBS), *GATA-1*. Mutant 2 showed a deletion of the sequence TGAGGT and were replaced by the sequence CAACAC, which included the TFBS, *Sp1*. Mutant 3 showed a deletion of the sequence ATCTGTTT and was replaced by CCACACCC, which included the TFBS, *GATA-3*. Except for the deleted nucleotides, the rest of the other sequences were in complete agreement. Five primer pairs were designed to amplify the mutants (table 2). Three mutants were produced and their activities were analysed as described earlier. The activities of each mutant was compared to that of the −224/+28 bp fragment. Figure 1b shows that the relative transcriptional activity of mutants 1 and 2 was statistically significant ($P < 0.01$).

The eukaryotic expression vector of pcDNA3.1-*GATA-1* and pcDNA3.1-*SP1* were constructed and were cotransfected with pGL3-basic-*Foxn1*. The relative luciferase activity of pcDNA3.1-*SP1* adds pGL3-basic-*Foxn1* was much higher than the others (figure 1c), which means the *Sp1* is one of the most important *cis*-acting elements of *Foxn1*. While the relative luciferase activity of pcDNA-*GATA-1* add pGL3-basic-*Foxn1* was same as pGL3-basic-*Foxn1* and means *GATA-1* is not the *cis*-acting element of *Foxn1*, there are some other factors among this region. *Sp1* is also a well-known transcription factor that has been implicated in a wide variety of essential biological processes such as cell growth, differentiation, apoptosis and carcinogenesis. *Sp1* activates the transcription of various genes that contain putative CG-rich Sp-binding sites within their promoters (Zhao *et al.* 2014; Vizcaíno *et al.* 2015; Yan *et al.* 2015).

To identify the most important *trans*-acting elements within the *Foxn1* promoter region, a series of fragments of different lengths between −224 bp and −620 bp were PCR amplified. Nucleotide positions −332, −438 and −530 bp with a common 3′-terminus at position +28 bp were cloned into the luciferase reporter vector pGL3-basic (D, E, F and R). The activities of each fragment was compared to that of the −620/+28 bp fragment. Figure 1d shows that the relative transcriptional activity between −332/+28 bp and −438/+28 bp were statistically significant differences ($P < 0.01$). This finding suggested the existence of *trans*-acting elements within the −332 to −438 bp region. Subsequently, when a similar method was used to detect the exact transcription factors,

Table 2. Primer pairs used to amplify the mutants.

Primer	Location (bp)	Sequence (5′–3′)	Size (bp)
mutant 1	−224 ∼ −6	CTA GCTAGC AGGCTATTTAACTGCTTCCTTCTGT	219
		CCC AAGCTT GGTCCTGGCCTCGAAGAAAGC	
mutant 2-1	−224 ∼ −140	CTA GCTAGC AGGCTATTTAACTGCTTCCTTCTGT	85
		AGAGAGAA**CCCACACC**AGAGACAGACAGA	
mutant 2-2	−166 ∼ +28	GTCTGTCTCT**CCACACCC**TTCTCTCTCTC	194
		CCC AAGCTT CAGACTGCGGCGGGAGTAGTGACA	
mutant 3-1	−224 ∼ −107	CTA GCTAGC AGGCTATTTAACTGCTTCCTTCTGT	118
		CTTCCCTTCCC**CAACAC**TCCCTGGGCT	
mutant 3-2	−106 ∼ +28	CCAGGGA**CACAAC**GGGAAGGGAAGCGG	134
		CCC AAGCTT CAGACTGCGGCGGGAGTAGTGACA	

The enzyme cut sites are in box; protect bases are underlined; the mutation sites are in bold.

no factors were found, which means there may be some new transcription factors which were not found.

Acknowledgements

This work was supported by the National Science and Technology Planning Project of 12th Five-Year in rural areas (2015BAD03B02-5); National Nature Science Foundation of China under grant (31301955); Modern Agricultural Technology System (CARS-36); Innovation ability raise of Scientific Research Institution, Heilongjiang (YC2016D001).

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Corresponding editor: Silvia Garagna