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Expression and localization of VCX/Y proteins and their possible involvement in regulation of ribosome assembly during spermatogenesis

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ABSTRACT

Variable Charge X/Y (VCX/Y) is a human testis-specific gene family that localized on X and Y chromosomes. In this study, VCY protein was expressed in E. coli in the form of glutathione-S-transferase (GST) fusion protein. With the purified fusion protein as antigen, the anti-GST-VCY antibody was generated and the localization of VCY protein in human testis was determined by immunohistochemistry. In the testis seminiferous epithelium, VCY proteins were highly expressed in nuclei of germ cells. Using propidium iodide staining and green fluorescent protein (GFP) tag technologies, VCY and VCX-8r proteins were mainly localized in the nucleoli of COS7 cells. In addition, the colocalization for VCY and VCX-8r in COS7 cells was also observed. With VCY cDNA as bait, a cDNA fragment of acidic ribosomal protein PO was obtained using yeast two-hybrid system. All the information above indicates that VCX/Y protein family might be involved in the regulation of ribosome assembly during spermatogenesis.

Key words: *VCX/Y protein, spermatogenesis, nucleoli, colocalization.*

INTRODUCTION

In previous studies, a systematic search of the non-recombining regions of the human Y chromosome (NRY) identified 12 novel genes or families [1]. One of them was named Basic Protein Y1 (BPY1) for the encoded protein being rich in basic residues (Lys and Arg). Using BPY1 as probe, a serious of additional cDNA clones were isolated. Many of these clones derived from close homologues of BPY1 on the human X chromosome and they are designated as Variable Charge X (VCX). Then BPY1 was renamed Variable Charge Y (VCY). All these genes compose of a human sex-chromosomal gene family [2]. These genes are the only active human XY homologues that are both expressed exclusively in testis[3]. Expression analysis (Northern blots and RT-PCR) shows that all copies of VCX and VCY are transcribed exclusively in the testis and most likely in germ cells[2]. Homo sapiens genome view build 30, software on medline, gives the localization of VCY and VCX (VCX-2r and VCX-8r) genes in human Y and X chromosomes: Yq11.1 and Xp22.3 (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/humsrch?chr=hum-chr.inf&query) (Fig 1). The predicted VCX/Y proteins encode 125-206 amino acids with an invariant highly basic amino-terminal segment. Both of them have a putative bipartite nuclear localization signal near the N-terminus, suggesting that they are nuclear proteins. The main difference among VCX-2r, VCX-8r, VCX-10r and VCY is that the protein products of the X-linked loci have varied

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tandem iteration of an acidic ten-amino-acid motif present singly in the Y homologues.

Another study shows that VCX-A (VCX-8r) is deleted in patients with X-linked nonspecific mental retardation, and so this suggests that VCX-8r has a highly distinctive spatial and temporal expression pattern[4].

However, the precise roles of VCX/Y proteins during spermatogenesis are still unknown.

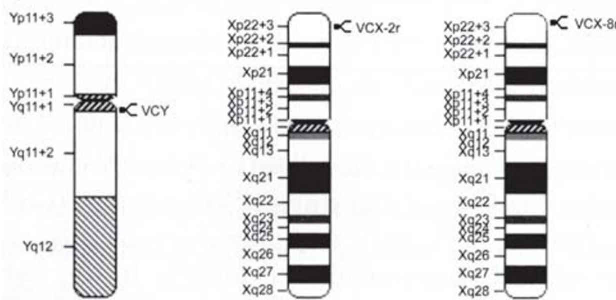

Fig 1. Diagrammatic representation of VCX/Y genes in human X and Y chromosomes.

In present studies, VCY protein was expressed in E.coli in the form of glutathione-S-transferase (GST) fusion protein. With the purified fusion protein as antigen, we immunized rabbit and the antibody of VCY-GST fusion protein was obtained. Immunohistochemical analysis was performed for the localization of VCY protein in human testis tissue. VCY cDNA was cloned into pEGFP-N1 and pDsRed1-N1 vectors. The *VCX8r* cDNA was cloned into pEGFP-N1 vector. pEGFP-N1 and pDsRed1-N1 vectors separately encodes green fluorescent protein (GFPmut1)[5] and red fluorescent protein (RFP)[6]. Genes cloned into the multiple cloning site of the vectors are expressed as fusions to the N-terminus of fluorescent protein. With GFP and RFP as fluorescent tags and propidium iodide (PI) staining as reference, localization and colocalization of VCX8r and VCY proteins in COS7 cell were identified. In addition, yeast two-hybrid screening also gave coincident result to the localization of VCX/Y proteins.

MATERIALS AND METHODS

RT-PCR clone of VCY cDNA

To make cDNA from the human testis biopsy sample, total RNA was first extracted using Trizol reagent (Gibco BRL), followed by cDNA synthesis using SuperScript™ First-Strand Synthesis System for RT-PCR (Gibco BRL). Following the RT reaction, the sample (25 μl) was diluted with 100 μl PCR mixture (final concentration: 1.5 m*M* $MgCl_2$, 0.5 m*M* Primers and 2.5 U Taq polymerase) and incubated at 94oC for 3 min before PCR, and then the cycles of amplification were performed using a Perkin-Elmer thermocycler for 35 cycles with each cycle consisting of 1 min at 94oC, 40 sec at 50°C and 50 sec at 72°C. The primers: P1 5'-GATGAGTCCAAAGCCGAGAGC-3' and P2 5'-ATCTCTGAGGTCTGGCGGCT-3'.

Expression and purification of GST-VCY fusion protein

VCY cDNA was ligated to pGEX-4T-3 (Amersham Pharmacia Biotech) to construct pGEX-4T-3-VCY vector. The recombinant plasmid was used to transform the E. coli strain BL21 (DE3). GST-VCY fusion proteins were produced in BL21 E. coli cells containing a recombinant pGEX-4T-3-VCY plasmid. Protein expression from a pGEX plasmid is under the control of the tac promoter, which was induced by 0.5 m*M* isopropyl-β-D-thiogalactoside (IPTG). The induced cultures lasted for 4-6 h and then cells were harvested and lysed by mild sonication. The bacterial lysate was cleared of cellular debris by centrifugation at 4000 g for 5 min. The GST-VCY fusions were purified by chromatography on the Glutathione Sepharose 4B column according to the manufacture's protocol.

Preparation of GST-VCY polyclone antibody

With the purified recombinant GST-VCY fusion protein as the antigen, according to a simply modified procedure[7], specific anti-GST-VCY serum was raised in one month.

Western blot

Specimens of human tissues were obtained from Hospital of Peking Union Medical College, Beijing, China. Samples (0.3 g) of human testes were homogenized in 1.5 ml of Buffer A (50 m*M* Tris-HCl, pH 7.4; 1 m*M* EDTA) to which a cocktail of protease inhibitors (4 μg/ml each of pepstatin, aprotinin, leupeptin and PMSF; Sigma) was added. The homogenate was centrifuged at 15,000 g for 20 min at 4°C. The supernatants were used and designated as testis extract.

Proteins in cell lysates containing GST-VCY fusions and testis extract were separated by SDS-PAGE using a 12% gel and transferred electrophoretically at 100 V for 1.5 h onto nitrocellulose membrane (Schleicher & Schnell). The blotted membrane was treated with a blocking solution: TBS-T solution containing 5% non-fat milk, 1% normal goat antiserum and 0.02% Tween 20, at an ambient temperature for 2 h. The membrane was subsequently further incubated at 4°C overnight with the rabbit anti-GST-VCY antiserum diluted at 1:500. After being washed, the membrane was then incubated with goat anti-rabbit IgG-AP conjugate at 1:1500 dilution (Boehringer Mannheim, Germany) at 37°C for 2 h and finally immunoreactive signals were detected with the NBT and BCIP color reaction[8].

Immunohistochemistry

Frozen sections (10 μm) of adult human testis were fixed in

Bouin's solution for 10 min, treated with 1% H_2O_2-methanol solution for 10-30 min, and blocked with 5% non-fat milk in 1% bovine serum albumin solution for 20-30 min at room temperature. Then, sections were incubated with anti-GST-VCY antiserum or normal rabbit serum (control) at 1:200 dilution at 4°C for 18 h, followed by incubation with biotinylated goat anti-rabbit IgG for 30 min and horseradish peroxidase-conjugated streptavidin at room temperature for 10 min according to the instruction of the Histostain-SP kit (Zymed Laboratory, Inc. USA). After completion of the conjugation reaction, the slides were placed in a substrate chromogen mixture (AEC) for 20-30 min and examined under a light microscope. The control was counterstained with Mayer's haematoxylin.

Construction of eukaryotic fluorescent expression vectors

Both pEGFP-N1 and pDsRed1-N1 are CLONTECH products. With the strategy of PCR and subcloning, VCY was cloned into eukaryotic expression vectors pDsRed1-N1 and pEGFP-N1 by Xho I/BamH I directional insertion. The structure of the recombinant vector, pDsRed1-N1-*VCY*, was verified by sequence analysis. With the same methods, VCX-8r was subcloned in-frame into pEGFP-N1.

Localization and colocalization of VCX/Y proteins in COS7 cell

COS7 cells, which were grown in the Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, were plated on glass coverslips at a density of 2-3$\times10^5$ cells per 35-mm dish. After attaching to the coverslips, COS7 cells were transiently transfected separately with the recombinant plasmids pEGFP-N1-VCY and pEGFP-N1-VCX-8r using the LipofectAMINE^{TM} 2000 Reagent (Invitrogen). After 24 h, COS7 cells that were grown on coverslips were fixed with 4% paraformaldehyde. After being permeated with 0.2% Triton X-100 (in PBS), the fixed cells were incubated in 100 μg/ml DNase-free RNase in PBS at 37°C for 20 min, and washed twice with PBS (pH 7.0). Cells were stained with PI (2 μg/ml in PBS) for 5 min in subdued lighting[7]. The coverslips were then soaked in PBS for 5 min followed by at least two rinses. At last, the coverslips were mounted onto glass slides using mounting medium for fluorescence. The distribution of the fluorescence was analyzed with LEICA, TCS NT laser confocal microscope.

The plasmids, pEGFP-N1-VCX-8r and pDsRed1-N1-VCY, were also used to cotransfected COS7 cells at the same time. After being fixed and mounted on slides, fluorescence of cells was observed using the confocal microscope.

Yeast two-hybrid system

The encoding region of VCY cDNA was amplified by the polymerase chain reaction (PCR) with a 5' EcoR I site introduced into the upper primers, and the lower primers corresponding to a BamH I site. After digesting with EcoR I and BamH I, VCY cDNA was inserted into the EcoR I/BamH I sites of pAS2-1 (Clontech) to construct the bait plasmid pAS2-1-VCY.

The bait plasmid was used to screen the MATCHMAKER cDNA library prepared from human testis using the MATCHMAKER Yeast Two-hybrid System 2 (Clontech). Yeast transformation and library screening were performed according to procedures provided by the manufacturer.

RESULTS

Expression and purification of GST-VCY fusion protein

GST-VCY fusions were expressed in E.coli strain BL21 (DE3). The molecular weight of the fusion protein was ~43 kD (Fig 2, lane 4). Densitometric scanning of lysate proteins prepared from the transformed cells showed that the expressed protein amounted to ~25.2% of the total protein content (data not shown). The fusion protein was located mainly in the cytoplasm, based on the electrophoretic analysis of the cell lysate fraction. The fusion protein in the cytoplasmic fraction was purified by affinity chromatography on a Glutathione-Sepharose 4B column (Fig 2, lane 5). The purity of the isolated GST-VCY protein was more than 95%, determined by densitometric scanning (data not shown).

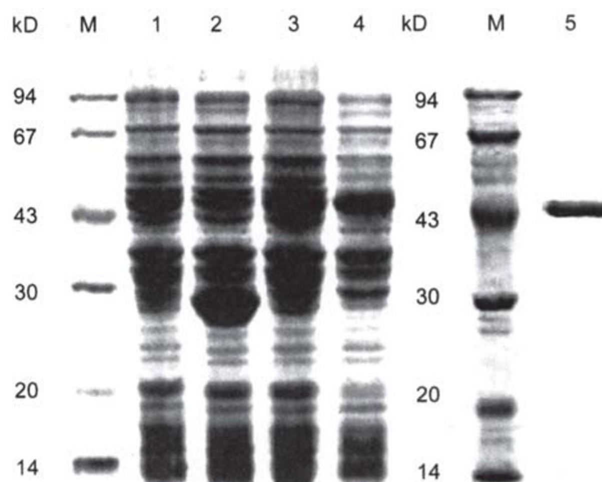

Fig 2. Sodium sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of proteins obtained from lysates from E.coli BL21 (DE3) transformed with pGEX-4T-3 (control) and pGEX-4T-3-VCY. Lane M, standard protein marker. Lane 1, pGEX-4T-3 cells incubated without isopropyl-b-D-thiogalactoside (IPTG). Lane 2, pGEX-4T-3 cells, induced with IPTG. Lane 3, pGEX-4T-3-VCY cells, incubated without IPTG. Lane 4, pGEX-4T-3-VCY cells, induced with IPTG. Lane 5, purified GST-VCY fusion protein by affinity chromatography on a Glutathione-Sepharose 4B column.

Preparation of anti-GST-VCY antibodies and immunohistochemical analysis

ELISA was used to determine the antibody titres of antiserum obtained from rabbits immunized with the purified fusion proteins expressed by pGEX-4T-3-VCY. The titres were > 1.6×10^5. Western blot analysis showed that the antiserum specifically reacted with the expressed polypeptide obtained from cells transformed with recombinant pGEX-4T-3-VCY (Fig 3, lane 1). Other immunreactive components of low mol. wt were detected in human testis extracts (Fig 3, lane 2) and they may be VCY and its homologues. After the reaction specificity of antiserum was confirmed by Western blot, immunohistochemical analysis for the localization of VCY protein in human testis was performed with anti-GST-VCY antibodies. Human testis section immunostained with anti-GST-VCY antibodies localized the antigen in the nuclei of germ cells of the seminiferous epithelium. Intense staining of spermatocyte and round spermatid occurred, and there was also some stronger staining region in some nuclei (Fig 4). While elongate spermatid were not stained. Normal rabbit serum was used in the control section.

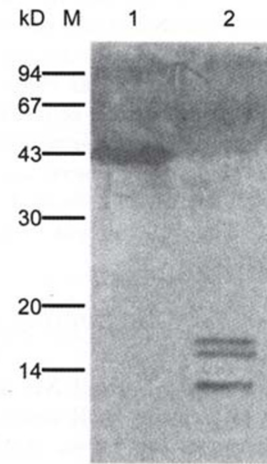

Fig 3. Immunoblot of expressed GST-VCY and testis extract stained with GST-VCY antiserum. Lane 1, cell lysates containing GST-VCY fusions. Lane 2, human testis extracts.

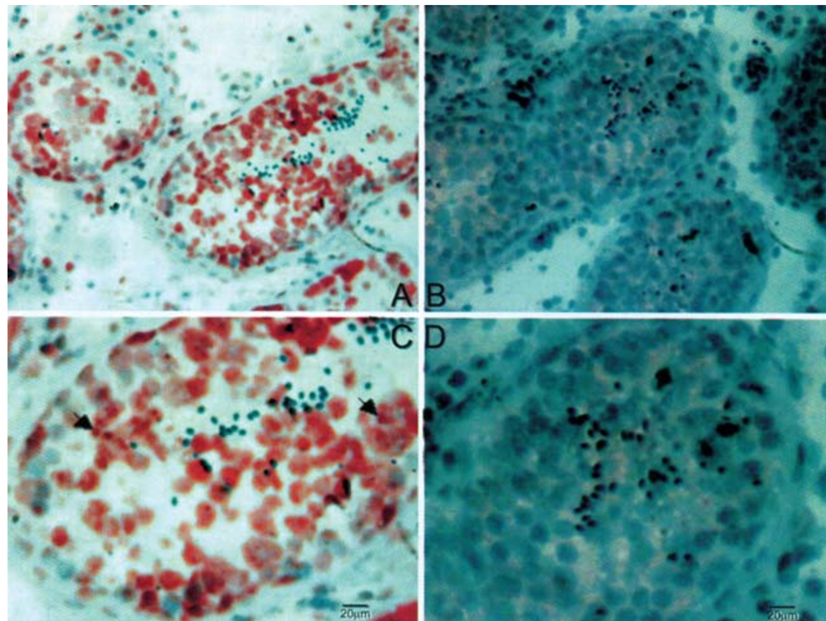

Fig 4. Immunolocalization of VCX/Y protein in germ cells of human testis. (A) and (C) Positive staining nucleus of germ cells of seminiferous epithelium with stronger staining spots in nucleus (arrowhead). (B) and (D) Control, counterstained with Mayer's haematoxylin. (Bar = 20 μm)

Localization of VCY and VCX-8r protein in COS7 cells

With the RT products as template that were obtained from total RNA of human testis tissue, VCY and *VCX-8r* cDNAs were cloned after PCR. The VCY cDNA was successfully subcloned into the plasmids pDsRed1-N1 and pEGFP-N1. We also con-

structed the conjugated *VCX-8r* plasmid with GFP at their carboxyl termini.

COS7 transformants expressing the GFP, VCY-GFP or VCX-8r-GFP fusions were fixed. To identify the major intracellular destination of these molecules, cells were co-stained with propidium iodide that binds specifically to nucleic acids. Images were observed with a confocal laser microscope (Fig 5). In cells expressing GFP that was not fused to another protein, the fluorescence of GFP dispersed throughout the cell, not concentrated in nuclei or nucleoli. However, VCY-GFP and VCX-8r-GFP fusions were concentrated uniformly in nuclei or nucleoli. The overlap of the two kinds of fluorescence of GFP and propidium iodide turned the color of nuclei yellow in the merged image. Moreover, the bright fluorescence spots of VCY-GFP and VCX-8r-GFP forming within each nucleus were also overlap with the bright spots of propidium iodide, which represent nucleoli.

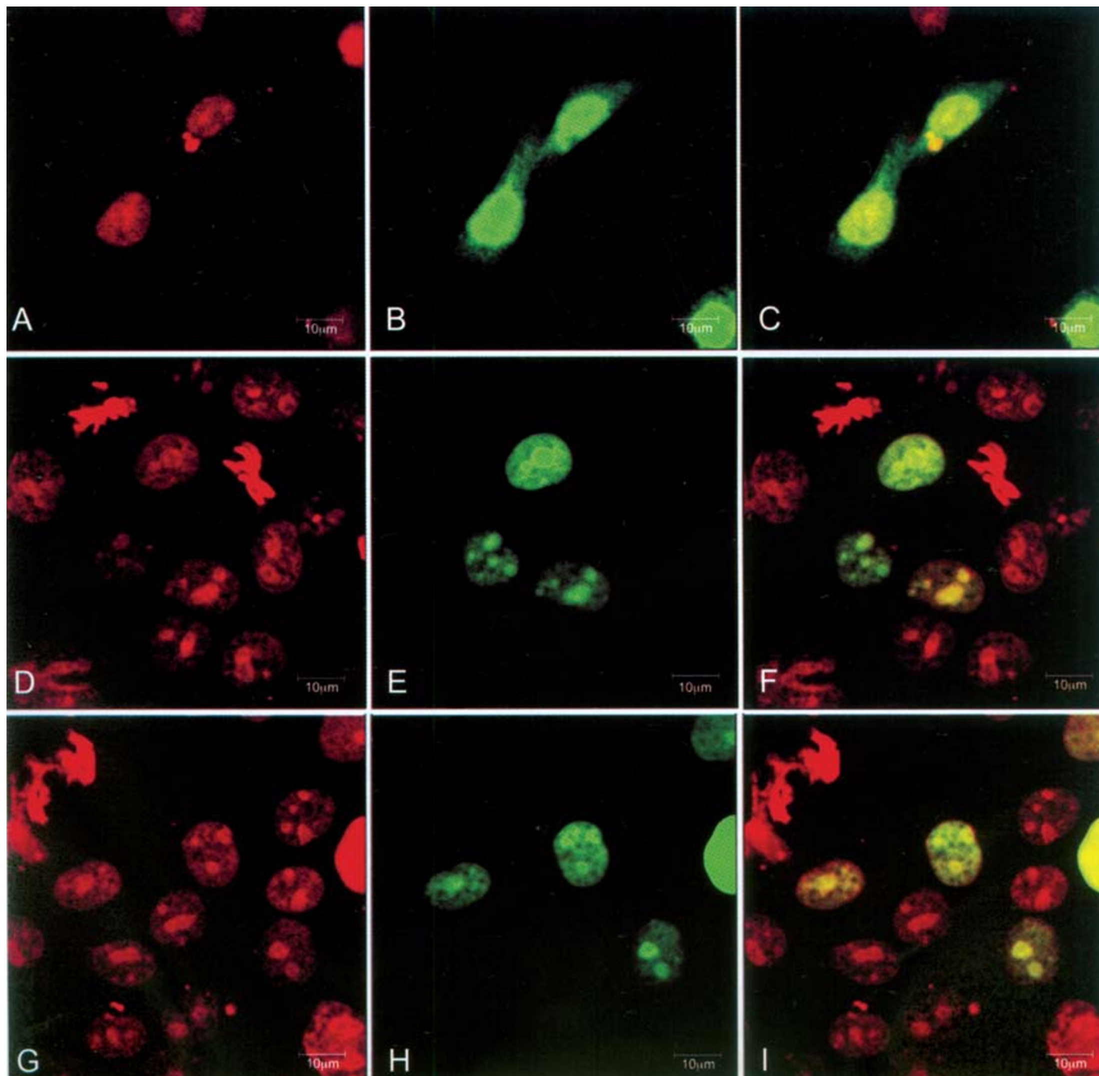

Fig 5. Localization of VCX/Y proteins in COS7 cell. DNA-staining with propidium iodide (A, D, G) and cells expressing GFP (B), VCX-8r-GFP (E) or VCY-GFP (H) proteins were observed under a confocal laser scanning microscope. These two images were merged in the right panels of each set (C, F, I) and the overlap of the fluorescences of GFP and propidium iodide showed yellow. The bright spots within each nucleus represent nucleoli. (Bar = 10 μm)

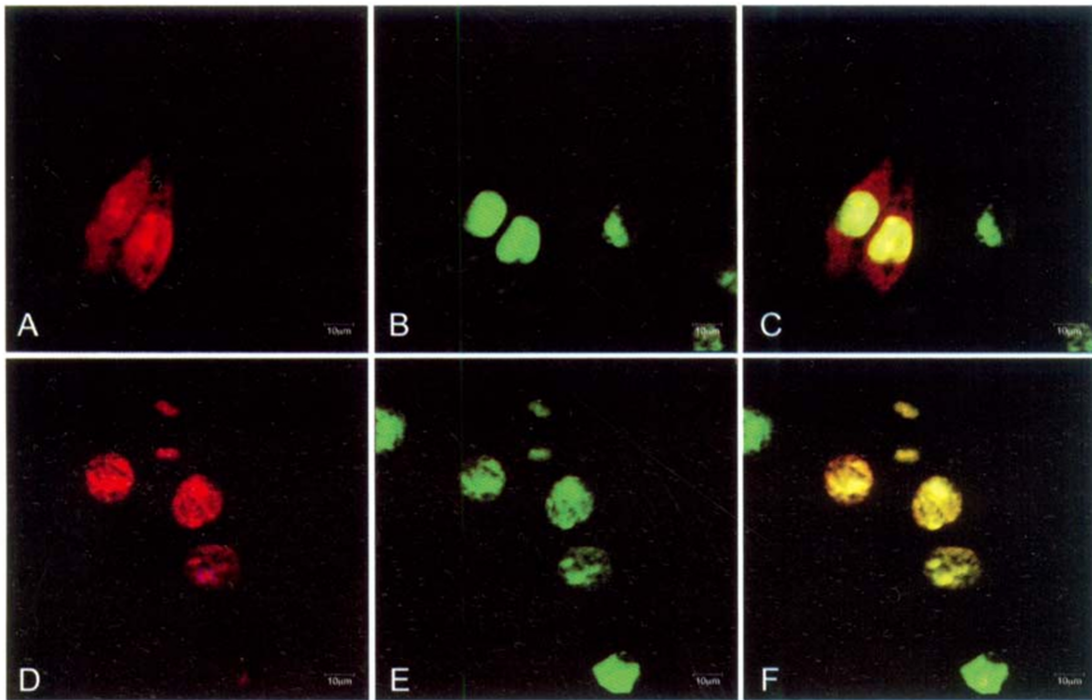

Fig 6. Colocalization of VCX/Y proteins in COS7 cells. Cells expressing RFP (**A**) or RFP-VCY proteins (**D**) and cells expressing VCX-8r-GFP (**B, E**) were observed under a confocal laser scanning microscope. These two images were merged in the right panels of each set (**C, F**) and the overlap of the fluorescences of GFP and RFP showed yellow. (Bar = 10 μm)

With the co-transfection of pDsRed1-N1 and pEGFP-N1-VCX-8r as control, the colocalization of VCX-8r and VCY protein were analysed by the co-transfection of pDsRed1-N1-VCY and pEGFP-N1-VCX-8r. According to the observed results (Fig 6), RFP is not localized in nuclei by itself but passively diffused in the cells just like the GFP. However, VCY-RFP and VCX-8r-GFP were colocalized in nuclei and nucleoli.

Yeast two-hybrid screening

From clones ($2.8 \sim 5.0 \times 10^6$) of the human testis library, we screened a range of positive colonies. After yeast mating to eliminate false positives, a positive clone was obtained. After being sequenced and evaluated by the Basic Local Alignment Search Tool (BLAST), it was identified to be a 420 bps cDNA fragment, which has high homology with 60S acidic ribosomal protein PO cDNA. The fragment encodes 44 amino acids of the C-terminus. However, the full lengthy PO protein contains 239 amino acids.

DISCUSSION

In present study, we showed the localization of VCY and VCX-8r proteins in human testis and COS7 cells. Besides of the repeat region, VCXs and VCY have high homology in amino acid sequence. So, the result of immunohistochemistry using anti-GST-VCY antibodies can represent the distribution of VCX/Y protein family. VCX/Y proteins were localized in the nuclei of spermatocyte and round spermatid of seminiferous epithelium, while vanishing during spermiogenesis. Nuclear localization can be explained by the putative bipartite nuclear localization signal near the N-terminus of VCX/Y proteins. It was reconfirmed that VCY and VCX-8r proteins are nuclear proteins by DNA-staining and confocal technology. VCX/Y protein molecules are small size and high charge, which resembles those chromatin-associated proteins, such as histones or protamines, the latter mediate condensed DNA packaging in sperm. Because of the two features, VCX/Y proteins had been speculated as components of chromatin in previous study[2, 3]. However, the results of immunohistochemistry and subcellular localization in COS7 cells

drop another hint. VCY and VCX-8r proteins' enrichment in nucleoli suggests that VCX/Y family should be nucleoli related rather than chromatin-associated proteins. Although ribosomal proteins were deemed having high false positive rate[8], yeast two-hybrid screening also gave coincident evidence to the result of subcellular localization. 60S acidic ribosomal protein PO has high homology with ribosomal P proteins that are generally considered to be associated with the 60S ribosomal subunit in eukaryotic cells[9]. Ribosomal P proteins have also been considered to be the stalk of the ribosomal 60S subunit[10]. It can be predicted that VCX/Y proteins may be involved in the regulation of ribosomal assemble during spermatogenesis, while the colocalization of VCX-8r and VCY in COS7 cell nucleoli suggests that VCX/Y protein family may form dimmer or polymer in this process. However, further investigation of VCX/Y protein family's true role in nucleoli during spermatogenesis is still needed.

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