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CHROMATIN STRUCTURE AND HISTONE MODIFICATIONS IN GENE REGULATION

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ABSTRACT

In the living cell, DNA is densely packed into a chromatin structure constituting nucleosomal arrays. One nucleosome core particle includes a disc shaped protein octamer consisting of pairs of histones H2A, H2B, H3 and H4. 146bp of DNA is wrapped in almost two turns around this protein complex. The N-terminal tails of the histone proteins protrude out from the nucleosome core. These tails are highly flexible and are targets for multiple covalent modifications coupled to gene regulatory events. A linker histone H1 binds at the DNA entry/exit site of the nucleosome and organises the linker DNA and influence higher order chromatin structure. In order to induce gene expression the transcription factors, coactivators and also the transcriptional machinery has to get access to the DNA. This is obtained by modulation of the chromatin structure by specific energy-dependent chromatin remodelling complexes that has the ability to locally unfold the chromatin. In addition, nucleosomes may be positioned to expose specific regulatory DNA segments.

The hormone-activated glucocorticoid receptor (GR) driven mouse mammary tumor virus (MMTV) promoter, fused to a thymidine kinase reporter gene was injected into *Xenopus laevis* oocytes. This system was used for studies on chromatin structure coupled to gene regulation. Specific histone acetylations at H3K9, H3K14 and H4K16 were found to correlate with hormone-induced chromatin remodelling but preceded the transcription step. These histone modifications were reversible upon sequential treatment of a hormone antagonist, RU486, which correlated with the disruption of chromatin opening event. Hormone-induction was also found to mediate transient trimethylation of H3K4 during the initiation phase of transcription.

Hyperacetylation of histones by Trichostatin A (TSA) mediated complex effects on the chromatin structure. TSA induced a general relaxation of chromatin and a specific rearrangement of nucleosomes in the distal part of the MMTV promoter. This correlated with an enhanced basal transcription. Hormone-activation was impaired by TSA treatment and this correlated with topological changes and an overall increased sensitivity to MNase digestion.

Nuclear factor 1 (NF1) was shown to bind to the non-induced MMTV promoter. Upon hormone induction, NF1 and hormone-activated GR formed an enlarged MNase-resistant structure that may function as a binding platform for general transcription factors. NF1 and octamer transcription factor 1 (Oct1) expressed together were found to cooperatively bind and to induce partial nucleosome positioning. This resulted in a faster and stronger hormone response and this also correlated with a faster induction of trimethylation of H3K4. Together this serves as a functionally MMTV chromatin presetting.

Linker histone H1 was expressed and incorporated into the MMTV chromatin where it dissociated specifically from the active locus upon hormone-induced remodelling. A subsaturated level of H1-specific nucleosome binding correlated with enhanced binding of hormone-activated GR and also with increased transcription. At this specific H1 concentration, a reduction of the general DNA access was seen thus indicating an H1-concentration dependent rearrangement of the chromatin fibre. Together this support a mechanism where H1-concentration dependent stimulation of GR binding is achieved through GR-induced disruption of H1 at active locus resulting in preferential H1 masking of the non-specific DNA.

LIST OF PUBLICATIONS

This thesis is based on the following papers that will be referred to in the text by their roman numerals:

I. **Carolina Åstrand**[1],Tomas Klenka[1], Örjan Wrange and Sergey Belikov
Trichostatin A reduces hormone-induced transcription of the MMTV promoter and has pleiotropic effects on its chromatin structure. *Eur.J.Biochem.*, 271, 1153-1162 (2004)

II. Sergey Belikov, **Carolina Åstrand**, Per-Henrik Holmqvist and Örjan Wrange
Chromatin-mediated restriction of Nuclear Factor 1/CTF binding in a repressed and hormone-activated promoter in vivo. *Molecular and Cellular Biology*, 24, 3036-3047 (2004)

III. Sergey Belikov, Per-Henrik Holmqvist, **Carolina Åstrand** and Örjan Wrange
Nuclear Factor 1 and Octamer Transcription Factor 1 binding preset the chromatin structure of the Mouse Mammary Tumor Virus promoter for hormone induction. *Journal of Biological Chemistry*, 279, 49857-49867 (2004)

IV. Sergey Belikov, **Carolina Åstrand** and Örjan Wrange
A subsaturating level of histone H1 enhances DNA binding of the hormone activated glucocorticoid receptor in vivo. Submitted

V. **Carolina Åstrand**, Sergey Belikov and Örjan Wrange
Histone modification and H1 binding to the inactive, preset and hormone-activated chromatin in the MMTV promoter. Manuscript

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LIST OF ABBREVIATIONS

bp	base pairs
ChIP	chromatin immunoprecipitation
DMS	dimethylsulfate
Fox	forkhead box
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HAT	histone acetyl transferase
HDAC	histone deacetylace
HMT	histone methyl transferase
MMTV LTR	mouse mammary tumor virus long terminal repeat
MNase	micrococcal nuclease
MPE	methidiumpropyl EDTA
mRNA	messenger RNA
NF1	nuclear factor 1
NRL	nucleosome repeat length
Oct1	octamer transcription factor 1
PAR	ADP-ribose in polymers
RNA pol II	RNA polymerase II
SRC	steroid receptor coactivator
ssDNA	single stranded DNA

1 INTRODUCTION

Every cell in the human body contains the same genetic material, DNA that is organised into protein-DNA complexes termed chromatin. Differences in the DNA sequence between two human individuals are displayed as heritable traits as for example hair- and eye colour but the DNA sequence itself can only partly explain the wide variety of all the individual properties. In concordance, monozygotic twins that carry identical DNA nevertheless display some phenotypical differences that tend to increase with age (Fraga et al., 2005). This is an example of how differential gene expression can develop into stably maintained responses in the organism (Wong et al., 2005). **Epigenetics** is the term collecting the complex regulatory mechanisms behind these phenomena. Epigenetic mechanisms are intimately linked to chromatin and are defined as heritable changes in gene expression that do not include the DNA sequence itself.

Fine tuned regulation of individual genes is crucial for correct development and cell maintenance. A failure in any of these mechanisms may lead to inappropriate or uncontrolled cell division. Recently massive effort has centred on studies of genome-wide effects of various modifications to chromatin and chromatin-related proteins (Murrell et al., 2005). In addition, we also need to understand the mechanistic aspects behind these events. Even though this thesis is focused at one gene, what we learn from this and similar studies will increase our knowledge about mechanisms governing different chromatin states. We are now in the beginning of unrevealing the molecular cause behind the epigenetic enigma of differential gene regulation.

1.1 CHROMATIN

DNA is organised into chromatin in an evolutionary well-conserved manner. In all eukaryotic cells DNA is wrapped almost two superhelical turns around a histone octamer complex that constitutes the nucleosome. The histone octamer complex consists of a tetramer of two hetrodimers of histone proteins H3/H4 that interacts with two hetrodimers of histone H2A/H2B and encomprising roughly 146 basepairs (bp) DNA (Luger et al., 1997) (reviewed in (Khorasanizadeh, 2004; Luger, 2003)). Arrays of nucleosomes are folded into 30nm chromatin fibres, which probably is the intracellular structure that participates in most DNA-related processes, however, the role and structural details of these fibres remains unclear (Robinson et al., 2006; Schalch et al., 2005). The chromatin may be additionally compacted into 100-400nm thick structures in the interphase nucleus. The histone proteins are folded into globular structures building the nucleosome core from where the histone N-terminal tails protrude. These histone proteins and the tails in particular, are subject to chemical modifications highly associated with gene regulation (discussed further below). The chromatin is a highly condensed structure and needs to be unpacked during the cellular processes to facilitate for molecules to get access to the DNA. This is accomplished by remodelling complexes containing ATPase subunits. These are known to move nucleosomes along the DNA, to participate in the replacement of histones, or in other ways alter the histone-DNA contacts and targeting of these complexes to chromatin contributes to specific gene regulation (Emerson, 2002; Smith and Peterson, 2005).

The DNA at the entry/exit site of the nucleosome is organised by the linker histone H1, which in fact is a family of closely related proteins. In mammalians these include the somatic H1a-e and $H1^{o}$, the testis specific H1t and the oocyte specific variant H1oo

(reviewed in (Khochbin, 2001)). H1 consists of a three-domain structure, the central globular domain, and the C- and N-terminal tails (Hartman et al., 1977). It is thought to bind asymmetrically and close to the dyad were the C-terminus brings together the two DNA strands into a structure that is more resistant to micrococcal (MNase) digestion called the chromatosome (Noll and Kornberg, 1977). The histone H1 proteins are highly mobile. Photobleaching experiments suggest that H1 is continuously exchanged between its binding sites at the nucleosomes (Lever et al., 2000; Misteli et al., 2000). H1 was generally regarded as a global repressor acting by compacting the chromatin and to block nonspecific transcription *in vitro* (Laybourn and Kadonaga, 1991), however, the role of H1 in the higher order organisms is not yet clear (Fan et al., 2005). A surprising feature of the linker histones is their high redundancy among the subtypes. Knockout mice lacking one or two H1 variants showed no phenotypic effect and the loss of H1 was compensated by the increase of expression of the remaining subtypes. However, H1 is essential in mammalians. This was clearly shown when three H1 subtypes were concomitantly deleted and the mouse embryos developed multiple abnormalities and died in midgestation early during embryonic development (Fan et al., 2003).

Traditionally, chromatin is divided into two major forms of structure, heterochromatin and euchromatin. Heterochromatin consists of densely packed, silent chromatin as opposed to the permissive or the actively transcribed euchromatin (Heitz, 1928). Heterochromatin differs in its composition in e.g. the pericentric- and centromeric heterochromatin, the protection of the ends in the chromosome as telomeric heterochromatin or in the silenced X-chromosome in females. Facultative heterochromatin describes the structural switch between the active and silenced chromatin that is set up in a tissue- and time specific manner throughout cell differentiation and development (Craig, 2005). Epigenetic mechanisms are closely linked to and differ in the above-described chromatin states. Particular epigenetic marks are associated with specific genes or whole regions of chromatin and are thought to determine the chromatin activation potential.

1.2 EPIGENETIC MECHANISMS

As stated above, epigenetics refers to the differences in the genome function that occur without change in the DNA sequence. This includes mitotically heritable patterns of DNA methylation as well as modifications of chromatin proteins i.e. covalent histone modifications and the exchange of histone variants. Each of these is reversible and highly dynamic in comparison to the static DNA structure but together form mitotically heritable patterns. These, so called epimutations can arise in somatic tissue within a lifetime and alter the phenotype of an organism. For example over the life span of a mouse it was estimated that certain epimutations occur much more frequently than DNA mutations (Bennett-Baker et al., 2003). In a number of eukaryotic species there is now clear evidence that epigenetic mechanisms are inherited also into the next generation but so far it remains unclear to what extent these mechanisms are transgenerational in mammals (reviewed in (Peaston and Whitelaw, 2006)). However, there is reason to believe that this actually occurs and one early study reporting this in humans concerned a paternal inheritance of a new functional centromere on chromosome 4 (Amor et al., 2004). No genetic mutation was found that could explain the original centromere inactivation or why the region that became the ectopic centromere was transmitted.

Possibly the most extensively studied epigenetic modification is DNA methylation. The DNA is covalent modified by methylation of cytosine residues that are followed by a guanine residue (CpG). Most often such DNA methylations are connected to gene repression but in some cases DNA methylation at a nearby sequence allows activation of a neighbouring gene (Zwart et al., 2001).

In addition to the histone H1 subtypes mentioned above, incorporation of different core histone variants may alter various cellular processes and have specialised functions (reviewed in (Kamakaka and Biggins, 2005)). Histone H2A has numerous variants with diverse functions, some involved in gene activation and others in silencing. Many of the histone H2B variants seem to take part in compaction of the chromatin structure during gametogenesis and development. The histone H3 variants consist mainly of H3.3, which is enriched in actively transcribed genes and the centromeric H3 (also termed CenH3 or CENP-A). H4 appears to be the only histone protein devoid of any variants. Apart from the exchange of the total histone protein by a variant, covalently modified residues of the histones are participating in gene regulation.

1.2.1 Histone modifications

The highly flexible N-termini of the histones constitute about one fourth of the total mass of the histone octamer and are thought to be involved in the packing of the DNA into 30 nm fibres by interaction of neighbouring nucleosomes (Dorigo et al., 2004) (Schalch et al., 2005). The histones proteins and variants are targets for a large number of covalent modifications. Such modifications include acetylation (ac), phosphorylation (ph), methylation (me), mono-ubiquitylation (ub), SUMOylation (su) and ADP-ribosylation (ar) (for more comprehensive reviews see (Biel et al., 2005) and (Millar and Grunstein, 2006)). Histone modifications are believed to participate in all processes involving the DNA such as gene regulation, repair, apoptosis and replication and although most modifications are evolutionary well conserved, they differ somewhat between yeast and higher eukaryotes (summarised in (Peterson and Laniel, 2004)). Collectively, the patterns of histone modifications direct cellular processes. This was postulated in the histone code model, suggesting that distinct patterns mark domains of chromatin for particular processes and serve as interaction sites for regulatory factors that lead to further downstream events (Strahl and Allis, 2000). Below follows a brief summary of some histone modifications, however, it is likely that new modifications interacting with complexes eliciting yet unknown functions remains to be found.

1.2.1.1 Histone acetylation

Histone proteins have numerous evolutionary conserved lysines (K) that are subject to acetylation. In mammals these include K9, K14, K18, K23, K27 at histone H3 and K5, K8, K12, K16 at histone H4. Also K5 at histone H2A and K5, K12, K15 and K20 at histone H2B are known to be acetylation sites in higher eukaryotes. Also, the linker histones have been reported to have multiple acetylation sites in both N- and C-terminal parts ((Phillips, 1963) and (Rall and Cole, 1971)). The histone acetylation is highly dynamic through the action of histone acetyltransferases (HATs) that transfer acetyl groups to histones and the histone deacetylases (HDACs) that sequentially removes these groups. Histone acetylation is generally found in euchromatin and has long been correlated with transcribing chromatin (Allfrey et al., 1964). Gcn5, which was the first nuclear HAT to be identified, connected histone acetylation to gene activation and transcription in *Tetrahymena* (Brownell et al., 1996). This discovery was followed by intense research and soon both general and specialised functions for a number of histone modifications were discovered. An example is H3K14ac and H3K9ac that

participated in the recruitment of transcriptional activators and proteins in the transcriptional machinery (Agalioti et al., 2000). It was also shown recently that a single acetylation of H4K16 could physically alter the chromatin structure by local dissociation of neighbouring nucleosomes *in vitro* (Shogren-Knaak et al., 2006).

1.2.1.2 Histone methylation

Different chromatin activation states correlate with specific methylated lysines that can be either monomethylated (me1), dimethylated (me2) or trimethylated (me3), which may have different functional outcome (Sims et al., 2003). These include K4, K9, K27, K36, K79 at histone H3 and K20 at histone H4. Also K25 at linker histone H1 was shown to be methylated (Garcia et al., 2004), at transcription repression during development (Kuzmichev et al., 2004). Histone lysine methyl transferases (HKMTs) are responsible for adding methyl groups to histone lysines e.g. the *Drosophila* SUV39 was found to recognise methylated lysine residues in the histone proteins for specific methylation of H3K9 (Lachner et al., 2001). This methyl mark is connected to silencing as it promotes binding of heterochromatin protein1 (HP1). The methylation of histones has been correlated with both positive and negative effects on transcription. H3K9me2/3, H3K27me1/2/3 and H4K20me3 are generally found in repressed chromatin (Bannister et al., 2001; Lachner et al., 2001; Schotta et al., 2004) whereas H3K4me2/3, H3K36me2/3 and H3K79me2 mark active chromatin domains (Margueron et al., 2005; Martin and Zhang, 2005). H3K4 methylation is generally associated with actively expressed genes and has been shown to interact with chromatin remodelling factors such as human chromodomain protein1 (hCHD1), nucleosome remodelling factor (NURF) and the WDR5 peptide in the WD40 repeat protein (Li et al., 2006; Sims et al., 2005; Wysocka et al., 2005). Methylation of histones is reversed by histone demethylases e.g. lysine specific demethylase1 (LSD1) specific for mono- and dimethylated residues (Shi et al., 2004).

Arginines (R) are targets for methylation by protein arginine methyl transferases (PRMTs). Arginines may be mono- or dimethylated and on top of this there is a discrepancy between the symmetric or asymmetric dimethylation. Some common sites for arginine methylation at histones are R2, R17, R26 at histone H3 and R3 at histone H4. The methylation of arginines and in particular H3R17me2 and H4R3me2 has been coupled to hormone activation and transcription (Chen et al., 1999; Metivier et al., 2003). Also arginine methylation is reversible by for example the conversion of mono- or unmethylated arginine into citrulline by peptidylarginine deiminase 4 (PAD4) (Cuthbert et al., 2004; Wang et al., 2004).

1.2.1.3 Histone Phosphorylation

Phosphorylation (ph) has been shown to occur in all histones at serines (S) and threonines (T). Like in many other proteins, linker histones phosphorylation has been found to participate in the cellcycle and peak during the cell division (Gurley et al., 1995). In addition to this, phosphorylation of histones is important during gene regulation, DNA repair and apoptosis (Nowak and Corces, 2004) and phosphorylation of H1 has been linked to hormone-induced gene regulation (Bhattacharjee and Archer, 2006).

1.2.1.4 Histone Ubiquitinylation

Ubiquitin (Ub), a small and highly abundant protein in the cell, serves as a mark for proteolytic degradation of proteins via the proteosome pathway. Unlike the poly-ubiquitin chains serving as degradation marks, specific lysines at H2A and H2B are mono-ubiquitylated and are involved in different epigenetic programming e.g. K123ub

at H2B which is involved in transcription activation. This mark seems to be important for histone methylation since impairing H2BK123ub prevented methylation of H3K4 and H3K79 (Peterson and Laniel, 2004).

1.2.1.5 Histone SUMOylation

SUMO (small ubiquitin related modifier) is structurally related to ubiquitin through secondary and tertiary elements. Together with many other proteins, histones can be SUMOylated (su), which is mostly connected with silencing (Shiio and Eisenman, 2003). It has been proposed that when acetylation of histone proteins increases, so does the SUMOylation of histone H4 and this would then serve as a signal for down regulation of gene expression(Nathan et al., 2003).

1.2.1.6 Histone ADP-ribosylation

It has long been known that ADP-ribosylation takes part in a number of chromatin processes and it was shown that the attachment of anionic ADP-ribose in polymers (PARs) of histones and linker histones H1 lead to altered chromatin structure (Huletsky et al., 1989) (Poirier et al., 1982). PAR polymerase-1 (PARP-1) is the most abundant member of a family that catalyzes the transfer of ADP-ribose units from NAD^+ to target proteins (Kim et al., 2005). Purified PARP-1 was shown to ribosylate mainly linker histone H1 but also the H2B tail. This promoted decondensation and destabilisation of nucleosomes and is thought to facilitate the binding of other factors to DNA (Rouleau et al., 2004). PAR-modifications have also been reported to occur during gene silencing. A model to explain this is based on PARP-1 action in concordance with neighbouring chromatin i.e. PARP-1 activates genes in a transcriptionally permissive environment but represses genes in a silent surrounding (Faraone-Mennella, 2005).

1.2.2 Imprinting

Genomic imprinting refers to specific gene inactivation events or the partial repression at e.g. on one of the two inherited parental alleles or the inactivation of one of the two X-chromosomes in female somatic cells. This might also include the control of lineage-specific maintenance of gene expression at different loci that are commonly organised in clusters and are evolutionary conserved among placental mammals (Delaval and Feil, 2004). These events include DNA methylation, covalent histone modifications and/or exchange of histone variants. One well-studied example of imprinting is the insulin-like growth factor-2 (IGF2) gene that is expressed from the paternally inherited allele in most tissues. This gene is regulated at a nearby DNA methylated imprinting control region (ICR) close to a neighbouring imprinted gene called H19, where allele specific DNA methylation is crucial for maintaining correct expression of the IGF2 gene (Zwart et al., 2001).

1.3 CHROMATIN REMODELLING AND NUCLEOSOME POSITIONING

A human cell nucleus of only a few micrometers in diameter must organise its almost 2m long DNA in a way that keep inactive genes silent and the active genes available for transcription. The packing of DNA into chromatin acts as a transcriptional barrier where the nucleosome masks transcription-binding sites (Lomvardas and Thanos, 2001). This is solved by nucleosome rearrangement upon activation over regulatory regions (Whitehouse and Tsukiyama, 2006). The nucleosomes might then be positioned along the DNA so that functional DNA sites are exposed for interaction with factors that initiate transcription and promote activation. The term 'translational positioning' is used to describe where the nucleosomes preferentially are placed according to the DNA

sequence during gene activation (Fig. 1) and 'rotational positioning' refers to DNA double helix orientation in relation to the histone octamer.

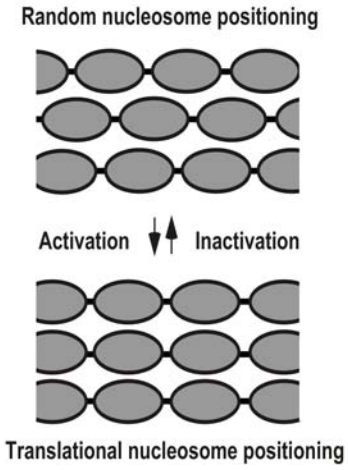

Fig.1 Randomly positioned nucleosomes may exhibit a transcriptional barrier. The nucleosome-positioning event followed by gene activation results in nucleosomes distributed at fixed positions over the DNA sequence, i.e. translational positioning. This state is reversed during gene inactivation.

In addition, specific nucleosome remodelling is proposed to function to facilitate stepwise formation of multiprotein complexes by uncovering DNA sites. This is often a dramatic, localized alteration in which one or few nucleosomes undergo structural change. Modulation of the chromatin structure is performed by energy-dependent chromatin-remodelling factors in complexes such as SWI/SNF (switch-independent non-fermenting chromatin remodelling complex), ISWI (imitation of SWI) and NURD (nucleosome remodelling and histone deacetylase) (Cairns et al., 1994; Tong et al., 1998; Tsukiyama et al., 1995). These complexes have been shown to recognise epigenetic modifications such as the specific histone acetylations and methylations.

1.4 TRANSCRIPTION FACTORS AND GENE ACTIVATION

Transcription induction is dependent on binding of transcription factors, coactivators and the basal transcription machinery that require access to binding sites in the promoter DNA. Specific transcription factors together with coactivators that enhance the action of both specific and general transcription factors may form a structural platform, a so called enhanceosome, for cooperative recruitment and binding of factors needed for stable gene induction (Bazett-Jones et al., 1994; Thanos and Maniatis, 1995). Conversely, the formation of a repressosome has been described in the establishment of repressor complexes over regulatory regions (Adhya et al., 1998; Geanacopoulos et al., 1999).

The term 'pioneer proteins' is used to describe the capacity of certain transcription factors to bind their cognate DNA site in chromatin and initiate the activation process. They are believed to participate in regulating genes in tissue- and cellular differentiation (Cirillo et al., 2002). Hormone receptors, important gene regulators have

this ability. Their activating ligands include steroid hormones, thyroid hormones, retinoids and vitamin D. These are small hydrophobic molecules that are carried from its produced organ through the bloodstream and interstitial fluid until it reaches its target cell. Common for the action of hormones is to bind to their corresponding receptors where they exert conformational changes critical for the receptors mechanism. A receptor binds to specific DNA sequences, so called hormone response elements where it recruits coactivators or corepressors for the gene regulation.

1.4.1 Glucocorticoid hormone and glucocorticoid receptor

The glucocorticoid hormone is derived from cortisol in the adrenal cortex and influence metabolism in nearly every cell in our body. The inactive glucocorticoid receptor (94kD) is stored in the cytoplasm in complex with heat shock proteins. The binding of the glucocorticoid hormone induces conformational changes and the receptor is translocated to the nucleus. It binds as a homodimer to palindromically arranged hexanucleotides (TGTTCT) that form a glucocorticoid response element (GRE) (Klock et al., 1987) and the mode of GR binding to the DNA is highly dynamic (McNally et al., 2000). GR is known to regulate transcription together with co activators such as steroid receptor coactivators (SRCs) that mediate recruitment of different HATs such as p300 and pCAF (p300/CBP associated factor) (Li et al., 2003).

1.5 MOUSE MAMMARY TUMOR VIRUS PROMOTER

The retroviral Mouse Mammary Tumor Virus (MMTV) causes tumours in the mammary tissue of mice (reviewed in (Gunzburg and Salmons, 1992)). The virus is activated by pregnancy hormones and is produced at high levels in the lactating gland from where it is transmitted through the milk from the mother to the pups or through the germline. The MMTV long terminal repeat (LTR) provides a model system for studying glucocorticoid hormone induction coupled to chromatin structure (Ringold et al., 1975). In tissue culture cells, the promoter is organised in six translationally positioned nucleosomes, termed A-F along its 1.2kb sequence (Fragoso et al., 1995; Richard-Foy and Hager, 1987; Truss et al., 1995). The B-nucleosome covers a cluster of four GREs and undergoes activation-dependent remodelling, triggered by GR binding (Fig. 2). This is seen as a hormone-induced DNaseI-hypersensitivity over this DNA segment (Richard-Foy and Hager, 1987; Truss et al., 1995; Zaret and Yamamoto, 1984). The MMTV promoter also contains a nuclear factor 1 (NF1) binding site and two octamer sites in close proximity of the TATA box. Both the transcription factors octamer transcription factor 1 (Oct1) and NF1 were shown to bind and to contribute to gene expression in tissue culture cells (Toohey et al., 1990). In addition, the MMTV promoter contains two forkhead box A (FoxA) transcription factor sites flanking the B nucleosome. FoxA1 was shown to bind and induce hypersensitivity primarily over the C-nucleosome (Holmqvist et al., 2005).

In opposite to the positioning of nucleosomes seen in tissue culture cells, nucleosome-mapping experiments on MMTV microinjected into *Xenopus* oocytes revealed a random positioning (Belikov et al., 2000). This came as a surprise as it was considered that the DNA sequence itself was causing the preferential positioning (Hager, 2001). However, hormone activation of the promoter in the oocytes displayed the six translationally positioned nucleosomes including the hypersensitive B-nucleosome, analogous to previously described. This nucleosome positioning obtained by hormone induction was then shown to be reversible upon sequential hormone antagonist treatment by the two glucocorticoid antagonists RU486 and RU43044 (Belikov et al.,

2001), schematically drawn in Figure 1. This showed that the MMTV LTR can adopt multiple translational frames of positioned nucleosomes in its inactive form *in vivo* and raised the question what, apart from the DNA sequence, is causing the nucleosomal positioning?

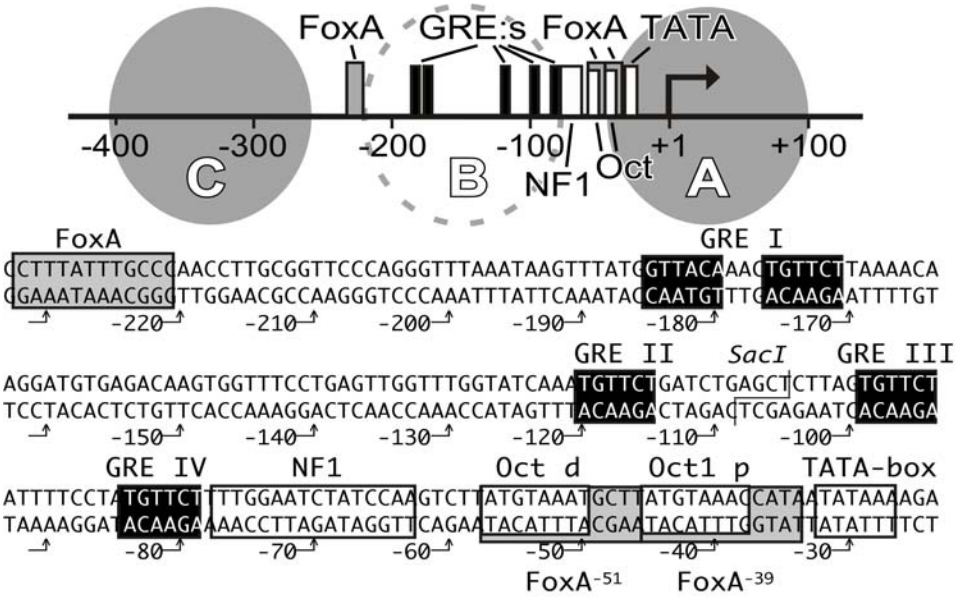

Fig. 2 The MMTV LTR with positioned nucleosomes A-C in an array of totally six nucleosomes. B-nucleosome (dashed line), containing the GREs (black), is remodelled upon hormone-activation. There is a NF1 site and also two Oct sites that are partially covering a double FoxA site in the linker region proximal to the TATAbox. A further FoxA site is situated upstream of the B-nucleosome.

2 AIMS OF THE THESIS

The aim of this thesis is to reveal how chromatin structure in the eukaryotic cell is cooperating with transacting factors during gene induction. The main objective is to reveal the molecular mechanisms of hormone-induced transcription activation of Mouse Mammary Tumor Virus (MMTV) promoter with special focus on the effects on the chromatin modifications. This has resulted in the following experimental studies:

1) Effects of the HDAC inhibitor, TSA-induced histone acetylation over the MMTV LTR (Paper I).
2) Interaction of NF1 and Oct1 with the promoter and their effect on chromatin structure and remodelling during gene induction (Paper II+III+V).
3) Functional implications of NF1- and Oct1 induced chromatin presetting (Paper III).
4) Effects of linker histone H1 on chromatin structure, protein binding and transcription of the MMTV promoter (Paper IV).
5) Effects on chromatin structure coupled to histone modifications in the inactive, the preset and the hormone-activated chromatin (Paper V).

3 MATERIAL AND METHOD

3.1 THE *XENOPUS* OOCYTE SYSTEM

Xenopus laevis or the South African 'clawed toad' oocytes and eggs have been studied since the late 1950s when early cloning experiments where performed, creating a full grown frog from genetic material isolated from an intestinal cell (reviewed in (Brown, 2004)). The *Xenopus* stage VI oocytes are large cells with a diameter of about 1.3mm. Their nucleus, called germinal vesicle, has a volume of 40nl (Hausen and Riebesell, 1991). Its size and the fact that it can be maintained for days makes the oocyte manageable as experimental system. An additional advantage is that it contains an excess of proteins stored for transcription and translation during the first 12 embryonic cell divisions. This enables the cells to chromatinise and express microinjected DNA. Multiple gene copies, up to a billion, can be injected in single stranded (ss) form into the cell nucleus where chromatin assembly is efficiently coupled to the second strand DNA synthesis (Almouzni and Wolffe, 1993). The high copy number enables *in vivo* studies on chromatin structure including nucleosome mapping and specific protein binding to the DNA by footprinting assays with high sensitivity and low background. In addition, the oocyte has the capacity to express proteins by injection of the corresponding mRNA into the cytoplasm.

We use the MMTV LTR fused to the herpes simplex virus thymidine kinase (HSV TK). This construct was inserted into the filamentous phage vector M13mp9 for production of ssDNA. Methods for detecting protein-DNA binding such as DMS and DNaseI footprinting, transcription assay by S1 analysis and nucleosome mapping experimental procedures concerning MNase and MPE hydroxyradical cleavage has been described previously (Belikov et al., 2000; Belikov et al., 2001; Gelius et al., 1999) and in the Papers I-V. Below follows a discussion of some methods that are especially important for this thesis.

3.1.1 Estimation of intranuclear concentration of expressed protein

Xenopus oocytes have a remarkable capacity to express any protein in up to pmol amounts upon mRNA injection. Below follows a typical experiment for estimation of intranuclear concentration of some coexpressed factors. Pools of oocytes coinjected with *in vitro* synthesised mRNA coding for GR, NF1, Oct1 and linker histone H1A into the cytoplasm were incubated overnight in medium containing [^{14}C]-lysine at concentration of 0.02 μCi/μl. The next day nuclei were manually dissected with a pair of forceps in pools of five and nuclear content was analyzed by SDS- PAGE (12%) electrophoresis (Fig. 3A). Relative amounts proteins were estimated by quantification on a Fuji BAS-2500 bioimaging analyzer using Image Gauge, version 3.3software, and with correction for the lysine content in the respective proteins (Fig. 3A, below). An aliquot was also analyzed by immunoblotting with GR antiserum together with known amounts of GR purified from rat liver (Wrange et al., 1979) to serve as a standard curve for calculation of absolute amounts of expressed proteins (Fig. 3B). We observed a linear correlation between injected RNA and translated protein at the concentration used (Paper II+IV) and Fig. 3A. Only when more than about 5ng mRNA was injected there were signs of saturation of the translational capacity.

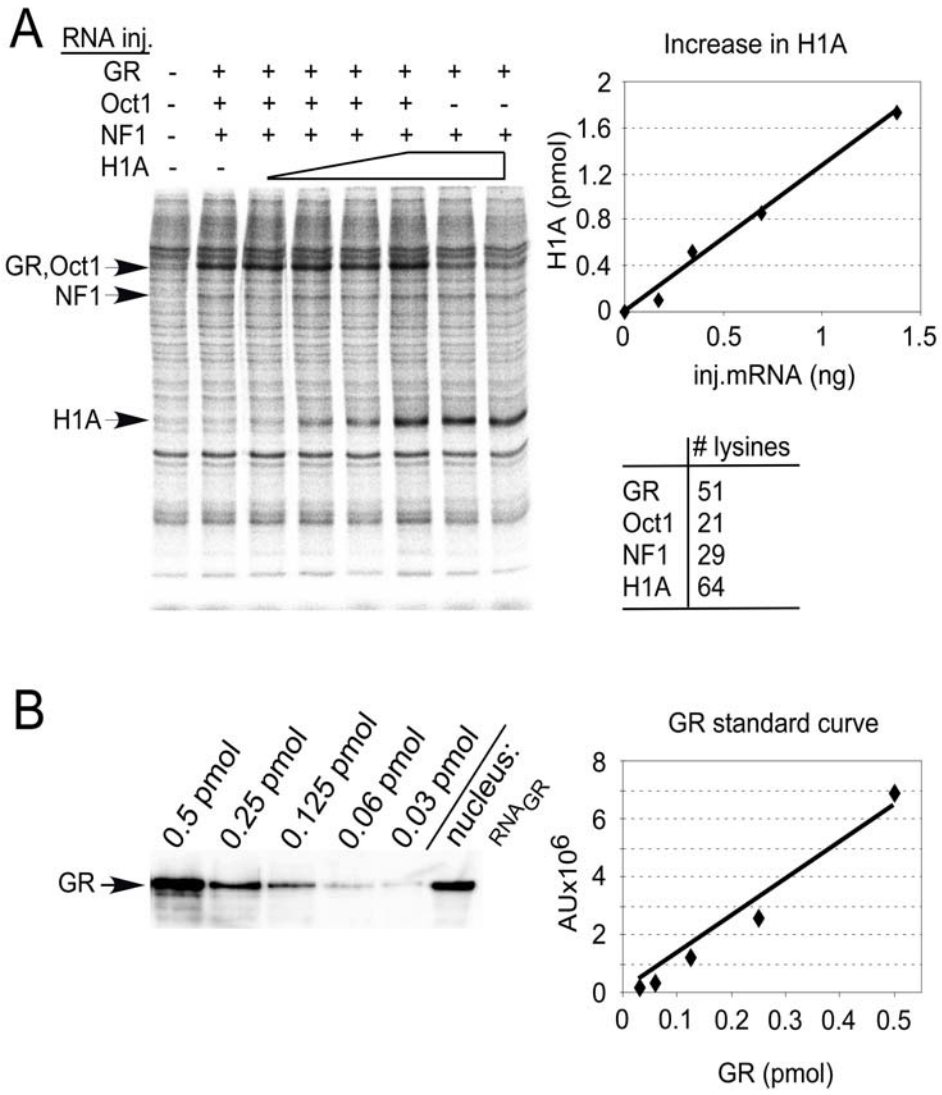

Fig. 3 Quantification of nuclear GR, NF1, Oct1 and H1A. Injection mRNA coding for the corresponding factors was followed addition of [^{14}C]-lysine in the growth medium. (A) Nuclear content was analysed by SDS-PAGE and relative amounts isoptope-labled proteins were estimated. (B) This nuclear content was also compared with a GR standard with known amount protein. (C) Number of lysines in the expressed proteins.

Furthermore, from the amount DNA injected in the nucleus and calculation of the number of nucleosomes (N) in the minichromosome (N=57 with NRL 179bp in the above experiment), estimation of total nuclear H1A/N can be made (Paper IV). The accuracy of H1A/N estimation is compromised by variation in the amount of DNA injected into each individual oocyte and this is measured by primer extension analysis. Endogenous oocyte DNA represent <0.6% of total DNA in a microinjected nucleus and is neglected in the above calculation. The estimated concentration is the total intranuclear amount H1A, i.e. the sum of specifically bound, free, and nonspecifically bound H1. It is therefore conceivable that an excess of intranuclear H1A is required to saturate the nucleosomes (Paper IV).

3.1.2 ChIP in *Xenopus* oocytes

Chromatin immunoprecipitation (ChIP) is a powerful method in linking a factor or a histone modification to a specific gene sequence. Although known for more than two decades ChIP has routinely been used only during the recent years and improvements are constantly made. However, some problems with the method and also concerning the presentation of ChIP data remain (for review see (Clayton et al., 2006)). Several important aspects such as antibody specificity and quality of different batches, occlusion of epitopes caused by crosslinking, variable quality of chromatin fragmentation and experimental setup regarding control genes might lead to confusion. The inclusion of proper controls to address the above mentioned issues is crucial to obtain reliable results.

In brief, this antibody-based method includes (with few exceptions):
Chromatin and associated proteins are (1) crosslinked to the DNA. After (2) shearing the chromatin into smaller fragments follows (3) an immunoprecipitation procedure with an antibody specific to the protein of interest. The antibody-bound protein crosslinked to the DNA is (4) collected and crosslinks are (5) reversed by heat treatment before (6) the DNA purification. The DNA is then generally (7) quantified by a PCR based technique.

For detailed method of ChIP in *Xenopus* oocytes, see Paper V, however, below are some aspects discussed. The highly manipulatable oocyte system has several advantages but also a few disadvantages as compared with other systems. Both DNA and the amount expressed protein is adjustable and results in easily obtained strong signal to noise ratio. The oocytes are well synchronised at the onset of treatment. Comparing treated oocytes with non-treated ones seems to be an important reference. Simultaneous injection of an additional reporter gene is not recommendable since this results in a systematic variation (Paper II) and is likely to have implications also on ChIP results. An obstacle is the large amount yolk protein in the oocyte cytosol that may interfere with the sonication, immunoprecipitation and detection. To avoid this by nuclear dissection after crosslinking is time consuming and therefore not ideal. However, the conditions used in Paper V, with a homogenisation buffer of 0.3% SDS was used to solve this and renders chromatin fragments below <500pb. In our experience the quantitation by the use of qPCR of the precipitated DNA is crucial for reproducible results on subtle effects seen in for example the preset chromatin state (Paper V). We also prefer to present ChIP data as immunoprecipitated DNA / total input DNA in percent (%), where the total input DNA is based on a standard curve from input chromatin aliquoted at the same time as the immunoprecipitation step was initiated.

4 RESULTS AND DISCUSSION

4.1 HISTONE MODIFICATIONS IN MMTV PROMOTER

4.1.1 Histone acetylation correlated with GR-induced chromatin remodelling

In order to investigate the connection between histone modifications and the state of chromatin activation, chromatin from MMTV-TK microinjected *Xenopus* oocytes was immunoprecipitated with antibodies to specific acetylated forms of histones. Hormone-activation correlated with a strong increase in acetylation of histone H3K14, H3K9 and H4K16 in both the promoter containing the GRE segment and the reporter gene. However, these modifications were not present on the vector DNA (Paper V). Specifically, H4K16ac was shown to increase over the promoter during the first hour of hormone-induction and then remained constant indicating that this modification is involved in a very early activation event. H3K14ac and H3K9ac over the promoter correlated in time with the previously shown B-nucleosome specific chromatin remodelling process detectable within 1h and is fully developed 4h after the addition of hormone (Belikov et al., 2000) . This was also reflective of GR binding as assayed by *in vivo* footprinting by DMS methylation protection (Paper III), but tended to precede RNA synthesis (Paper III+V), thus indicating a role in early steps of the induction process. Previous studies in *Xenopus* oocytes demonstrated that histone acetylation by p300 facilitated recruitment of SWI/SNF complex (Huang et al., 2003). This is a possible link of a chromatin-remodelling complex in *Xenopus* oocytes known to be recruited by the H3 acetylation seen in GR-activated MMTV activation.

4.1.2 Hormone-induction mediates transient H3K4me3

We observed transient hormone-induced H3K4me3 associated with the transcribing region (Paper V). It has been reported that trimethylation of histone H3K4 occurs after the general transcriptional complex is assembled on the promoter (Pavri et al., 2006). In line with this, the H3K4me3 in the MMTV microinjected oocytes appeared later that the histone acetylation and chromatin remodelling of the B-nucleosome but instead correlated in time with early transcription events (Paper V).

The functional role of methylation at H3K4 is debated (Sims and Reinberg, 2006). This modification has been shown to be recognised by nucleosome remodelling complexes (Li et al., 2006; Sims et al., 2005; Wysocka et al., 2005) and suggested to participate in the positioning and maintenance of the chromatin structure at 5'-ends of genes. Methylation of H3K4 has also been suggested to serve as a memory tag of newly transcribed genes (Ng et al., 2003). The transient nature of this H3K4me3 modification seen in the *Xenopus* oocyte system is intriguing. It cannot be excluded that this is caused by antibody specificity by occlusion of the methyl mark or a change in epitope by introduction of nearby modifications. However, preliminary data indicate that dimethylation of this site also has a turnover that alternates with the trimethylation (unpublished results). This phenomenon requires further investigation.

4.1.3 Histone acetylation is reversible by a hormone antagonist

The addition of the GR antagonist RU486 disrupts the remodelling at the B-nucleosome and to randomize previously hormone-positioned nucleosomes in the MMTV promoter (Belikov et al., 2001). Here, we found that the hormone-induced

acetylation of H3K14 and H3K9 was partly reversed by addition of the hormone-antagonist RU486 (Paper V). Our results show that the major part of histone acetylation is reversible within a short time period when also the chromatin is transformed into an inactive state. This links histone deacetylation to modulation of chromatin structure during the gene inactivation.

4.2 EFFECTS OF TSA-INDUCED HISTONE ACETYLATION

HDACs exist in large multiprotein complexes and catalyse the cleavage of acetyl groups, thereby generating deacetylated chromatin. The histone deacetylase inhibitor, Trichostatin A (TSA) is commonly used in research and nowadays also as an antitumor drug (Finnin et al., 1999). We used this compound to study the effects of inhibition of HDACs on transcription and chromatin structure of the MMTV promoter (Paper I).

Treatment of microinjected *Xenopus* oocytes with 16nM TSA resulted in increased acetylation of bulk histones, as well as histones in the minichromosomes as revealed by Western blotting and ChIP, respectively. We noted a reduced transcriptional response by glucocorticoid hormone in the presence of TSA. We also observed TSA-dependent effects on topology such as introduction of negative supercoils and an increased access for MNase digestion. Interestingly, HDAC1 was recently shown to act also as a coactivator in MMTV transcription (Qiu et al., 2006).

In addition, TSA enhances constitutive transcription of the MMTV (Paper I). This correlated with a relaxation of the minichromosome as compared with the non-treated inactive state. This topological shift occurred at an early stage since there was no further development of relaxation of the minichromosome between short treatment (4h) and long term treatment (24h) hours. In addition, modulation of the chromatin structure in the promoter region was observed. Specifically, a positioning of initially randomly placed nucleosomes along the distal MMTV LTR was seen by MPE hydroxyradical cleavage. In particular the C-E nucleosomes were focused at specific positions along the DNA, however, no remodelling of the B nucleosome was seen by TSA treatment as opposed to in the hormone-induction (Fig. 4).

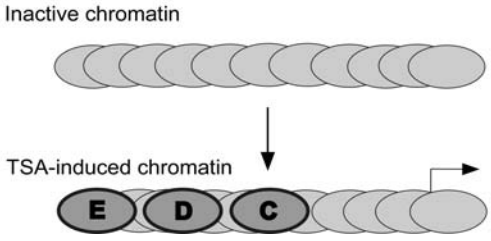

Fig 4 Model of TSA induction at the inactiv MMTV promoter.
Nucleosomes C-E are partially positioned during TSA treatment.

Although it cannot be excluded that TSA acts indirectly, it is plausible that the increased level of histone acetylation causes the specific translational and topological modulation of the chromatin in the MMTV promoter. No change by addition of TSA was detected in nucleosomal structure proximal to the transcriptional start site neither by MPE nor by a restriction enzyme cutting assay. This indicates that the activating signals are not dependent on B-nucleosome remodelling. In conclusion, hyperacetylation of histones is likely to reduce the repressive function of the chromatin in the oocyte and causes a permissive chromatin structure with enhanced basal transcription and a partial translational nucleosome positioning. On the other hand the hormone-activated transcriptional response is impaired by inhibition HDACs. Possibly, part of this inhibition mediated by physically corrupting the chromatin including the introduction of distinct topological changes corresponding to a more closed chromatin structure.

4.2.1 Constitutive NF1 binding

An initial step in gene regulation is the binding of a pioneer protein(s), with the capacity to access DNA embedded chromatin and to induce chromatin remodelling. This in turn is thought to lead to the subsequent binding of other transcription factors and co-activators (Cirillo et al., 2002). Previously, a two-step model for activation of the MMTV promoter has been proposed (Hager, 2001). This model was based on studies in tissue culture cells where the first step was the binding of a ligand-activated glucocorticoid receptor to a GRE. This recruited the remodelling complexes that generated an opening of the chromatin structure and enabled binding of the additional transcription factors NF1 and Oct1 (Cordingley et al., 1987; Truss et al., 1995).

However, when NF1-C1 was expressed in *Xenopus* oocytes a low degree of constitutive and concentration dependent NF1 binding was detected to obtain its cognate site in the MMTV promoter (Paper II). Detection of this binding was possible to in the *Xenopus* oocyte system due to a high signal to noise ratio by the high gene copy number.

4.2.2 Hormone-dependent NF1 binding

NF1 is expressed in most tissues as one or several of the variants that recognise the same DNA binding sequence (Gronostajski, 2000) where it binds as a homodimer (Kruse and Sippel, 1994). The *Xenopus* oocyte system also renders the possibility to semi-quantify the concentration of a factor in an oocyte nucleus (see material and methods). Furthermore, it is possible to correlate this to factor binding to the specific DNA sites (Paper II).

We expressed NF1 in increasing amounts and monitored the hormone-dependent NF1 binding to the MMTV promoter. In microinjected *Xenopus* oocytes, NF1 is present as monomers and as dimers, bound specifically or non-specifically to the DNA in the nucleus. When the total NF1 concentration reached a critical level there was a drastic increase in DNA binding that rapidly reached a plateau of saturation. Knowing that NF1 dimer has a considerably higher affinity than the NF1 monomer, we hypothesise that the transition point of half maximal DNA binding reflects the concentration where NF1 dimerises (Paper II).

4.2.3 A functional role for NF1

NF1 isoforms are highly redundant but has been reported to have diverse roles in development (das Neves et al., 1999; Grunder et al., 2002; Steele-Perkins et al., 2003). In *Xenopus* oocytes NF1 expressed alone or together with GR acts neither as an activator nor a repressor to MMTV transcription. None of the NF1 subtypes tested (pig NF1-C1, murine NF1-B2) had significant increase on transcription as was previously proposed (Chaudhry et al., 1998). Our studies indicate that NF1 by itself is a seemingly neutral transcription factor, and instead has a structural role in the context of surrounding molecules (Paper II+III).

The hormone-dependent NF1 binding to its cognate DNA site in the MMTV promoter was estimated to be ~50-fold higher than the constitutive binding (Paper II). DMS footprinting revealed GR and NF1 cooperativity both in terms of DNA binding and chromatin remodelling of specific DNA segment at the B-nucleosome. The hormone dependent component of NF1 binding was fully dissociated by addition of the GR antagonist RU43044. However, treatment with RU486, an antagonist that supports partial GR-DNA binding but abolishes transcription, also maintained partial NF1 binding. As this antagonist repositions nucleosomes into random frames, this suggests that the GR-NF1 cooperativity is independent of agonist-driven chromatin remodelling but is rather dependent on GR-DNA binding (Paper II).

NF1 induced the formation of an enlarged MNase resistant protein-DNA complex containing an additional 10bp covering the NF1 site (Paper II). This enlarged sub-nucleosomal particle indicates a structural role for NF1 that may serve as a binding platform at the nucleosome. This might enhance recruitment and stabilize the binding of additional factors and coactivators for transcription. Indeed, enhanced binding and cooperativity with both GR and Oct1 was shown (Paper II+III). Together this indicates that NF1 has a structural role and acts in concert with the surrounding environment rather than to have a specific activating function. We speculate that hormone-independent NF1 binding may be involved in maintaining transcriptional competence and establishment of tissue specific gene networks.

4.2.4 A NF1- and Oct1-induced preset chromatin state

When the MMTV LTR is integrated in the genome of a mammalian cell it harbours six specifically positioned nucleosomes (see introduction) (Fragoso et al., 1995). The mechanism of this nucleosome arrangement remains unclear. Previous *in vitro* reconstitution of nucleosome(s) on small segments of the MMTV LTR suggested that the DNA-sequence was decisive for the nucleosome arrangement. However, expression of MMTV LTR in *Xenopus* oocytes rendered randomly distributed nucleosomes (Belikov et al., 2000). One possible explanation for this is that oocytes lack factor(s) that induce the nucleosome positioning at the MMTV LTR seen in other cells. In paper III, we demonstrated that specific and concomitant binding of NF1 and Oct1 to their cognate sites within the MMTV promoter together induces a nucleosome rearrangement. This was then further developed by hormone-induced GR binding into a distinct nucleosome positioning. The NF1-and Oct1-induced nucleosome positioning was not transcription dependent as this was seen also in the presence of α-amanitin, a powerful RNA pol II inhibitor.

Oct1, but not NF1, was found to increase both basal and hormone-induced-transcription. NF1 and Oct1 expressed together reciprocally facilitate each other's

binding to the DNA in the absence of hormone (Paper III) and render an enhanced basal transcription (Paper II). The NF1 and Oct1 binding also facilitate hormone-dependent GR binding and generate a faster and stronger hormone response (Paper III). As described above hormone-induction mediated transient H3K4me3 that was associated with the transcribed reporter gene (Paper V). The time required to initiate the first cycle was reduced by expression of NF1 and Oct1, thereby reflecting the faster hormone response of the preset chromatin. Since NF1 and Oct1 generate an intermediary state of nucleosome positioning and enhance the hormone-induced response, we refer to this as a preset chromatin structure. We propose that this NF1- and Oct1-induced chromatin mimics the early steps of transcription factor induced presetting and chromatin remodelling that is involved in cellular differentiation during the formation of specific patterns of gene expression.

4.2.4.1 Histone modifications at the preset chromatin state

The NF1- and Oct1-induced preset chromatin state was characterised by clear structural changes as revealed by topology assay (Paper V) and nucleosomal rearrangements (Paper III). However, only a mild increase in histone acetylation at H3K14, H3K9 and H4K16 was detected by ChIP, predominantly over the promoter region (Paper V). In the preset chromatin state the majority of the templates harbour positioned nucleosomes, as revealed by MPE (Paper III) and a large portion of the minichromosomes are forming a topologically different structure (Paper V). Together this indicates that the overall increase in histone acetylation seems to be of less importance in creating a permissive chromatin environment (Paper V). This indicates a mechanism where NF1- and Oct1-binding directly pushes the nucleosomes in position or that they act by recruitment of cofactors independently of histone-modifications. Recent studies suggest a dynamic model where specific nucleosomes exhibited rapid turnover of acetylation in the preset chromatin rather than an overall increased level of acetylation (Hazzalin and Mahadevan, 2005). This is an alternative explanation for the low level of histone acetylation in the preset MMTV chromatin.

4.2.5 Functional significance of chromatin structure for viral propagation and transmission

One may speculate that the different gene activation steps such as the NF1- and Oct1 presetting described in this thesis are important for the progression of the MMTV virus in its infectious cycle. As the retrovirus infects a new host it is dependent on the cellular environment and combination of transcription factors for its propagation in the different tissues. In addition to the hormone receptor dense mammary tissue, the lymphoid cells are known to exhibit non-hormone dependent high MMTV expression. Oct2 is expressed exclusively in lymphoid cells and exhibit high similarity to Oct1 (Herr et al., 1988) and has been shown to bind and contribute to MMTV expression (Prefontaine et al., 1998). Hence, Oct2 might participate in the maintenance of enhanced basal expression by modulating the chromatin structure in these cells. Furthermore, it is proposed that nearly every tissue of the infected mouse have some low viral expression (reviewed in (Gunzburg and Salmons, 1992)). To accomplish this the integrated retrovirus is likely to establish a preset chromatin state that is maintained by the help of the cellular factors such as NF1 and Oct1 to counteract chromatin silencing.

4.3 ROLE OF H1 DURING GLUCOCORTICOID HORMONE-INDUCTION

4.3.1 A subsaturating level of histone H1 enhances GR-binding and transcription

The oocyte lacks somatic linker histones but contain an oocyte specific variant called histone B4 (H1M) (Dimitrov et al., 1993). The B4 is replaced by the somatic linker histone H1A during early embryogenesis. The injection of mRNA for *Xenopus* histone subtype H1A (from now on called H1 for simplicity) resulted in H1 incorporation into MMTV chromatin (Paper IV). This was seen by MNase digestion as the appearance of a chromatosome stop and by an increased nucleosome repeat length (NRL) and also by ChIP analysis. Gradual H1-dependent increase in NRL from ~164 to ~173bp reached a plateau showing that H1 binding to chromatin was saturable, confirming earlier *in vitro* data (Fyodorov and Kadonaga, 2003). Based on these findings we addressed the effects of H1 on GR binding and hormone induction.

Subsaturating level of H1 to its binding site was found to correlate with enhanced hormone-dependent binding of GR to the GREs and also with enhanced hormone-induced MMTV transcription. This positive effect was lost at saturating levels of H1. Moreover, the same subsaturating level of H1 that enhanced GR binding was coinciding with reduced DNA access in chromatin as monitored by MNase digestion. MNase preferentially cleaves DNA in the linkers between the nucleosomes and this indicates the formation of an H1 concentration-dependent rearrangement of the chromatin fibre to occur within the same H1 concentration range that stimulates GR binding.

4.3.2 H1 dissociation correlates with chromatin remodelling

ChIP and MNase analysis revealed a hormone dependent dissociation of histone H1 from the activated chromatin domain (Paper IV+V), in accordance with earlier data (Bresnick et al., 1992) (Vicent et al., 2002). This H1-dissociation by hormone-activation coincided with histone acetylation and remodelling of the MMTV promoter (Paper V). Sequence specific transcription factors such as NF1 and Oct1 participating in nucleosome positioning but not in B-nucleosome remodelling were not capable of inducing H1 dissociation (Paper V). Similarly, we could not detect any H1 dissociation by treatment with the glucocorticoid hormone antagonist RU486 (Paper V), although this antagonist induces a low level of GR binding (Paper II) and a low level of histone acetylation (Paper V). In addition, TSA-induced histone acetylation did not cause any H1-dissociation (Paper V). Together, this suggests that the remodelling of the B-nucleosome and possibly the general opening of the chromatin is the major determinant of H1 dissociation in MMTV rather than histone acetylation as such. Furthermore, it is likely that H1 dissociation requires a certain threshold of chromatin remodelling, perhaps caused by the disrupting the H1 nucleosomal binding site by a pioneer protein similar to the hormone activated GR in complex with a hormone agonist which leads to recruitment of coactivators.

In conclusion, these results indicate that the mechanism of H1 stimulation of GR binding is based on GR-induced dissociation of H1 from the GRE-containing DNA segment. Since H1 remains bound to the inactive chromatin domain, where it reduces the DNA access, this results in that the free GR concentration in the nucleoplasm is increased and that the binding equilibrium is shifted from unspecific DNA contacts to the specific DNA binding sites.

5 SUMMARY OF MMTV CHROMATIN ACTIVATION

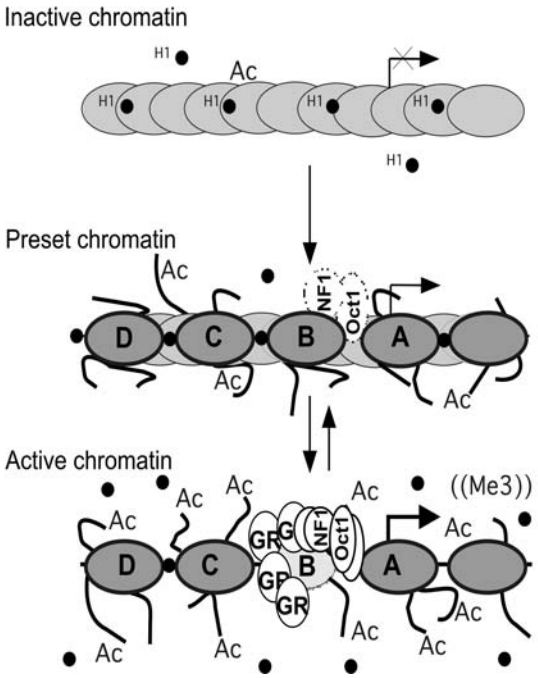

Fig. 5 The stepwise chromatin changes during NF1- and Oct1 mediated chromatin presetting and hormone-activated GR induction.

The initially random nucleosome position in the inactive chromatin is altered by the formation of a more focused translational positioning by weak binding of transcription factors NF1 and Oct1 that also enhances basal transcription. Linker histone H1 binding is not yet disrupted and only mild histone acetylation is seen in this chromatin state.

Hormone-activated GR binding then triggers a more distinct translational positioning and a specific remodelling over the GRE containing segment in the B-nucleosome is seen. NF1, Oct1 and GR bind cooperatively to their cognate sites. Binding of GR is increased by a focusing effect of H1 dissociation over the active chromatin, which in turn further stimulates the cooperative binding of NF1 and Oct1. This chromatin state is characterised by enhanced histone acetylation and strong transcription. In addition, transient H3K4me3 is observed in the transcribing region.

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