Quantitative Chromatin Immunoprecipitation Using the LightCycler® 480 System

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Introduction
Antigen processing and presentation play a critical role in immune surveillance. Peptides derived from self, viral, or tumor-associated antigens are generated in the cytoplasm by the proteasome. Transporter associated with antigen processing (TAP), a member of the ATP-binding cassette (ABC) transporter family, functions by transporting these peptides from the cytoplasm to the lumen of the endoplasmic reticulum (ER), where each peptide forms a ternary complex with beta-2 microglobulin (ß2m) and major histocompatibility complex (MHC) class I heavy chain. These complexes are then transported to the cell surface and recognized by cytotoxic T lymphocytes (CTLs), which eventually kill cells that present non-self antigens.

Studies have shown that metastatic cancer cells have defects in components of the antigen presentation pathway that allow them to evade immune surveillance. Absence or downregulated expression of TAP-1 molecule is a feature common to many tumors that impairs the ternary complex formation in the lumen of ER. This results in a lack of tumor antigen presentation on the cell surface by MHC class I. As a consequence, specific CTLs are unable to recognize and destroy the malignant cells.

The mechanisms underlying TAP-1 deficiency in tumor cells has not been fully elucidated. Several studies reported that TAP-1 down-regulation in many cancer cells occurs at the mRNA level, suggesting possible defects in the transcriptional process. Recruitment of the RNA polymerase (pol) II complex to gene promoters is an important event in transcription initiation [1]. In this study, we tested the hypothesis that the impairment of TAP-1 transcription is due to the lack of RNA pol II recruitment to the TAP-1 promoter. By chromatin immunoprecipitation (ChIP) assay and real-time PCR, we compared the levels of RNA pol II recruitment to the TAP-1 promoter in TAP-expressing and in TAP-deficient cells. Furthermore, we aimed to investigate the effects of drug treatments on the levels of RNA pol II recruitment to the TAP-1 promoter.

Real-time quantification of ChIP products has several advantages over the more commonly used semi-quantitative PCR method. The quantitative real-time method allows greater accuracy in determining fold differences between products than estimation of the intensity of bands on gel. The LightCycler® 480 System allows experimenters to run and analyze up to 96 samples simultaneously. Data from independent experiments can then be pooled and presented all together.

Materials and Methods
Cell lines and reagents
A primary, TAP-expressing murine tumor cell line (TAP+) and its metastatic, TAP-deficient derivative (TAP-) were grown in DMEM media supplemented with 10% heat-
inactivated fetal bovine serum (FBS). Each cell population was divided into three groups: treated with drug X, treated with drug Y, or left untreated.

**Chromatin immunoprecipitation assays**

Chromatin immunoprecipitation experiments using 20 million cells per sample were done as previously described [2]. Protein A Sepharose CL-4B beads (Amersham Biosciences) and 5 µg of anti-RNA pol II (N-20, sc-899, Santa Cruz Biotechnology Inc.) polyclonal antibody (Ab) were used for immunoprecipitation. Mock co-precipitations without antibody were performed for each sample, in parallel with the co-immunoprecipitations, to detect any non-specific co-precipitation of genomic DNA with Protein A Sepharose beads. Levels of endogenous TAP-1 promoter co-immunoprecipitating with the antibody from each sample were quantified by real-time PCR using primers specific for the 3’-end of the TAP-1 promoter: TTCTTCCTCTAAACGCCAGCA (forward); CGAGCTGAGCTGTCGAGTAGCT (reverse). Serial dilutions of plasmid containing the murine TAP-1 promoter [3] were amplified following the same protocol to generate a standard curve (Ct vs. log [copy number]). Copy number of each ChIP product (eluate, background (without antibody) and input) was therefore determined using the following formula: ((Ct – y-intercept)/(slope)). The background product was then subtracted from the eluate. Relative RNA pol II levels were determined as the ratio of copy numbers of the eluted TAP-1 promoter and copy numbers of the corresponding inputs (total amount of DNA present in different samples).

**Real-time quantitative PCR analysis**

Purified genomic DNA was used as template for amplifications using 500 nM of each primer and 1 µl SYBR Green I PCR Master Mix in a 10 µl total reaction mixture. Forty cycles of denaturation (5 seconds, 95°C), annealing (5 seconds, 61°C), and elongation (20 seconds, 72°C) were performed using a LightCycler® 480 Instrument.

**Results and Discussion**

The recruitment of RNA pol II to the TAP-1 promoter is lower in the TAP-deficient cells than in the TAP-expressing cells.

In order to investigate whether the lack of TAP-1 mRNA expression in TAP-deficient cells is due to an impairment of TAP-1 transcription, we compared the levels of RNA pol II within the TAP promoter in TAP-deficient (TAP-) and TAP-expressing (TAP+) cells by means of ChIP. We found that the level of RNA pol II recruitment to TAP-1 promoter of TAP- cells was approximately 2.5 times lower than in TAP+ cells (Figure 1), indicating that deficiency in transcription is, at least partially, underlying TAP deficiency in the metastatic cancer cells.

Drug X significantly enhanced the levels of RNA pol II recruitment to TAP-1 promoter of TAP-deficient cells, while drug Y increased the levels in both TAP-expressing and TAP-deficient cells.

We further tested the effects of 2 drugs, X and Y, in RNA pol II recruitment to TAP-1 promoter of TAP+ and TAP- cells. We found that 48 hours of incubation of TAP- cells with drug X prior to ChIP analysis enhanced the recruitment of RNA pol II complex to the TAP-1 promoter, up to levels similar to those in TAP+ cells (Figure 2). Furthermore, 48 hours of treatment of both the TAP+ and TAP- cell lines with drug Y significantly increased the levels of RNA pol II recruitment to TAP-1 promoter by 3- and 8-fold, respectively (Figure 2). These results indicate that drug Y is a more potent inducer of TAP-1 promoter activity than drug X, although treatment with drug X is sufficient to restore the level of binding of RNA pol II to the TAP-1 promoter in this particular TAP- cell line.

**Conclusions**

We were able to run multiple ChIP samples and analyze the copy numbers of the eluates, the backgrounds (no antibody) and the inputs, simultaneously using the LightCycler® 480 System. This system enabled us to do quantitative comparisons of the levels of protein
binding to a specific DNA sequence between multiple cell lines, as well as between untreated and treated samples. For the drug treatment experiment described above, there were a total of 18 samples to be analyzed at the same time (2 cell lines, each was treated with 2 different drugs or left untreated, and each sample was divided into eluate, background and input). If we were to add one additional cell line into the experiment, this would mean an addition of 9 samples; therefore, the total number of samples to analyze would quickly add up to 27 with all controls included. The LightCycler® 480 System allows us to do quantitative, multiple ChIP analyses in a time- and cost-effective manner.

Acknowledgments
We thank Robyn Seipp for critical review of the manuscript.

References

Product | Pack Size | Cat. No.
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LightCycler® 480 Instrument 1 instrument (96 well) | 04 640 268 001
1 instrument (384 well) | 04 545 885 001