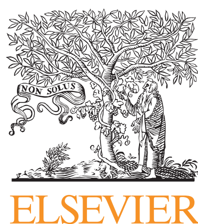
EXPERIMENTAL CELL RESEARCH 319 (2013) 3160–3169



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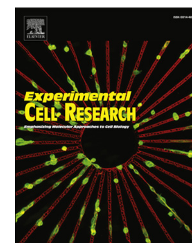

Research Article

Chromatin dynamics at the hTERT promoter during transcriptional activation and repression by c-Myc and Mnt in Xenopus leavis oocytes ☆

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ARTICLE INFORMATION

Article Chronology:
Received 30 April 2013
Received in revised form
3 July 2013
Accepted 5 July 2013
Available online 13 July 2013

Keywords:
c-Myc
Mnt
Chromatin remodeling
Transcription
hTERT promoter
Histone modification

ABSTRACT

The transcription factors c-Myc and Mnt regulate gene expression through dimerization with Max and binding to E-boxes in target genes. While c-Myc activates gene expression via recruitment of histone modifying complexes, Mnt acts as a transcriptional repressor. Here, we used the *Xenopus leavis* oocyte system to address the effect of c-Myc and Mnt on transcription and chromatin remodeling over the E-box region in the *human telomerase reverse transcriptase (hTERT)* promoter. As expected we found elevated and decreased levels of *hTERT* transcription upon exogenously expressed c-Myc/Max and Mnt/Max, respectively. In addition, we confirmed binding of these heterodimers to both E-boxes already enriched with H3K9ac and H4K16ac. These chromatin marks were further enhanced upon c-Myc/Max binding followed by increased DNA accessibility in the E-box region. In contrast, Mnt/Max inhibited Myc-induced transcription and mediated repression through complete chromatin condensation and deacetylation of H3K9 and H4K16 across the E-box region. Importantly, Mnt was able to counteract c-Myc mediated activation even when expressed at low levels, suggesting Mnt to act as a strong repressor by closing the chromatin structure. Collectively our data demonstrate that the balance between c-Myc and Mnt activity determines the transcriptional outcome of the *hTERT* promoter by modulation of the chromatin architecture.
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Introduction

Myc exerts its biological functions by regulating genes important for cellular processes such as cell growth and division, cell cycle progression, apoptosis, and differentiation. In human cancer, c-*Myc* is the most frequently deregulated oncogene [1]. The c-Myc protein as well as Mxd and Mnt belongs to the family of basic-helix-loop-helix-leucine-zipper (bHLHZip) transcription factors

Abbreviations: Ac, acetylation; AU, arbitrary units; bHLHZip, basic helix loop helix leucine zipper; bp, basepair; ChIP, chromatin immunoprecipitation; DMS, dimethylsulfate; DNase I, deoxyribonuclease I; dsDNA, double stranded DNA; EMSA, electrophoretic mobility shift assay; HAT, histone acetylase; HDAC, histone deacetylase; HEK293T, human embryonic kidney 293T; *hTERT*, *human telomerase reverse transcriptase*; ssDNA, single stranded DNA; WB, Western Blot.

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http://dx.doi.org/10.1016/j.yexcr.2013.07.004

and binds specifically to E-box sequences, CACGTG, as heterodimers with Max [2]. c-Myc/Max complexes bind preferentially to promoters enriched for euchromatin marks such as H3K4me2, H3K4me3, H3K79me2, H3K9ac, H3K18ac and H3K27ac [3–6]. In addition, Myc was found in genomic regions containing CpG islands, which generally are associated with transcriptionally active promoters [7]. Myc was recently shown to accumulate in the promoter regions of active genes where it acts as an amplifier of expression rather than being an on-off specifier of gene activity [5,6]. However, the on-off switch might be controlled by c-Myc indirectly since c-Myc interacts with 10–15% of human promoters (reviewed in [8]).

The accessibility of the chromatin is crucial for gene expression. Nucleosomes, consisting of DNA wrapped around the histone octamer (histone H3, H4, H2A and H2B), are packed into higher order chromatin structures restricting protein binding to DNA. Thus, local modulation of DNA accessibility influences the transcriptional outcome [9]. Once c-Myc/Max is bound to the promoter additional histone modifications are induced by recruitment of various cofactors including histone acetyltransferases (HATs) Tip60 [10], GCN5/PCAF [11,12] and p300/CBP [13], HAT associated proteins such as Transactivation/transformation domain associated protein (TRRAP) [11,14], members of the SWI/SNF chromatin remodeling complex (SNF5 and BRG1) (reviewed in [15–17]), histone methyltransferase mixed-lineage leukaemia 1 (MLL1) [18] and ATPase/helicase TIP48 and TIP49 ([19,20].

Myc induced transactivation is antagonized by binding of Mxd/Max or Mnt/Max heterodimers to the same E-box sequence resulting in establishment of an inactive chromatin conformation through recruitment of mSin3 containing corepressor complexes possessing histone deacetylase (HDAC) activity [21,22]. HDACs remove acetyl groups from histones, leading to decreased histone acetylation, chromatin condensation and, hence, transcriptional repression [23].

We have previously analyzed the promoter of the c-Myc/Mxd/Mnt target gene *human telomerase reverse transcriptase* (*hTERT*), and found a switch from c-Myc/Max to Mad1(Mdx1)/Max binding during differentiation of human promyelocytic leukemia (HL60) cells resulting in decreased promoter histone acetylation followed by reduced *hTERT* expression [24]. In contrast to c-Myc and Mxd, which are expressed in proliferating and differentiating cells, respectively, Mnt is ubiquitously expressed and Mnt/Max complexes are present at the promoter during both cellular stages [24,25]. However, we found that Mnt mediated transcriptional repression is inhibited through phosphorylation of the protein in proliferating cells [25]. Furthermore, since Mnt has been suggested as a key regulator of the Myc network as well as a potential tumor suppressor (reviewed in [26]) we wanted to study how c-Myc and Mnt regulate gene expression by remodeling of the chromatin structure across the E-box region in the *hTERT* promoter. To this end, we reconstituted the promoter in the *Xenopus leavis* oocyte system [27]. Oocytes from the African clawed frog are large cells allowing cytoplasmic microinjection of *in vitro* synthesized *c-Myc*, *Max* or *Mnt* mRNA(s) and nuclear injection of the corresponding expression vector(s) [28]. Reporter DNA containing the *hTERT* promoter is provided by injection into the oocyte nucleus where it undergoes chromatin assembly within a few hours. In this way, the interaction between newly assembled chromatin of the *hTERT* promoter and c-Myc/Max or Mnt/Max as well as chromatin dynamics during transcriptional activation/repression by c-Myc and Mnt can be analyzed at high precision.

Materials and methods

Ethics statement

Ethic permissions N61/09, #C10/10 and N21/12 were approved by the Stockholm regional ethical committee for animal research.

Xenopus oocyte microinjection and plasmids

Defolliculated stage VI *Xenopus laevis* oocytes were prepared by collagenase treatment [29]; microinjections of DNA and mRNA into the oocytes were performed as described previously [30]. The *c-Myc*, *Max*, *Mnt* cDNAs were cloned between the BamH1/Not I sites in the RN3P vector, used for mRNA production as described [31]. The plasmids were linearized with Acc651 and *in vitro* transcribed using the mMESSAGE mMACHINE kit (Ambion). Obtained mRNAs were purified on a spin column (MEGAclear, Ambion). Indicated amount of *c-Myc*, *Max* or *Mnt* mRNA were injected in 23 nl into the cytoplasm. 5 to 7 h after mRNA injection, 3 ng single stranded (ssDNA) or double stranded (dsDNA) of the *hTERT* promoter plasmids *hTERT* A (p1009, [32]), *hTERT* B (p330, [32]), *hTERT* C (pBT-255, [33]), or minM4tkLuc [34] in 18.4 nl were injected into the oocyte nucleus. For expression of the *hTERT* containing reporters as ssDNA, −1009/+360 bp fragment (*hTERT* A) or −330/+360 bp fragment (*hTERT* B) were cloned into M13-mp19 bacteriophage vector using KpnI and SacI. For some experiments DNA expression vectors for c-Myc (pSp-*Myc*), Max (pSp-*Max*), Mnt (pRc-*Mnt1A*) were used to express the corresponding protein. In this case the plasmids were co-injected into the nucleus together with the *hTERT* or minM4tkLuc reporter. Injected oocytes were incubated in OR2 medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES pH 7.8) containing 10 μg/ml gentamicin (Sigma-Aldrich) at 19 °C for 24 h before analysis (Fig. SI1A). Pools of 7–10 injected oocytes of each group were used and all experiments were performed in duplicates or triplicates.

Analysis of intracellular amounts of exogenously expressed proteins

Xenopus oocytes injected with c-*Myc*, *Max*, *Mnt* mRNA, or expression vectors for Mnt or Max were incubated in OR2 medium containing ^{14}C-Lysine. 24 h post injection the oocyte nuclei were manually dissected in nuclear isolation buffer (20 mM Tris–HCl pH 7.5, 0.5 mM $MgSO_4$, 140 mM KCl) and homogenized in 10 mM Tris–HCl pH 7.5 containing complete protease inhibitor cocktail (Roche). Protein extracts were separated in 8 or 10% SDS PAGE followed by Western blot or by autoradiography of the dried gels. Western blotting was performed as previously described [35]. Membranes were probed with anti-c-Myc antibody (N262), anti-Max (C17) or anti-Mnt (M132), all from Santa Cruz Biotechnology Inc, or anti-actin (A2103) from Sigma. HRP-conjugated anti-rabbit antibody (Amersham Biosciences) was used as secondary antibody. Membranes were developed by enhanced chemiluminescence (ECL, Amersham).

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