Features of the mammalian cyclin C promoter's function

Ph. D. thesis summary

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Introduction

Cyclin C (CCNC) is an exceptionally conserved cyclin among animals which is characterized by strong pleiotropy. It takes part in the regulation of cell cycle, messenger RNA transcription and cell adhesion. It activates serine-threonine cyclin-dependent kinases (CDKs) by binding to them and together they form heterodimeric enzyme complexes. Cell biology functions of CCNC can be described mainly by its protein partners and its roles in biochemical pathways. Two mechanisms are known by which it regulates the cell cycle. Cyclin C binds to CDK2 and together they phosphorylate serine 309 residue of LSF (late *Simian vacuolating virus 40* factor) transcription factor which prevents this protein from inducing typical S-phase genes in G1. CCNC/CDK3 enzyme complex contributes to the transition to S-phase by phosphorylating pRb (Retinoblastoma) at 807/811 serines. This way it activates E2F (adenovirus E2 promoter factor) by detaching from pRb. This results in the gene expression profile alteration that eventually makes the cells pass through G0/G1/S transition. Cyclin C can either up- or downregulate gene transcription as well. These regulatory effects are elicited together with CDK8 kinase partner in the CDK subunit of the RNA transcription regulator Mediator complex. CCNC/CDK8 is proven to phosphorylate Ser-2 and Ser-5 amino acids of RNA polymerase II's CTD (carboxy terminal domain), Ser-5 and Ser-304 of TFIIH's (general transcription factor IIH) cyclin H subunit and the ICD component of Notch. Moreover, it cooperates functionally with c-Myc in cell adhesion *via* a kinase independent way. In the background, parallel activation of VCAM-1 (vascular cell adhesion molecule 1) and VLA-4 (very late antigen 4) integrins is found. Regulation of gene transcription, cell cycle and cell adhesion are core events of developmental programs. Direct and indirect evidence are also accumulating that emphasize the important role of CCNC in the development of eukaryotic organisms. It mediates the timing of slime mold's (*Dictyostelium discoideum*) early cell fate determination. It is an essential regulator in progression from the early pupal stages of *Drosophila*

melanogaster and in house mouse (*Mus musculus*) it is also found to be indispensable during the initial stages of embryonic development. In addition to fruit fly in case of mice it may also play a role in cell fate decision.

There is little known about the functions of cyclin C at the tissue/organ level. Tracking of spatiotemporal gene expression is needed in order to reveal these functions. Furthermore, factors that influence CCNC expression must also be identified. It is demonstrated *in vitro* on human cell cultures that vitamin D_3, ATRA (all-trans retinoic acid) and cAMP (cyclic adenosine monophosphate) regulate the gene's transcription thorough their corresponding nuclear elements by binding to the promoter of cyclin C. These elements are known to have their effect mostly through the first 3 kilobase of CCNC promoter but no evidence exists whether this DNS fragment is sufficient for the full physiological regulation of cyclin C's expression. There is no information on what other elements can affect this process.

Aims of the study

1. Can we identify novel elements in the CCNC promoter, that influence gene transcription in mammalian cells?
2. Is the upstream 3.6-kilobase DNA segment (consisting of a 3.4-kb promoter fragment and 173-bp 5'UTR) flanking the translation startpoint of CCNC gene sufficient to mimic the spatiotemporal expression characteristics of cyclin C?
3. Is it suitable to use this DNA fragment in a reporter system to follow the expression of the cyclin C spatiotemporally?
4. It has also been planned to determine the spatial transcription pattern of the cyclin C1 and C2 isoforms, compare it with the reporter-generated and endogenous human pattern.

In order to get answers to these questions we ligated DNA fragments of different lengths from the upstream flanking region of CCNC gene to hrGFP reporter gene. The resulting reporter constructs were used to assess their expression *in vitro* in mammalian cells and *in vivo* in transgenic mice.

Methods

1. Construction of reporter plasmids (plasmid DNA purification, restriction endonuclease and DNA ligation reactions, agarose gel-electrophoresis, DNA fragment purification, transformation of bacteria and growth on selective plate, DNA sequence design and alignment with software).
2. Maintenance and transfection of mammalian cell cultures *in vitro.*
3. Establishment of transgenic mouse model (vasectomy of male mice and generation of pseudo pregnant surrogate mothers, extraction of living zygotes from pregnant females and *in vitro* injection with micromanipulators, embryo transfer into the oviducts of pseudo pregnant female mice).
4. Genomic DNA purification from mouse tail.
5. Polymerase chain reaction (PCR), primer design and optimization.
6. Southern hybridization (alkaline transfer onto DNA-binding membrane, generation of radioactive probe, DNA-DNA hybridization, radioactive signal detection).
7. Fluorescent *in situ* hybridization (metaphasic chromosome preparation of mouse spleen white blood cells, DNA-DNA hybridization, immunodetection and signal amplification).
8. RNA purification from mouse organs and quality assessment.
9. Reverse transcription coupled quantitative real-time PCR (DNase treatment of RNA samples, cDNA synthesis, qPCR).
10. Protein extraction from mouse organs and immunoblotting.
11. Fluorescent microscopy, digital photography and image processing.
12. Transcription factor motif search with JASPAR software.

Results and discussion

We have proven that first 3.4 and 10.4 kilobases of cyclin C promoter plus 173 nucleotides of the 5'UTR are capable of eliciting expression in mammalian cells. In contrast, deletion of the first 58 base pairs and 5'UTR significantly reduces expression. We think that the loss of the first 58 nucleotides of the CCNC promoter alone (and not the loss of 5'UTR) causes this phenomenon since this region may contain the binding site of RNA polymerase II, however this has not been analyzed. Our original aim was to track the spatiotemporal expression characteristics of cyclin C. For this the already tested 3.6-kb promoter fragment – 5'UTR – hrGFP construct was used to establish four different, homozygous transgenic mouse lines. During the analyses of transgenic mice, they were turned out to be inappropriate for our original plans. Reporter transgene was found to express very weakly and its messenger RNA transcription is limited mainly and consistently to testes. However, similarly to human samples RNAs of cyclin C1 and C2 isoforms are detectable in all examined organ specimens. We also conclude that other components are needed besides the isolated CCNC promoter and 5'UTR fragment for the proper regulation of CCNC's spatial expression. In addition, we identified CRE motifs in the 3.4-kb sequence of the mouse cyclin C promoter. The first 3 kilobases of human cyclin C promoter also contains four functional CRE sites. Since testis-specific promoters were proven previously to contain more CRE motifs than other promoters, we assume that CRE binding factors significantly contribute to the maintenance of testicular expression of cyclin C as well. In summary, the demonstrated results suggest a unique, but still unknown testis-specific role of cyclin C.

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