Screening of Chicken Genes Related to Germ Cell Development

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닭에서 생식세포 발달에 관여하는 유전자 검색

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요 약

본 연구에서는 Germonline 싸이트에 존재하는 유전자를 닭에 적용함에 따라 생식세포 발달과 관련된 TCs (tentative consensus sequences)의 발현 패턴을 닭의 다양한 조직에서 관찰하였다. 따라서 생식세포 분화와 관련된 전체 84개(생쥐 10개, 쥐 71개, 사람 3개)의 유전자 집단에 대해 BLAST 검색을 시행한 결과, 닭에 상응하는 84개의 TC중에서, 42개의 TC가 높은 유사성을 나타냄을 확인하였다. 42개의 TC 가운데에서 Hmgcs2와 Sycp3는 각각 정소와 생식기관 특이적으로 발현됨을 알 수 있었다. 또한, Crmp4, Cyct, Ldhc, Epha7, Pcsk4, Dnmt3a은 뇌, 정소, 난소에서만 발현되었다. 닭 유전자를 대상으로 생식세포 발달과 관련된 유전자 탐색을 수행한 본 연구는 생식세포 분화의 메커니즘을 규명하는데 유용한 정보의 원천으로써 제공될 수 있을 것이다.

(**Key words** : Chicken, Germ cell, GermOnline, Hmgcs2, Sycp3)

Ⅰ. INTRODUCTION

Germ cells possess special properties due to their unique roles. Since germ cells ultimately give rise to all the tissues of a developing animal, they must remain pluripotent, also they are differentiated into highly specialized gametes. Therefore, the research to verify the mechanism of genes involved in germ cell development is highly valuable. The recent developments of large-scale DNA sequencing techniques, micro-array technology and bioinformatics have been enable scientists to identify potential genes, study their patterns of expression and analyze their promoters at the genomic level (Lockhart and Winzeler, 2000). GermOnline being accessible at http://www.Germonline.org is a gateway for germ cell growth and gametogenesis (Wiederkehr et al., 2004a, 2004b). It provides a rapid access to curated information about genes as well as

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microarray expression data implicated in germline development, the mitotic and meiotic cell cycle, gamete formation, and gamete function in currently 11 model species such as Homo sapiens, Mus musculus, Rattus norvegicus, Danio rerio, Drosophila melanogaster, Caenorhabditis elegans, Arabidopsis thaliana, Schizosaccharomyces pombe and Saccharomyces cerevisiae.

However, little is known of molecular requirements for specification of chicken germ cells. Thus, we suggested how many genes related to germ cell lineage in mouse, rat and human are conserved in chicken and the expression patterns of the genes in various chicken tissues.

Ⅱ. MATERIALS AND METHODS

1. Tissues

White Leghorn chickens used in this study were kept at the University Animal Farm in Seoul National University. Six tissues including brain, spleen, liver, muscle, testis and ovary were obtained from sexually matured chickens (25 weeks old). The tissues, excluding testes and ovaries, were obtained from both male and female chickens.

2. Extraction of mRNA and performance of RT-PCR

According to the manufacturer's protocol, samples of chicken tissues were homogenized and total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). For each tissue analyzed, cDNA was synthesized from 0.5 μg total RNA in 20 μl reactions by reverse transcription with the Superscript III First-Strand Synthesis System (Invitrogen).

Touchdown PCR was performed to prevent the formation of spurious bands. Each PCR reaction was performed using 0.5 U of Taq polymerase (Biotools, Madrid, Spain) with 2 μl cDNA per 20-μl reaction volume. An initial denaturation step at 94°C for 2 min was followed by 5 rounds of touchdown cycles. The primer annealing temperature was 62°C in the first cycle and decreased by 0.5°C in every subsequent cycle. In the next 28 cycles, amplification was performed by using 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. The thermal cycling program ended with incubation at 72°C for 7 min to ensure complete extension. PCR amplification of all cDNA samples was also performed by using primers specific for the house-keeping gene, glycerol-dehyde-3-phosphate dehydrogenase (GAPDH) as a control for RNA extraction and cDNA synthesis.

3. Search for ESTs and primer design

To investigate possible germ cell-specific marker, we searched the pool of 84 genes related to germ cell lineage in mouse(10), rat(71) and human(3) from GermOnline site and found 84 chicken homologous TCs (tentative consensus sequences) from Gallus gallus Gene Index (GgGI) at the Institute for Genomic Research (TIGR) as performing a tBLASTn search. We selected 41 Chicken homologous TCs having Coverage of 60% and Identities and Positives of 60~70% from the result of a BLAST search. Primer pairs for RT-PCR were designed for each of the 41 TCs using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Ⅲ. RESULTS AND DISCUSSION

We searched the pool of 84 genes related to germ cell lineage in mouse(10), rat(71) and human(3) from GermOnline site and found 84 chicken homologous TCs from GgGI. Of those, 42 chicken homologous TCs had Coverage of 60% and Identities and Positives of 60~70% in performing a BALST search (Table 1). This suggested that many genes involved in germ cell development were conserved in chicken. We selected 41 chicken homologous TCs except for SOX9 because SOX9 has been reported frequently about expression pattern and its function in bird (Healy et al., 1996; Kent J et al., 1996)

As one of results, Hmgcs2 and Sycp3 were shown to be expressed in a testis-specific manner and a reproductive organ (testis and ovary)-specific manner by RT-PCR analysis, respectively (Fig. 1). 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (Hmgcs2), a HMG-CoA cycle enzyme catalyzing a step in ketone-body biosynthesis (Gil-Gomez et al., 1993), is expressed in rat testis (Schlecht et al., 2003, 2004). SYCP3/ SCP3, a component of the synaptonemal complex (Lammers et al., 1994), is highly expressed in spermatocytes and round spermatids (Schlecht et al., 2004). SYCP3 appears to have an essential meiotic function in human spermatogenesis because it was found to be mutated in patients displaying azoospermia (Miyamoto et al., 2003). As a result, the expression patterns in testis of chicken Hmgcs2 and Sycp3 mean that the function of chicken Hmgcs2 and Sycp3 may be similar to mammal, on the other hand, it is interesting that Sycp3 is weakly expressed in ovary. Therefore, future research will be needed to elucidate their functions and cell-specific expression in chicken testis.

Crmp4, Epha7, Cyct, Ldhc, Pcsk4 and Dnmt3a expressed in brain, testis, and ovary (Fig. 1). Collapsin response mediator protein 4 (Crmp4), which may regulate the plasticity of the adult nervous system (Wang and Strittmatter, 1996) and Eph receptor A7 (Epha7), which may mediate developmental effects in the nervous system (Valenzuela et al., 1995), are highly expressed in purified spermatids (Schlecht et al., 2003, 2004). Testis specific cytochrome c (Cyct), a component of the mitochondrial electron transport chain (Shima et al., 2004), is expressed in pachytene spermatocytes and round spermatids (Schlecht et al., 2003, 2004). Lactate dehydrogenase C chain (Ldhc), a member of the lactate dehydrogenase family which catalyzes the conversion of lactate to pyruvate (Sakai et al., 1987), is highly induced in spermatocytes and post-meiotic spermatids (Schlecht et al., 2003, 2004). Dnmt3a, encoding a de novo DNA methyltransferase essential for paternal and maternal imprinting (Reik and Walter, 2001; Hata et al., 2002), is required for spermatogenesis. Although Crmp4, Cyct, Ldhc, Epha7 and Dnmt3a genes are highly expressed in spermatogenesis system, it is known that these genes play important roles in nervous system, cellular function as a component of the mitochondrial electron transport chain and a catalyzer of the conversion of lactate to pyruvate and DNA methylation. Therefore, the patterns of gene expression in chicken might be related to various functions of these genes.

Proprotein convertase subtilisin/kexin type 4 (Pcsk4), which has a possible role in the developmental maturation of germ cells, is highly abundant in the rat testis (Seidah et al., 1992). However, in performing our BLAST search, Pcsk4 in rat had the highest similarity with trans-golgi network protease, Furin, in chicken. It may

Table 1. The list of the chicken tentative consensus sequences (TCs) by performing a BLAST search with GermOnline genes

	Gene symbol	The result of a BLAST search at GgGI				Biological process
		Chicken TC	% Coverage	% Identities	% Positives	
1	Adcyap1	TC106496	100	68	78	insulin secretion
2	Aqp8	TC108639	67	55	70	water transport; spermatogenesis
3	Ccnb1	TC101755	90	50	65	cell cycle; regulation of cell cycle
4	Chrne	NP347482	98	50	64	transport; synaptic transmission; ion transport
5	Crmp4	TC102225	100	88	93	neurogenesis
6	Cst8	TC121340	71	29	58	
7	Cyct	TC121703	99	87	92	electron transport
8	Fbp1	TC123026	99	74	87	gluconeogenesis; carbohydrate metabolism
9	Gpd2	TC130234	100	78	89	glycerol-3-phosphate metabolism; electron transport
10	Hmgcs2	TC129011	61	72	85	ketone body biosynthesis; cholesterol biosynthesis; acetyl-CoA metabolism
11	Ide	TC115179	23	90	94	proteolysis and peptidolysis
12	Mak	TC109360	93	71	79	spermatogenesis; protein amino acid phosphorylation
13	Mcsp	TC118837	15	59	63	sperm motility; fertilization (sensu Animalia)
14	Pctp	TC135038	99	70	84	lipid transport; transport
15	Pim1	TC124577	75	83	89	protein amino acid phosphorylation; cell growth and/or maintenance
16	Pkib					negative regulation of protein kinase activity
17	Polb	TC127027	100	85	93	base-excision repair; pyrimidine dimer repair
18	Prm2	BU419008	40	42	59	spermatogenesis; spermatid development; nuclear organization and biogenesis; mitotic chromosome condensation; chromosome
19	Slc2a3	TC124181	100	66	78	transport; carbohydrate transport
20	Sycp3	TC106186	73	65	78	synaptonemal complex formation; homologous chromosome segregation; male meiosis chromosome segregation; male meiosis I; spermatogenesis
21	Tcf2	TC131449	45	60	70	regulation of transcription, DNA-dependent
22	Tnp1					sperm chromatin condensation
23	Aif1	TC122955	92	59	80%	macrophage activation
24	Atp1a4	TC99944	96	77	86%	sperm motility; metabolism; potassium ion transport; sodium ion transport
25	Chn2	TC124818	94	92	96	intracellular signaling cascade; acrosome formation
26	KRP2	TC124520	92	65	76	meiotic chromosome movement; meiotic spindle assembly; meiosis
27	Tlbp	TC109311	100	61	79	acrosome formation
28	Cdc20	TC121594	100	75	84	proteolysis and peptidolysis; regulation of cell cycle; mitotic cell cycle; cell cycle

Table 1. The list of the chicken tentative consensus sequences (TCs) by performing a BLAST search with GermOnline genes (cont'd)

	Gene symbol	The result of a BLAST search at GgGI				Biological process
		Chicken TC	% Coverage	% Identities	% Positives	
29	Top2a	TC123405	86	79	86	DNA topological change; DNA metabolism
30	Adam1	NP959246	80	37	53	binding of sperm to zona pellucida; fusion of sperm to egg plasma membrane; fertilization (sensu Animalia)
31	Tpt1	TC120477	84	99	100	spermatogenesis; cell proliferation
32	Prkcd	TC124695	98	80	88	apoptosis
33	Aqp9	TC130395	91	61	77	water transport; transport
34	Ppp3r2	TC100990	97	81	89	calcium ion sensing; calcium-mediated signaling
35	Adam2	NP959246	94	31	47	fertilization (sensu Animalia); regulation of cell adhesion
36	Ldhc	TC121498	99	67	83	glycolysis
37	Sycp2	CD761525	9	59	79	sperm chromatin condensation; spermatogenesis
38	Dll3	TC130861	78	33	45	
39	Epha7	TC108720	72	95	96	protein amino acid phosphorylation; transmembrane receptor protein tyrosine kinase signaling pathway
40	Hao3	TC132280	91	56	73	electron transport
41	Prim2	TC130308	100	66	81	DNA replication
42	LOC171574	TC110889	47	85	92	
43	Crem	TC123032	83	73	81	
44	Muc1	BU432980				
45	Odf2	TC132010	59	66	80	spermatid development
46	Tnp2	TC122100	14	56	75	sperm chromatin condensation
47	Itmap1	TC132472	59	52	70	hormone mediated signaling
48	Pci	TC108772	90	38	57	negative regulation of blood coagulation
49	Amsh	TC110841	63	52	67	cytokinin mediated signaling
50	Sp10					spermatogenesis; sperm axoneme assembly
51	Sp17	TC108005	92	31	48	binding of sperm to zona pellucida
52	Pcsk4	TC127481	81	61	72	
53	Rcl	BU263678	55	33	44	cell growth; anti-apoptosis; regulation of apoptosis
54	Spam	TC129291	78	43	59	binding of sperm to zona pellucida
55	RABIN3	TC126722	87	75	82	
56	Akap4	TC108607	20	50	90	fertilization (sensu Animalia); sperm motility
57	Map17	TC131806	85	35	53	glucose transport; mannose transport
58	Plcd4	TC127627	62	37	53	intracellular signaling cascade
59	Krp5	TC123073				meiosis
60	Tsga10	BU212235				
61	Veli1	CN217431	89	90	90	
62	Adam3	NP959246	90	31	47	regulation of cell adhesion
63	Adam4	NP959246	47	32	48	regulation of cell adhesion

Table 1. The list of the chicken tentative consensus sequences (TCs) by performing a BLAST search with GermOnline genes (cont'd)

	Gene symbol	The result of a BLAST search at GgGI				Biological process
		Chicken TC	% Coverage	% Identities	% Positives	
64	Adam5	NP959246	75	33	50	regulation of cell adhesion
65	Adam6	NP959246	87	29	47	regulation of cell adhesion
66	Tpx1	TC119031	93	30	46	fertilization (sensu Animalia); cell-cell adhesion
67	ODF3	NP346652				cytoskeleton organization and biogenesis
68	Hsd3b1	TC108855	100	52	67	
69	Ces3	TC102409	99	42	63	
70	LOC286978	TC100072	89	57	60	
71	LOC59320	TC138688	10	25	46	G-protein signaling, adenylate cyclase activating pathway
72	Ube2b	TC101466	100	100	100	spermatogenesis
73	G6pd2	AI981687	25	77	87	glucose metabolism; spermatogenesis
74	Clgn	TC121819	91	57	74	fertilization; binding of sperm to zona pellucida
75	Ccna1	TC109538	58	85	91	spermatogenesis; meiosis I; regulation of cell cycle
76	Sycp3	TC106186	74	63	79	meiosis; spermatogenesis; female gamete generation; synaptonemal complex formation; meiotic chromosome condensation
77	Dnmt3l	TC101864	73	41	57	imprinting; spermatogenesis; DNA methylation during gametogenesis
78	Rad18	TC124491	78	48	62	imprinting; female gamete generation; spermatogenesis
79	2310009E 07Rik	TC127036				DNA repair; response to DNA damage stimulus
80	Zfp145	TC129585	100	83	87	negative regulation of transcription; cell growth; cell growth and/or maintenance; skeletal development; limb morphogenesis; male germ-line stem cell division; germ-line stem cell maintenance; spermatogenesis
81	Dnmt3a	TC101864	84	86	88	imprinting; spermatogenesis; DNA methylation during gametogenesis
82	SOX9	TC106036	94	83	86	primary sex determination; male sex determination; sex determination, establishment of X : A ratio
83	FOXL2	TC136944	30	84	86	primary ovarian follicle growth; ovarian follicle atresia; morphogenesis of follicular epithelium
84	SRY	TC108068	77	45	67	male sex determination

The TC in the gray areas has Coverage 60%, Identities and Positives 60~70%.

Appendix 1. The list of the primer pairs used in this study.

#	Gene	Primers		Product size
1	Adcyap1	Left Right	GCTCTTAGCCCTCCTGGTCT CGTTCCGTCACTCTGGAAAT	638
2	Aqp8	Left Right	CATCCTGACCACCTTCCTGT TTACAGTGGCTGCTGTGCTC	563
3	Ccnb1	Left Right	TCCAGGTCCACTCAAGGTTC GACGTTCTTGGCCATATGCT	533
4	Chrne	Left Right	AAGATCATCAATTCAGGGCG TCATCAGTCCATCCCTCTCC	580
5	Crmp4	Left Right	CCAGGAGAAAGATGGCAGAG CCACAAGCATTCAACACCAC	566
6	Cyct	Left Right	ACGTCGTGACGTCAGTCTTG ACACTTCCATTGCCATCTCC	621
7	Fbp1	Left Right	CACTTCTGCTGGAGGTGTCA GCTTTAAGGCATTCCTTCCC	539
8	Gpd2	Left Right	TTGCTCGTTCAAGGTGACTG GCTGCTCGCTGGGTTATTAG	596
9	Hmgcs2	Left Right	TGTGGAGCAGTCTGAGTTGG TCTGAGGACAGGTCAGGCTT	546
10	Mak	Left Right	AGAGTGACTGGCCAGAAGGA TCCAAATTCAGCATCATCCA	614
11	Pctp	Left Right	TGCCTGCTTTCTTGAAGGAT AGGGATTGATTCACTGACGC	538
12	Pim1	Left Right	TGACTTCATCACAGAACGGG AGCTGTGCAGGTGGATCTCT	530
13	Polb	Left Right	CCTGACACGAGTTACTGGCA AACTCCAGTGACACCCAAGG	622
14	Slc2a3	Left Right	TCCATGCTGTTGGTGAATGT GAGCTGCAGTGTGATGGAAA	558
15	Sycp3	Left Right	AATTTCGTTATGGCACCGTC CTCCTGCTGTTGAGTGTCCA	685
16	Aif1	Left Right	CTGCCCTTCTTGCATTTCTC GTCTTTGCCTTGTAGCCAGC	630
17	Atp1a4	Left Right	TGCTCGACTCAACATTCCTG TTTCTTGGCTGCCTCTTCAT	579
18	Chn2	Left Right	GAAAGCCCACAATACCCAGA CAGAAATTGAAGTGCAGCCA	691
19	KRP2	Left Right	GCAATGATTTCTCCAGGCAT GTTTGCTTCTCTCAGGCACC	604
20	Tlbp	Left Right	AGGACCACTGCCTCACAAAG GCATGAAGCTTTGGTCACTG	501
21	Cdc20	Left Right	CCACCACCATGATGTCAGAG GTCGTAGCGTTTCATCAGCA	539

Appendix 1. The list of the primer pairs used in this study (cont'd).

#	Gene		Primers	Product size
22	Top2a	Left Right	ATGGTTACGGAGCCAAACTG AAGAGCTCGAGGTGTTTCCA	563
23	Tpt1	Left Right	TCATCTACCGGGACCTCATC AGCCAATTATGGTGACAGGC	552
24	Prkcd	Left Right	TGGACACACCTCACTACCCA GCACAGAAGAACACTGCCAA	508
25	Aqp9	Left Right	GGCATGTTGTGTTGGTTCTG AAATGCTCCCAGGAATTGTG	573
26	Ppp3r2	Left Right	GCAGTTCTCCAGTCCTTTGC AGATACAACCACTGGCTGGG	572
27	Ldhc	Left Right	GGCAGCAGCTACATCAAACA CATTCCTACGGCACCACTTT	678
28	Epha7	Left Right	GCAGGGTGAATGTGTTCCTT TCTGTTCACTGGAAACAGCG	518
29	Hao3	Left Right	AAGCAATGGGCACCTGTTAC CACGCAGAATCCTCAGAACA	693
30	Prim2	Left Right	TCGCTTCCATGAACTGACTG ACGGAGGTGATGGTTCTCAC	517
31	Crem	Left Right	GGGCTCTGCATAGTCCTCAG GCAACACGACTCTCCAGACA	540
32	Pci	Left Right	TGAGGTTGCACAAGAAGCAC GCTGGCAGAATTAGAAACGC	651
33	Amsh	Left Right	AAGAAGCAAGAACTGGCTCG CAGAAACTTCAAGCATGCCA	646
34	Pcsk4	Left Right	GGCTGCTGGAGATTGAGAAC TCCTCTCATTGTGCACTTCG	696
35	RABIN3	Left Right	CATCAATGTTACCGACGCAG CGATAGGTCAGCCTCTTTGC	556
36	Veli1	Left Right	CACGCTGGACAGAGATGTTG CCTTGGGAGTGTAGCGTACC	506
37	Hsd3b1	Left Right	CCTGGTCACTCTGCTGAACA GCTATCAAGTGGCTTCTGCC	586
38	Ube2b	Left Right	GATGCGGGACTTCAAGAGAT GGTTTCCTAAACTGGAGGGC	583
39	Clgn	Left Right	GACAAAGGGCTTGTGTTGGT TCCTCATCCCAATCTTCAGG	686
40	Zfp145	Left Right	GCCAAAGCATTTGTCTGTGA CAGATGGTGCACTGGTATGG	533
41	Dnmt3a	Left Right	ACCAGGCCACCTACAACAAG CAGCAGATGGTGCAGTAGGA	559

be caused by the fact that Pcsk4 in mouse contains a bacterial subtilisin-like catalytic domain closely related to that of Furin in mouse (Nakayama et al., 1992). Thus, it suggests that in chicken, Pcsk4 might not exist and there may be unusual mechanism involved in convertase

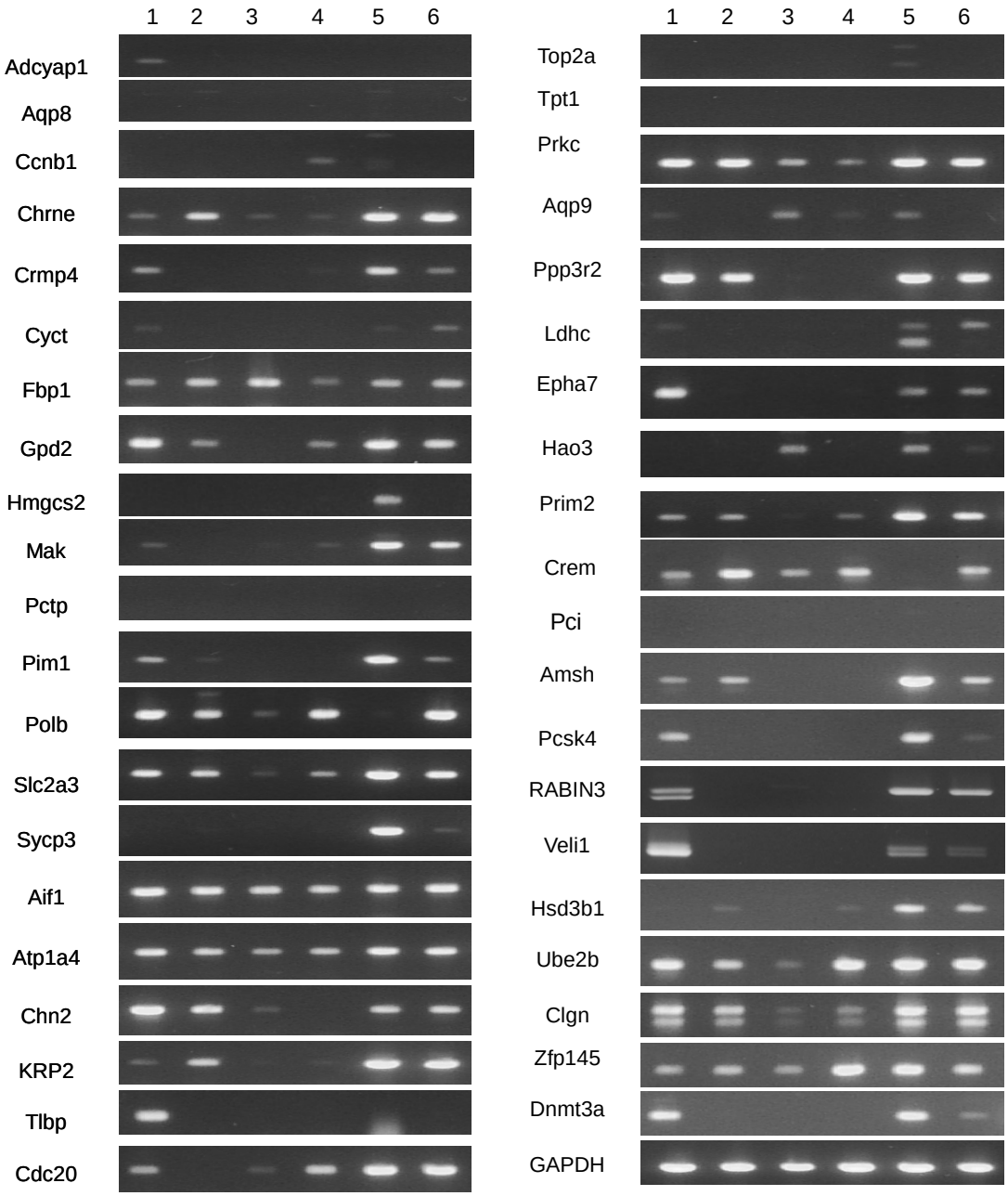

Fig. 1. The expression pattern of the chicken tentative consensus sequences (TCs) originated with GermOnline genes by RT-PCR. Lane 1: brain, lane 2: spleen, lane 3: liver, lane 4: muscle, lane 5: testis, lane 6: ovary.

subtilisin/kexin. Also, it may be related to the fact that chicken Furin is expressed in reproductive organ as well as brain tissue.

RABIN3 and Veli1 apparently expressed as alternatively spliced transcripts in the tissues examined (Fig. 1). The PCR products amplified from the brain were of the predicted size, whereas bands of unexpected sizes were generated from the RNA extracted from testis and ovary tissues, indicating possible reproductive organ-specific alternative splicing. RABIN3, a protein that associates with the Ras-like GTPase Rab3A and Veli1 (lin-7-Ba), a gene that may mediate the formation of cell-cell junctions (Irie et al., 1999), are highly induced in spermatocytes and post-meiotic spermatids (Schlecht et al., 2003, 2004). Interestingly, in performing our BLAST search, Veli1 in rat had the highest similarity with the unknown transcripts (Gene Bank No. CN217431) in chicken. Therefore, future studies will be needed to elucidate the unknown transcripts and bands of unexpected sizes which were expressed in testis and ovary tissue.

Adcyap1 and Tlbp were also shown to be expressed in a brain-specific manner and Chrne was specific in muscle tissue (Fig. 1). The 9 numbers of candidate TCs (Ccnb1, Fbp1, Scl2a3, Aif1, Atp1a4, Prkcd, Ube2b, Clgn, Zfp145) were expressed in all tissue. There were 2 TCs (Polb, Crem) shown to be not expressed especially in testis and Chn2 shown to be not expressed especially in muscle tissue. In addition, 11 TCs (Gpd2, Mak, Pim1, KRP2, Cdc20, Aqp9, Ppp3r2, Hao3, Prim2, Amsh, Hsd3b1) were shown to be randomly expressed in various tissues.

Gene expression pattern in each tissue or organ is associated with the formation, differentiation, and development of the organ. Thus, gene expression profiling in various tissues can be used as a part of the process of screening for genes controlling the differentiation and development. In further investigation, it will be necessary to profile the spatial and temporal expression of various genes to verify the differentiation mechanisms of germ cells, the formation of genital organ and analysis of their functions.

Ⅳ. ACKNOWLEDGMENTS

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Ⅴ. ABSTRACT

We examined the expression patterns of the chicken TCs (tentative consensus sequences) originated from GermOnline genes in various chicken tissues, applying information from GermOnline to chicken organisms. 42 TCs among 84 chicken homologous TCs from the pool of 84 genes related to germ cell lineage in mouse(10), rat(71) and human(3) had high homology based on a BLAST search. Of these, Hmgcs2 and Sycp3 was shown to be expressed in a testis-specific manner and a reproductive organ (testis and ovary)-specific manner, respectively, by RT-PCR analysis. Crmp4, Cyct, Ldhc, Epha7, Pcsk4 and Dnmt3a are expressed in brain, testis, and ovary. The characterization of chicken genes originated from GermOnline in this research may give an enormously useful source of information related to germ cell development.

(**Key words**: Chicken, Germ cell, GermOnline, Hmgcs2, Sycp3)

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