# Testis-specific transcripts in the chicken

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## ABSTRACT

Sequences of candidate chicken testis-specific genes were analyzed in order to develop a resource for functional genomic studies of the testis and male germ cells. Tentative consensus sequences (TCs) containing ESTs expressed in testis libraries only were selected from the TIGR *Gallus gallus* Gene Index, resulting in a total of 292 TCs. The transcriptional expression of these genes were evaluated in a variety of chicken tissues, including testis and ovary. Of the panel of 292 TCs, 110 were expressed in a testis-specific manner. The correlation between the number of ESTs assembled into each TC and the number of testis-specific TCs was not significant. Annotation of the TCs using the Gene Ontology database terms showed that the proportion of testis-specific TCs that were classified as having catalytic activity (within the Molecular Function branch) was larger than the proportion of total chicken TCs classified in the same way. Our results might facilitate the investigation of testis-specific genes and their functional analysis in the chicken, as well as in other avian species.

▸ **Key words** : testis-specific, EST, chicken, TIGR

## INTRODUCTION

The testis is the organ that produces sperm, and during spermatogenesis transcriptional regulation within germ cells is carefully orchestrated [1]. Sperm develop in association with highly specialized somatic testicular cells, such as Sertoli cells and Leydig cells. During the differentiation of germ cells into spermatozoa, a complex paracrine dialogue with Sertoli cells occurs [2]. Endocrine activity such as testosterone secretion by Leydig cells promotes germ cell differentiation [3]. Thus, it has been speculated that the testis has specialized transcription complexes that coordinate the differentiation program of spermatogenesis. In birds, the female is the heterogametic (ZW) sex, but genes on the W chromosome do not influence gonadal development in the way that the SRY gene on the mammalian Y chromosome does. No sex-chromosome-specific *SOX* gene homologous to the mammalian sex-determining gene *SRY* has been found in birds [4]. However, SRY-like HMG-box gene 9 (*SOX9*) may influence gonadal development by the initiation of transcription of anti-Müllerian hormone (AMH) during the early stages of chick gonad differentiation [5]. The avian *DMRT1* gene is located on the Z chromosome [6] and is expressed more strongly in male than in female embryonic gonads [7-9].

It is therefore thought that numerous genes affect male germ cell development in birds and that some of these may be expressed in a testis-specific pattern. The chicken is one of the most important model organisms for the study of germ-line development, as its embryonic development occurs *in ovo*. In this study we analyzed EST sequences from chicken testis libraries. A total of 292 tentative consensus (TC) sequences were found containing 100% of the ESTs from libraries related to "testis" in the latest release

of the TIGR *Gallus gallus* Gene Index (GgGI). The expression patterns of all of these TCs were examined in various chicken tissues.

## MATERIALS AND METHODS

### *Tissues*

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

*Extraction of mRNA and performance of RT-PCR*

Samples of chicken tissues were homogenized and total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. 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 25 cycles, amplification was performed 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 using primers specific for the house-keeping gene glycerol-dehyde-3-phosphate dehydrogenase (*GAPDH*) as a control for RNA extraction and cDNA synthesis.

### *Search for ESTs and primer design*

To investigate possible testis-specific TCs, we searched the GgGI at TIGR for ESTs expressed exclusively in chicken testis libraries and assembled those found into TCs. Primer pairs for use in RT-PCR were designed for each of the TCs using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

### *Gene Ontology annotation and bioinformatics analysis*

The testis-specific genes were categorized using terms from the Gene Ontology (GO) database. Since a large portion of these chicken sequences have not yet been annotated, gene annotation was performed by 1) extracting information with the term already annotated, and 2) tentatively annotating based on a protein family database search (Pfam). The latter method is based on indirect experimental evidence. The term assignments were based on the protein domain using GO terms. Protein sequences were generated from the 110 testis-specific genes using the ESTScan program [10]. The protein sequences were subjected to protein domain searches using HMMER 2.3.2 (http://hmmer.wustl.edu/) and Pfam-A family matrices (http://www.sanger.ac.uk/Software/Pfam/). The search parameters were set at an E-value 1 and trusted cutoff and noise cutoff were also used. The assignment of terms was based on the Pfam2Go translation tables, which associate protein domains with GO terms.

The testis-specific gene sequences were aligned against chicken genome draft sequences from the University of California Santa Cruz Genome Browser. The distribution of the number of testis-specific genes on each chromosome was compared to the estimated number of genes on each chromosome. The Chi-square test was used to test the differences in the distributions. The number of exons in each gene was determined from alignment of the testis-specific transcript and the genome sequences.

## RESULTS

### *Tissue specificity*

A total 292 tentative consensus sequences (TCs) were found in libraries related only to chicken testis

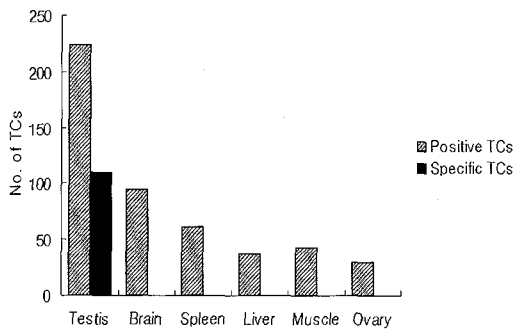


**Figure 1.** The tentative consensus sequences (TCs) yielding positive reactions by RT-PCR for several chicken tissues. The total numbers of amplified TCs for each tissue were compared with the numbers of tissue-specific TCs. There were 110 testis-specific TCs, whereas there were no tissue-specific expression of any the TCs in other tissues.

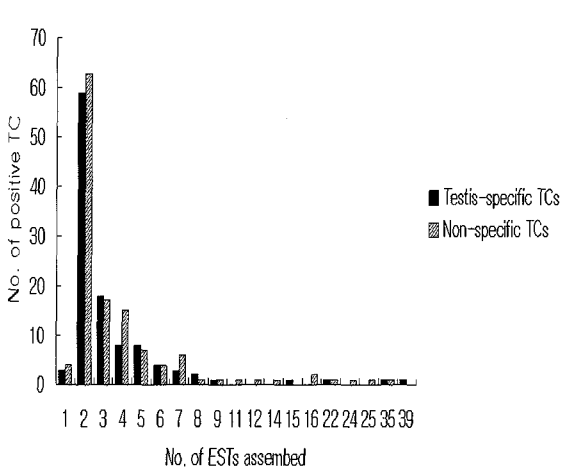


**Figure 2.** The positive TCs classified by the number of assembled ESTs consituting each TC.

in the GgGI from TIGR . Of these, 110 (38%) were shown to be expressed in a testis-specific manner by RT-PCR analysis. The largest number of candidate TCs were expressed in the testis, followed by the brain, spleen, muscle, liver, and ovary, in descending order. However, organ-specific TCs appeared only in the testis (Fig. 1).

Large proportions of the candidate TCs and testis-specific TCs were assembled from two ESTs (51% of the total candidate TCs and 48% of the testis-specific TCs; Fig. 2), whereas 48 of the testis-specific TC sequences contained more than 3 ESTs. Three of the nine singleton sequences were testis-specific. There was no significant correlation between the number of ESTs assembled into the TCs and the number of testis-specific TCs.

### *Gene Ontology annotation and bioinformatics analysis*

We found 26 TCs that could be classified according to the Gene Ontology Index. The testis-specific TCs were distributed in three branches of the GO Index: Molecular Function, Biological Process, and Cellular Component (Fig. 3). Of those classified in the Biological Process branch, 54% of the cDNA sequences encoded proteins involved in physiological processes, 31% encoded proteins involved in cellular processes, and 15% encoded proteins involved in development (Fig. 3A). Of those classified in the Molecular Function group, 46% of the TCs were involved in binding and 42% were involved in catalytic activity (Fig. 3B). Almost all (94%) of the TCs classified as Cellular Component were distributed in the cell (Fig. 3C). The distributions of the testis-specific TCs and total chicken TCs in the Biological Process and Cellular Component branches of the GO index were similar, although only simple patterns were evident. However, among the annotated testis-specific TCs classified in the Molecular Function branch, 42% were classified as having catalytic activity and 46% as being involved in binding; these percentages were higher than those found for the total chicken (22% and 36%, respectively; Fig. 4).

Fifty-five of the 292 candidate TCs were not amplified by RT-PCR, and 46 of these (84%) were assembled with less than two ESTs. It is supposed that TCs assembled from more ESTs could provide more reliable sequence information for the screening of EST libraries.

The chromosomal distribution of the testis-specific genes was not significantly different from that of the total number of genes on each chromosome (two-tailed Chi-square test, $P$ = 0.165). The number of single-exon testis-specific genes was not significant;

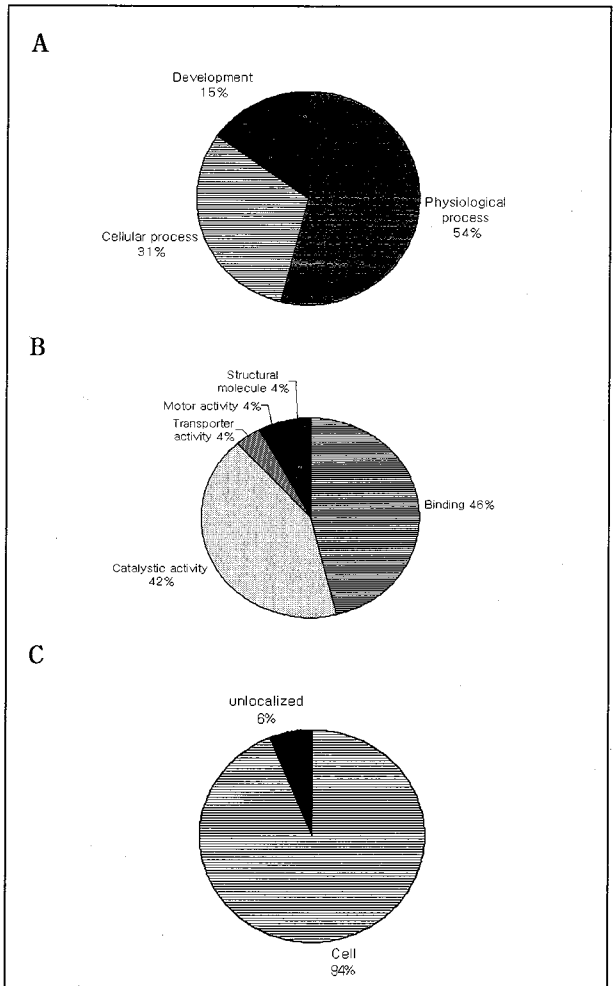


**Figure 3.** The percentage of TCs classified in different categories of the Gene Ontology Index. The cDNAs were classified according to GO categories for Biological Processes (A), Molecular Functions (B), and Cellular Components (C).

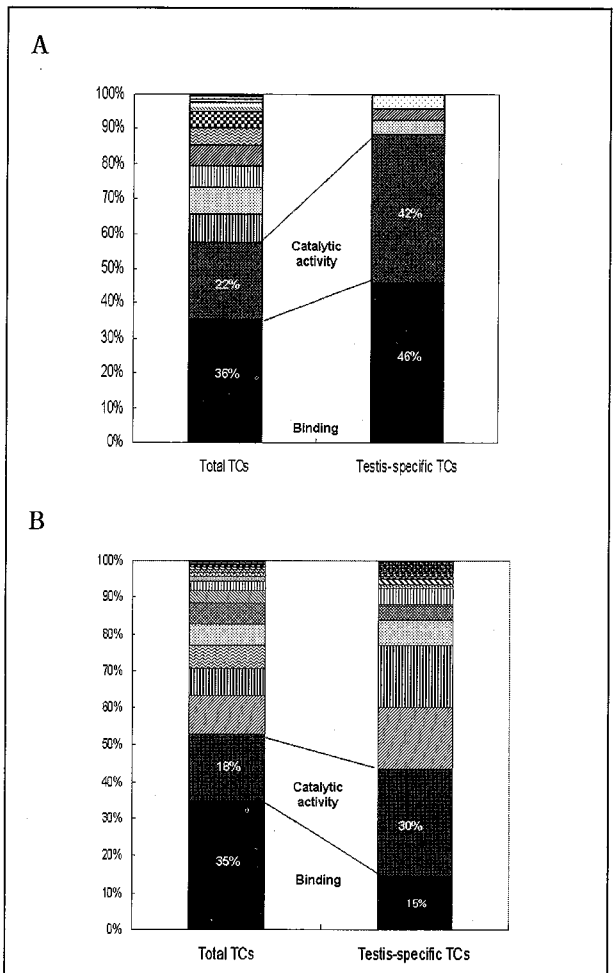


**Figure 4.** The distributions of the testis-specific TCs and total chicken TCs were compared for the Molecular Functions category of the Gene Ontology Index for chicken (A) and human (B).

however, in a previous study, we identified a set of intronless testis-specific genes [11].

## DISCUSSION

Genes expressed in a tissue-specific manner are usually associated with the formation, differentiation, and development of the tissue or organ. Therefore, organ- or tissue-specific expression can be used as part of the process of screening for genes controllingthe formation and/or differentiation of specific cells in an organ. In the testis, several types of cells are present in addition to the different developmental stages of male germ cells. Spermatogenesis is a complex process involving specific interactions between the developing germ cells and their support cells, the Sertoli cells, within the seminiferous tubules.

In the present study, we analyzed the testis-specific EST sequences expressed in tissues from sexually mature chickens. A considerable portion of the assembled ESTs examined (38%) were demonstrated to be testis-specific genes, indicating that the comparison of assembled sequence databases established from different tissues is an effective method for screening for genes that have a high likelihood of being specifically expressed in a particular tissue. Moreover, none of the candidate TCs found using

these methods proved to be specific to a tissue other than the testis (Fig. 1). This conclusion supposes that the single tissue-related ESTs in the database did not give false-negative results for expression.

The Gene Ontology classification of the testis-specific TCs was different from that of total chicken TCs in the category of Molecular Function. The proportions of TCs that were classified as having catalytic activity or binding activity were larger for the testis-specific TCs than for the total chicken TCs. In the human, the percentage of testis-specific TCs annotated as having catalytic activity was also larger than that of the total human TCs. In testis, there are special cell-to-cell interaction, hormone balancing, and catalytic action for the establishment of microenvironment for the male germ cell development. Therefore, this might be a particular characteristic of the testis; however, much more investigation is needed to confirm this conclusion. In humans, the findings for the proportions of TCs represented in the binding category of the GO classification exhibited the opposite pattern of that found for the chicken (Figure 4).

The testis-specific ESTs verified by RT-PCR in the present study might represent an enormously useful source of information concerning organogenesis and germ cell development. Therefore, further research on these presumed testis-specific genes could define mechanisms involved in the development of the testis and the process of spermatogenesis. As examples, ten testis-specific TCs that have interesting tentative annotation with high similarity are listed in Table 1. Some of these have been reported as being predominantly, if not exclusively, expressed in the testis or germ cells in mammals or avian species.

Members of the phosducin-like protein (PhLP) family are preferentially expressed in male and female germ cells. During spermatogenesis, a stage-specific expression of high levels of PhLP RNA and protein was demonstrated in pachytene spermatocytes and round spermatids. In female germ cells, the level of PhLP expression was low in whole ovary extracts, whereas higher expression levels were detected after the induction of superovulation [12]. Our results showed that phosducin-like 2 (PDCL2) was expressed in a testis-specific manner; however, further studies are necessary to verify the germ-cell specificity of PDCL2 expression in birds.

Although testis-specific genes are not always germline-specific, they might be related to male germ cell development. For instance, testis-specific serine kinase (tssk)-1 and -2 were expressed only in the testis of sexually mature males and were expressed exclusively during the cytodifferentiation of late spermatids to sperm [13]. On the other hand, expression of a third member of this family, tssk-3, was induced at puberty, persisted during adulthood, and was restricted to the interstitial Leydig cells of post-pubertal males [14].

Double sex and mab-3 related transcription factor 1 (DMRT1) is expressed exclusively in the testis in humans and in the genital ridge of early male and female murine embryos [7]. Nanda et al. [6]proposed that the dose of Dmrt1 expression could affect sex determination in the avian embryo. However, its expression becomes testis-specific after the onset of sexual differentiation [9].

Zona pellucida binding protein competed with proacrosin for binding to the zona pellucida and showed abundant testis-specific expression in the pig [15].

Our results also demonstrated the testis-specific expression of several other genes with various functions in cells: RICS (RhoGAP involved in the β-catenin-N-cadherin and NMDA receptor signaling), dual-specificity phosphatase-like protein, pantophysin (synaptophysin-like protein), cytoplasmic dynein light intermediate chains, THAP proteins, and the lamin B receptor. However, these genes have not been reported elsewhere to have testis- or germ cell-specificity. These genes play important roles in cellular functions as GTPase-activating proteins, participating in phosphorylation, as synaptic vesicle proteins, and as components of the cell membrane. However, future research will be needed to elucidate their functions and expression in chicken testis.

During embryonic development and sexual maturation, germline cells and gonads undergo a series of morphological changes including cellular differentiation.

Therefore, future investigations of the spatial and temporal expression of the testis-specific genes during embryonic development will provide more clues about

Table 1. Testis-specific TCs with tentatively annotated genes of interest.

| TC | Tentative annotation | Hit coverage | % ID | % Sim |
|---|---|---|---|---|
| TC100296 | RICS protein | 81% | 71.26 | 85.44 |
| TC106523 | PDCL2 (Fragment) | 94% | 74.55 | 89.29 |
| TC109549 | Dual-specificity phosphatase-like protein (Homo sapiens) | 61% | 76.7 | 84.47 |
| TC134123 | Testis specific serine kinase-3 (Mus musculus) | complete | 79.42 | 88.89 |
| TC107637 | Pantophysin (Synaptophysin-like protein) (Homo sapiens) | 65% | 69.23 | 80.47 |
| TC135326 | Zona pellucida binding protein | 52% | 71.27 | 84.53 |
| TC110609 | Dynein light intermediate chain 2 cytosolic (LIC53/55) (LIC-2) (Homo sapiens) | 54% | 83.71 | 91.67 |
| TC138275 | THAP domain protein 1 (Homo sapiens) | 48% | 82.35 | 94.12 |
| TC138514 | Lamin B receptor (Gallus gallus) | 30% | 98.96 | 98.96 |
| TC134235 | Doublesex and mab-3 related transcription factor 1 (Gallus gallus) | complete | 100 | 100 |

the mechanisms of formation of the genital organs and the differentiation of germ cells. To our knowledge, this is the first report using EST sequences extracted from a comparison of tissue-specific libraries and analyzing whole sequences to confirm tissue-specificity. This method might facilitate the identification of species-, tissue-, and cell-specific genes and analysis of their functions.

## 적 요

본 연구에서는 닭의 정소 및 정자에 대한 기능 유전체 연구를 위한 자원을 확보할 수 있도록 정소 특이적 유전자로 예상되는 후보 염기서열을 분석하였다. TIGR Gallus gallus Gene Index 상의 데이터베이스에서 닭의 정소에서만 나타나는 것으로 공개된 EST 염기서열을 검색하여 나온 총 292개의 서열을 선택하였으며, 이와 같이 선별된 서열들에 대하여 닭의 정소와 난소를 포함한 다양한 조직에서 전사체의 발현을 검증하였다. 결과에서, 총 292개의 염기서열 중 110개가 정소 특이적인 발현을 나타내었다. Tentative consensus sequence (TC) 상에서 집합된 EST의 수와 정소 특이적으로 발현하는 TCs의 수 사이의 상관관계는 발견되지 않았다. Gene Ontology 데이터베이스 용어를 이용하여 분류한 결과에서는 정소특이적인 TC는 닭의 전체 TC를 분류한 것과 비교하면 catalytic activity (Molecular Functionbranch)의 카테고리에 많은 수의 TC가 포함된 것으로 나타났다. 본 연구의 결과는 닭의 정소특이적 유전자에 대한 연구와 그 기능 분석을 보다 더욱 촉진시킬 수 있을 것이다.

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