Successive increases in human cyclin A1 promoter activity during spermatogenesis in transgenic mice

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Abstract. The human cyclin A1 gene is highly expressed in pachytene spermatocytes and is essential for spermatogenesis. To analyze mechanisms of cyclin A1 gene expression in vivo, we cloned a 1.3 kb fragment of the promoter upstream of the cDNA of enhanced green fluorescent protein (EGFP). Four lines of transgenic mice were generated that carried the transgene. Cyclin A1 promoter activity in the organs of the transgenic mice was analyzed using fluorescence microscopy and flow cytometry. Expression of EGFP was seen in male germ cells of all four murine lines. Spermatogonia at the basal membrane expressed low levels of EGFP, but bright green fluorescence was present in spermatocytes entering meiosis. Interestingly, a further sharp increase in EGFP expression was found in spermatocytes at the stage of the first meiotic division. EGFP levels stayed high thereafter and EGFP was present in mature spermatozoa. A portion of c-kit expressing cells in the testis also expressed EGFP indicating cyclin A1 promoter activity in a subpopulation of spermatogonia. These data suggest that cyclin A1 is active not only in pachytene spermatocytes but also in earlier phases of spermatogenesis.

Introduction

Cyclins regulate crucial steps in the progression of the cell cycle of somatic cells (1), and cyclins have been found to be important for the meiotic divisions during spermatogenesis and oogenesis (2). Cyclins are named for their oscillating expression throughout the cell cycle. They range in size from about 35 to 90 kDa with the sequence homology between different cyclins being concentrated in the cyclin box (3). The cyclin box is necessary for the interaction with cyclin dependent kinases (cdk), which are the main interacting partners for the cyclins. Binding of a cyclin to a cdk leads to activation of the kinase activity and determines the substrate specificity of the kinase (3). The variations in the abundance of different cyclins critically determines cell cycle progression, since cdk levels are held relatively constant during the cell cycle. Most cyclins are ubiquitously expressed, but a few show tissue-specific expression and function. For example, cyclin D2 is expressed in spermatogonia and has been shown to be involved in the pathogenesis of testicular cancer (4).

Cyclin A1 is a recently discovered second A-type cyclin that is highly expressed in testis (5). Male mice with a homozygous deletion of the cyclin A1 gene are infertile, proving the essential role of cyclin A1 in spermatogenesis (6). Besides testis, cyclin A1 is highly expressed in myeloid leukemia cells from patients as well as established cell lines (7,8). We have shown that cyclin A1 directly interacts with E2F-1, B-Myb and the RB family of proteins (9). These findings implicate a role for cyclin A1 in the progression of the mitotic cell cycle. To determine the regulation of cyclin A1 expression during spermatogenesis and in acute myeloid leukemia, we have cloned the human cyclin A1 promoter (10).

Using this promoter in vitro, we have shown that the Spl and myb family of transcription factors as well as histone acetylation status of the gene play important roles in the expression of cyclin A1 (10-12). The myb family of transcription factors influence the expression of a number of genes (13) including the proto-oncogene c-kit (14). In the mouse, c-kit is allelic with the W locus (15-17). Mice homozygous for mutations at the W locus are deficient in three classes of cells: germ cells, some hematopoietic cells (stem cells, erythroblasts and mast cells) and melanocytes (18,19). Expression of c-kit may be important for both spermatogenesis and hematopoiesis (20,21). In the testes, c-kit is
expressed in the spermatogonia and the preleptotene spermatocytes (20).

To analyze the regulation of the cyclin A1 promoter in vivo, we have generated mice that are transgenic for the cyclin A1 promoter driven expression of enhanced green fluorescent protein (EGFP). Here, we show that the human cyclin A1 promoter is active during spermatogenesis and that promoter activity increases step by step from spermatogonia to the first meiotic division and stays high thereafter.

Materials and methods

Generation of the construct and the transgenic mice. The cyclin A1 promoter-EGFP expression plasmid was constructed by inserting a 1445 bp (-1299 to +145) fragment of the cyclin A1 promoter into the BglII and HindIII sites of the promoterless EGFP-1 plasmid (Clontech). The vector sequences were removed from the cyclin A1 promoter-EGFP construct by restriction digestion and the DNA fragment was purified. Transgenic mice were generated using standard techniques (22). Fertilized eggs were harvested from the oviduct of B6D2F1 (C57Bl/6JxDBA)F1 females and the DNA was micro-injected into the pronuclei of fertilized eggs. The injected eggs were incubated in KSOM (Speciality Media) at 37°C, 5% CO₂ and then were transferred to the oviduct of pseudopregnant females. About 10% of the offspring had the transgene. PCR and Southern blot analysis confirmed the presence of the transgene. The expression of the transgene did not impair either survival or fertility of the mice.

Preparation of cells for flow cytometric analysis and cell sorting. The tunica was removed from the testis and the tubules teased out and spread in a 5% collagenase solution in HEPES buffered modified HTF medium (Irvine Scientific) for 15 min at 37°C. The tubules were lifted into 1 ml/testis of enzyme mixture (containing 1% trypsin, 0.5% collagenase, 1% DNAse) and minced using sterile needles. The minced tubules were incubated at 37°C for 15 min. The undigested tubules were allowed to settle and the cell suspension was aspirated and washed three times by centrifugation at 2000 rpm and resuspended with 6 ml of modified HTF. The cells were resuspended in 1 ml HTF and passed through a nylon mesh. Monoclonal antibodies directed against murine surface antigens were purchased from PharMingen. Antibody staining was carried out according to the recommendations of the manufacturer.

Southern blotting of the transgene. Mouse tail DNA (10 μg) was digested overnight with HindIII and XhoI restriction enzymes. Digests were loaded onto an agarose gel, electrophoresed and blotted onto a positively charged nylon membrane. Non-radioactive hybridization was performed with a digoxigenin random labeled probe derived from the EGFP cDNA. Hybridization products were detected by digoxigenin antibodies coupled to alkaline phosphatase (Boehringer, Mannheim). Chemiluminescence (CDP-Star, Tropix, Bedford, MA) was recorded on film.

Sample preparation and microscopy methods. Organs were dissected and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) buffer pH 7.4 for 48 h at 4°C. The tissue was washed three times with cold PBS and placed in 20% sucrose in PBS for 24 h at 4°C. The tissue was frozen in OCT and 8 μm sections were cut on a cryostat. Prior to viewing, the sections were brought to room temperature and layered with PBS and covered with a coverslip. Confocal microscopic analyses were performed using a Zeiss LSM 410 or a Leica TCS/SP system. Excitation and emission wavelengths used were in the narrow band, at appropriate recommended ranges, in order to record EGFP-specific signals.

Results

In transient transfections in tissue culture, the cyclin A1 promoter was active in all cell lines analyzed. These findings were contrary to the highly restricted cyclin A1 expression in vivo. To analyze whether the isolated 1.3 kb of the human cyclin A1 promoter would be sufficient to direct tissue-specific expression of cyclin A1, we generated mice that were transgenic for a cyclin A1 promoter-EGFP construct (for details see Materials and methods). Four founder lines were obtained that harbored the transgene as determined by Southern blotting (Fig. 1). Frozen sections from organs of the transgenic mice were analyzed by fluorescence microscopy. All transgenic lines expressed EGFP in the testis, and the patterns of expression were consistent with those obtained in tissue culture. The expression of EGFP was found in interstitial Leydig cells. Leydig cells exhibited autofluorescence of a yellow hue that was characteristic of the human Leydig cells (Fig. 2a). Confocal microscopy (Fig. 2b-f) was used to analyze expression during spermatogenesis in greater detail. Low levels of expression were found in early spermatogonia located at the basal membrane. EGFP expression increased in two successive steps reaching a maximum of expression during the stage of the first meiotic division. These stepswise increases were evident when testes sections of young males (11-day-old) were used (Fig. 2f). Also, EGFP expression remained high even in mature spermatozoa (Fig. 2e).
Figure 2. Expression of EGFP in male germ cells by microscopy. Frozen sections were obtained from testis of transgenic or control mice and analyzed by either (a) conventional fluorescence microscopy or (b-f) by confocal laser scanning microscopy. (a) Shows a section through the testis of a control mouse and the small, inserted picture depicts expression of EGFP in a transgenic animal. Please note, the 'yellowish' green of the Leydig cells differed from the EGFP color. These differences were not seen by analysis with the confocal microscope; and interstitial cells, therefore, appear green in (b) and (c). (b), Expression of EGFP in testis of a transgenic mouse, magnification x40. (c), EGFP expression during spermatogenesis increased stepwise (x100). (d), Very low expression was seen in type A spermatogonia located at the basal membrane. The figure shows a higher magnification of the area indicated by the white box in (c). (e), Expression of EGFP was detected in all stages subsequent to the first meiotic division. Shown here are mature spermatocytes. The lumen of the seminiferous tubulus is located on the right side of the picture. EGFP expression appears to be excluded from the nucleus of the mature sperm. (f), EGFP expression in testis of a 11.5-day-old mouse. No mature sperm were seen, but three different degrees of cyclin A1 promoter activity (none, weak, strong) were observed.
Further analysis of the EGFP expressing cell types was performed using two-color flow cytometry. A single cell suspension from an adult transgenic mouse testis was prepared and stained with anti-c-kit antibody labeled with phycoerithrin (Fig. 3). Interestingly, the majority of c-kit expressing cells also expressed EGFP. On the other hand, a subpopulation of c-kit expressing cells did not show cyclin A1 promoter activity.

The analyses of somatic tissues revealed that EGFP was not expressed in heart, lung, gut, skin, muscle, liver, brain or in blood vessels (data not shown). Also, no detectable expression was seen in the ovary of adult females. One transgenic murine line showed expression of EGFP in a subpopulation of cells in the kidney, in bone marrow, spleen, thymus and blood. Flow cytometry revealed that many of these cells showed a B cell phenotype (CD19+, CD45R/B220+, CD117−, CD5+, CD45R/B220−, GL7+). No significant expression of the endogenous cyclin A1 was found in mature B cell populations that were sorted by flow cytometry and analyzed by RT-PCR (data not shown). The transgene in this murine line remained non-methylated in all tissues analyzed (12). None of the other three transgenic lines expressed EGFP outside the testis.

Discussion

In the current study, we have analyzed the activity of the cyclin A1 promoter in vivo by generating mice that were transgenic for a cyclin A1 promoter-EGFP construct. The EGFP reporter was chosen for several reasons. First, EGFP can be easily detected in frozen sections without the need to manipulate the slides further. Second, no non-specific fluorescence occurs due to antibodies binding to unrelated targets or absorption by Fc-receptors. Third, EGFP can be visualized not only by fluorescence microscopy but also by flow cytometry. Flow cytometry allows the use of double- or triple-staining of cells and thus enables a far more detailed analysis of the phenotype of single cells than other methods. The transgenic murine lines were initially used to analyze the role of CpG methylation in directing tissue specific activation of the cyclin A1 promoter (12).

Our data indicated that the isolated 1.3 kb cyclin A1 promoter fragment contained all necessary elements to direct specific expression of EGFP during spermatogenesis. While early A-type spermatogonia expressed low levels of EGFP, cyclin A1 promoter activity increased stepwise during spermatogenesis and reached a maximum of activity during the first meiotic division. Strong green fluorescence was detected in all subsequent stages of spermatogenesis and high levels of expression of EGFP were found in mature spermatozoa. However, assessment of cyclin A1 promoter activity in mature sperm is difficult, since EGFP has a relatively long half-life, and the protein might be present in the absence of promoter activity. No expression of EGFP was found in the interstitial Leydig cells confirming the specificity of the cyclin A1 promoter. Our findings regarding cyclin A1 promoter activity during spermatogenesis are in agreement with the previously reported immunohistochemistry and in situ-hybridization data (5,23). However, the successive increases in cyclin A1 promoter activity have not been reported previously. Also, promoter activity appears to be more widespread during spermatogenesis than cyclin A1 protein levels as detected by immunohistochemistry (23). One possible explanation might be that the isolated promoter fragment used to generate the transgenic mice is less stringently controlled than the endogenous genomic region of the cyclin A1 gene. On the other hand, the cyclin A1 promoter was adequately repressed in almost all tissues that do not express cyclin A1. In addition, endogenous cyclin A1 expression as measured by Western blot analysis of flow cytometry sorted cell populations exactly matched the EGFP expression pattern (12). The most likely explanation for the observed differences is that the successive increase in cyclin A1 promoter activity and subsequent cyclin A1 expression become so strong that the weak expression in earlier stages is easily missed. Detection of EGFP by confocal microscopy may also be more sensitive than histochemical methods. We propose that cyclin A1 promoter activity albeit weak is present in earlier stages of spermatogenesis than previously reported. Expression probably starts when cells enter the prophase of the first meiotic division.

Another interesting finding of our study is that the expression of c-kit appears to be concomitant with cyclin A1 promoter activity in the testis. The majority of c-kit expressing cells in the testis also expressed EGFP. Expression of c-kit has been shown to be important in spermatogenesis as well as in hematopoiesis (23,24).

Expression of EGFP in hematopoietic progenitors and in lymphocytes did not match expression of the murine endogenous cyclin A1. However, we have previously found that human hematopoietic progenitors do express cyclin A1 (7). Therefore, the expression pattern of the murine and human cyclin A1 may differ in somatic tissues. Further analyses are necessary to clarify this point.

Taken together, we have shown that a 1.3 kb fragment of the cyclin A1 promoter is sufficient to direct testis-specific expression in vivo. Cyclin A1 promoter activity increases during spermatogenesis in successive steps. The use of EGFP as a reporter gene has enabled us to isolate and analyze cyclin A1 expressing cell populations by flow cytometry. More than half of the c-kit expressing spermatogonia also expressed EGFP.

Figure 3. Two-color flow cytometry identifies EGFP expression in c-kit expressing spermatogonia. Testis cells were enzymatically disaggregated and stained with PE labeled anti-c-kit antibody. The left dot plot shows staining with an irrelevant control antibody. Simultaneous expression of c-kit and EGFP is shown in the right dot plot. About 70% of the cells in the testis expressed EGFP. Expression of c-kit was detected on the surface of cells in EGFP-expressing and non-expressing populations.
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References