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The closer one looks at the performance of matter in living organisms the more impressive the show becomes.

Max Delbrück (1906 - 1981)

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Contents

1. Introduction

The genetic information of cellular organisms is encoded in DNA, and realized by transcription into RNA, followed by translation into proteins. In eukaryotes, most of DNA is organized as linear double-stranded helices with single strands in opposite orientation and base complementarity, forming chromosomes compartmentalized within cell nuclei. Additionally, genetic information is located in cell organelles (mitochondria, and in plants also plastids). *In vivo*, DNA is associated with proteins and RNA in a complex recognized as chromatin.

The proper functioning of the genome is controlled at several levels: 1) by DNA regulatory elements in *cis* (transcription factor binding sites etc.); 2) by epigenetic mechanisms (i.e., heritable influence on gene activity not accompanied by a change in DNA sequence) mediated mainly by DNA methylation and histone modifications; and 3) by higher order structure of chromatin (degree of DNA packaging, spatial arrangement of chromatin within nucleus). Chromatin organization and epigenetic phenomena are interrelated.

1.1. General chromatin structure

In contrast to the mostly circular prokaryotic genomes of 10^4-10^7 bp, eukaryotic nuclear genomes, can be several orders of magnitude larger (10^8-10^{11} bp) and consist of a species-specific set of linear chromosomes. Because chromosomes (Waldeyer, 1888; from Greek *chroma* – colour, *soma* – body) could be stained and recognized individually only during nuclear division, the majority of cytogenetic work since 19th century has been done on dividing nuclei (either mitotic or meiotic).

Each metaphase chromosome consists of two sister chromatids attached at the centromere, the region where a proteinaceous complex, the kinetochore, interacting with microtubules and responsible for chromosome movement, is assembled prior to nuclear division. After chromatid separation and movement towards spindle poles during anaphase and telophase, the chromosomes start to decondense, when daughter nuclei enter interphase.

In some species, the chromosomes maintain their polar anaphase orientation also during interphase resulting in location of centromeres and telomeres at the opposite nuclear poles (Dong and Jiang, 1998; Abranches et al., 1998). This so-called Rabl orientation (Rabl, 1885) is usually observed in plants with larger (>5000Mb) genomes (Dong and Jiang, 1998). Non-Rabl orientation of interphase chromosomes was often detected in mammals.

Mammalian interphase chromosomes occupy distinct, non-overlapping territories as shown by chromosome painting (fluorescence *in situ* hybridization with chromosome-derived DNA probes) (Cremer et al., 1993; Cremer and Cremer, 2001). This is also true for Arabidopsis chromosomes (Lysak et al., 2001; Lysak et al., *in press*).

The chromosomal DNA associates with proteins and undergoes hierarchical folding. The basic unit of chromatin organization ubiquitous for all eukaryotes is the nucleosome, consisting of: (i) a nucleosome 'core' formed by two molecules of each of the histone proteins H2A, H2B, H3, H4 (histone octamer), (ii) ~146 bp of DNA being wrapped around the core, and (iii) 15-55 bp of linker DNA connecting the adjacent nucleosome core particles (Luger et al., 1997). The nucleosome array known also as 'beads on the string' (11-nm chromatin fibre) is further folded (with participation of linker histone H1) into 30-nm chromatin fibre. At this level, the compaction ratio of DNA is ~30-40-fold. The further levels of chromatin fibre folding into large chromatin domains and chromosome territories are still poorly understood (Belmont et al., 1999).

At microscopical level, three distinct chromatin domains in interphase nuclei can be distinguished. The lowest chromatin density is found in the nucleolus, where rRNA gene transcription and ribosomes assembly takes place (Lamond and Earnshaw, 1998). Based on cytological observations, the remaining chromatin is made up of faintly stained and partially decondensed euchromatin and intensely stained and highly condensed heterochromatin (Heitz, 1928). The distinction of eu- and heterochromatin was initially inferred from staining properties. Later it was found that eu- and heterochromatin differ for instance in gene density, content of repetitive DNA sequences, meiotic recombination frequency, replication timing, chromatin composition, nucleosome spacing and accessibility to nucleases (Henikoff, 2000a; Table 1).

Table 1. Summary of euchromatin and heterochromatin features (Henikoff, 2000a)

	euchromatin	heterochromatin
interphase appearance	less condensed	highly condensed
sequence composition	mostly non-repetitive	repetitive
gene density	high	low
replication timing	throughout S-phase	late S-phase
meiotic recombination	normal frequency	low frequency
nucleosome spacing	variable	regular
nuclease accessibility	variable	low

The biological relevance of constitutive heterochromatin, mainly composed of tandemly and dispersed repetitive DNA sequences, is still a matter of debate (Henikoff, 2000a). It is widely accepted that heterochromatinization serves as a kind of defense mechanism against mobile elements such as transposons and retrotransposons by silencing their potentially deleterious transcriptional and transpositional activity. Only in a few

exceptional cases genes are expressed even when located within constitutive heterochromatin (e.g. in Drosophila, Lu et al., 2000 and references therein). Moreover, it has been argued that heterochromatin often located in the vicinity of centromeres (pericentromeric heterochromatin), might be required for proper centromere function (Henikoff, 2000a). Indeed, protein components of heterochromatin, like for example HP1 (heterochromatin protein 1) and its homologues (Eissenberg and Elgin, 2000) were proven to be essential for correct chromosome segregation (Kellum and Alberts, 1995; Ekwall et al., 1995; Bernard et al., 2001; Nonaka et al., 2001).

There is increasing evidence that (the interplay of) at least two factors are involved in heterochromatin assembly: (i) repetitive sequences, capable to trigger the assembly of silenced chromatin (Fourel et al., 2002), and (ii) histones, the most abundant proteins within chromatin, which contribute to heterochromatin assembly by specific post-translational modifications.

1.2. Histones and the functional importance of their post-translational modifications

The ubiquity of nucleosomes as the basic structure of eukaryotic nuclear genomes is paralleled by the high evolutionary conservation of core histones (Thatcher and Gorovsky, 1994). Histone H4 differs by only two residues between cow and pea (DeLange et al., 1969). Histones H4 (102 amino acids) and H3 (135 amino acids), are about 10-fold less divergent than H2A and H2B, while the linker histone H1 is a rather variable molecule (van Holde, 1989; Thatcher and Gorovsky, 1994). Sequence-variant subclasses were identified for all core histones except H4 (Brown, 2001). Some of them have acquired special functions, like for example H2A.X (involved in DNA repair in human, Paull et al., 2000), H2A.Z (essential role in early mammalian development, Faast et al., 2001), or macroH2A (preferentially located at transcriptionally silent X chromosomes of mammals, Constanzi and Pehrson, 1998). Specific histone H3 variants occur at nucleosomes of active centromeres and contribute to the distinct chromatin organization within these chromosomal regions in yeasts (Cse4 in *S.cerevisiae*, Meluh et al., 1998; SpCNP-A or Cnp1 in *S.pombe*, Takahashi et al., 2000), Drosophila (Cid, Henikoff et al., 2000b), human (CENP-A, Sullivan et al., 1994), and Arabidopsis (HTR12, Talbert et al., 2002).

The central part of a histone molecule is a globular domain formed by three helices, also known as histone fold (Luger et al., 1997). The N-terminal ‘tails’ contain high amounts of basic amino acids such as lysine and arginine, resulting in a positive net charge of the tail at physiological pH. Tails of core histones were found to mediate internucleosomal contacts

(Luger et al., 1997) and change their interactions when the chromatin fibre undergoes folding or compaction, suggesting that specific tail interactions are correlated with specific fiber conformations (Wolffe and Hayes, 1999). The influence of histone variants on nucleosome structure and/or folding properties of nucleosomal arrays is still largely unknown (Horn and Peterson, 2002).

Aminoacid residues at specific positions within histone tails are subject to a number of post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitination or ADP-ribosylation (van Holde, 1989; Smith et al., 1995; Spencer and Davie, 1999) (Figure 1). Modifiable aminoacids occur also within globular domains, as for example lysine 79 of H3 (Feng et al., 2002; van Leeuwen et al., 2002). Data accumulated during the last two decades have shown that histones are important 'players' in regulating chromatin functions via their modifications (see below).

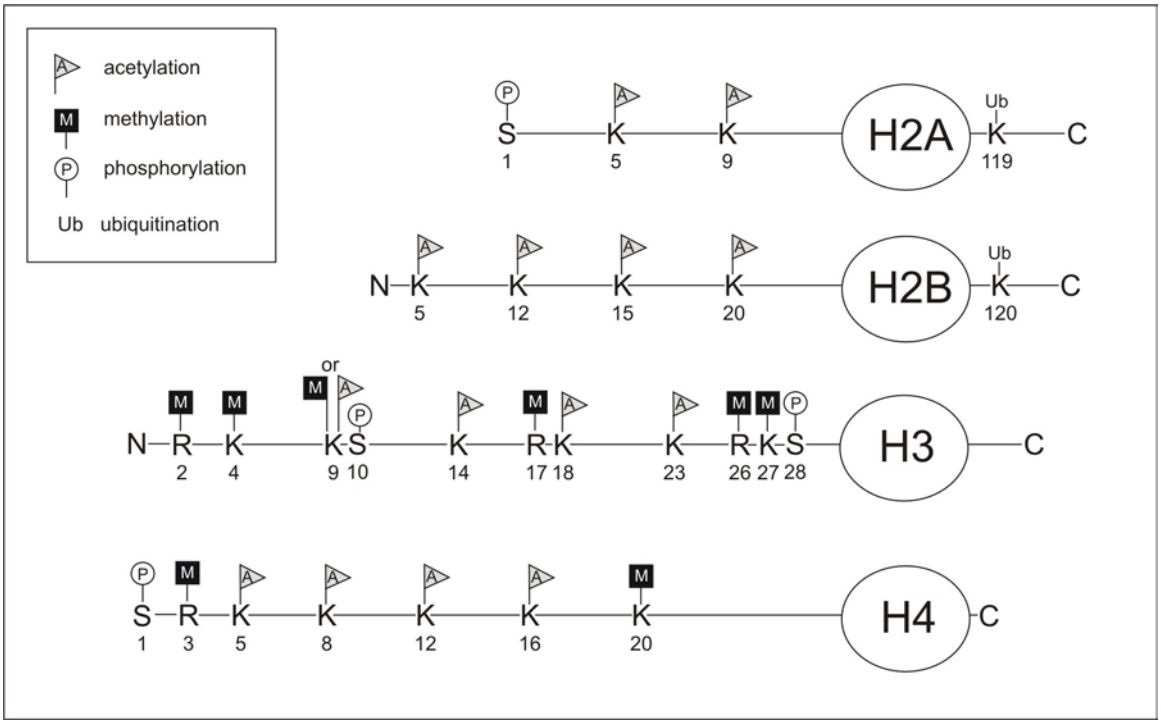

Figure 1: Sites of post-translational modifications on the histone tails (adopted from Zhang and Reinberg, 2001; Richards and Elgin, 2002). K9 of histone H3 can be either acetylated or methylated. In plants, K20 of histone H4 is not methylated but acetylated (Waterborg, 1992). Additionally, within the globular domain of H3 K79 can be methylated (Feng et al., 2002; van Leeuwen et al., 2002).

1.2.1. Histone acetylation

Histones can be acetylated at specific lysine residues of histone H3 (K9, 14, 18, 23) and H4 (K5, K8, K12, K16, and in plants also at K20, see Fig.1), as well as of H2A and H2B (Fig.1). Since the initial observation of histone acetylation (Philips, 1963), this modification was studied intensively. The association of acetylated histones with transcriptionally active chromatin was proposed soon after the recognition of this modification by Allfrey et al. (1964) and is now well-documented (e.g., Hebbes et al., 1988; Grunstein, 1997; Struhl, 1998). Acetylation of histones causes a decrease of the net positive charge of the tail resulting in less condensed chromatin structure and increased accessibility of regulatory factors to DNA. Many transcriptional activators possess intrinsic histone acetyltransferase activity (Brownell et al., 1996; Struhl, 1998). Histone acetylation is a reversible process and deacetylation, mediated by histone deacetylases, is generally required for transcriptional silencing (Grunstein, 1997; Spencer and Davie, 1999).

In agreement with this, transcriptionally silenced heterochromatic domains are usually less acetylated than euchromatin. For example in yeast telomeres, histone hypoacetylation and heterochromatin assembly is mediated by the Sir-proteins complex in a step-wise process (Grunstein, 1998; Moazed, 2001). In mammals, the inactivated X chromosome appears as facultative heterochromatin and is largely free of acetylated histones (Jeppesen and Turner, 1993). Also heterochromatic regions of mitotic plant chromosomes are usually less acetylated than euchromatin (Houben et al., 1996, Belyaev et al., 1997) but certain heterochromatin fractions may contain specifically acetylated histone isoforms (for example, H3Ac9/18 and H3Ac14 in *Vicia faba*, Belyaev et al., 1998). Similarly, Drosophila heterochromatin is enriched in H4 acetylated at K12 (Turner et al., 1992). Nucleolar organizers (NORs) of Drosophila and mammals usually do not contain large amounts of acetylated histones, while in *V.faba* and other plants NORs belong to the most intensely acetylated regions of mitotic chromosomes (Belyaev et al., 1997). Despite the fact that transcription is downregulated during mitosis, strong acetylation at euchromatic regions of the mitotic chromosomes is largely maintained, and therefore histone acetylation at euchromatin during mitosis was suggested to represent an epigenetic mark at potentially transcriptionally active regions transmitted to daughter nuclei (Jeppesen, 1997).

During replication, diacetylated histone H4 is incorporated into newly formed nucleosomes, e.g. in *Tetrahymena thermophila* (acetylated at lysines 4 and 11), Drosophila and human (acetylated at lysines 5 and 12; Sobel et al., 1995). This phenomenon could be traced also at the level of large chromatin domains. Studies in plants have shown that histone deacetylase blocked for more than 2h before mitosis mediated strong acetylation of

histone H4 at heterochromatin suggesting reversible alterations of the histone acetylation status during interphase (Belyaev et al., 1997). In mammals, heterochromatin becomes highly acetylated during its replication and deacetylated towards mitosis (Taddei et al., 1999).

A chromatin structure relaxed by histone acetylation and therefore accessible to regulatory factors, was found to correlate with DNA recombination and repair processes as well (McBlane and Boyes, 2000; McMurry and Krangel, 2000; Ikura et al., 2000; Bird et al., 2002).

1.2.2. Histone methylation

The evidence for histone methylation was first demonstrated by Murray (1964). Histone methylation concerns arginine (R2, R17, R26 of H3, and R3 of H4) and lysine residues (K4, K9, K27, K79 of H3, and in animals K20 of H4) (Fig.1). Mono- or dimethylated arginines and mono-, di-, or trimethylated lysines were reported (Bannister et al., 2002). While arginine methylation is largely connected with transcriptional activation (Zhang and Reinberg, 2001), the situation is more complex for lysine methylation.

Methylated K4 of H3 was found at transcriptionally active chromatin in fission yeast (Noma et al., 2001), budding yeast (Bernstein et al., 2002) and chicken (Litt et al., 2001). However, it is also required for rDNA silencing in budding yeast (Briggs et al., 2001).

High amounts of methylated K9 of H3 were first detected in transcriptionally silenced domains of fission yeast (Noma et al., 2001) but interestingly this modification is not present in budding yeast (Strahl et al., 1999; Briggs et al., 2001). Heterochromatin contains high amounts of H3 methyl K9 also in Drosophila (Schotta et al., 2002) and mammals (Peters et al., 2001). The methyl-group of K9 provides a binding site for HP1 (heterochromatin protein 1) (Bannister et al., 2001; Lachner et al., 2001) thus it is involved in assembly of heterochromatin (Nakayama et al., 2001). The heterochromatic state can ‘spread’ from an initial ‘mark’ by self-association of HP1 molecules (Eissenberg and Elgin, 2000).

At lower levels, H3K9 methylation is detectable also in Drosophila (G. Reuter, personal coomunication) and mammalian euchromatin where it is involved in the transcriptional repression of developmental genes (Tachibana et al., 2002), and was found to be involved also in cell cycle-dependent downregulation of cyclin E expression via HP1 recruitment. In contrast to constitutive heterochromatin, H3K9 methylation is restricted to a single nucleosome at the cyclin E promoter (Nielsen et al., 2001). Although an increasing number of histone methyltransferases has been described since Rea et al. (2000) have first shown that mammalian homologues of Drosophila Su(var)3-9 encode H3K9-specific methyltransferases (reviewed in Lachner and Jenuwein, 2002), histone demethylases that

would actively remove methyl groups (analogously to histone deacetylases), are not yet identified. For the active turnover of methylated histones, mechanisms including histone replacement (Ahmad and Henikoff, 2002) and/or tail clipping have been suggested (Bannister et al., 2002).

1.3. Cross-talk between histone modifications and the 'histone code' hypothesis

The high diversity of histone modifications, as well as the high number of residues that can be modified within histone tails, and the correlation of individual modifications with various nuclear processes, lead to the hypothesis that specific combinations of histone modifications provide a histone 'code', which after 'translation' by downstream factors determines specific chromatin functions (Turner, 1993; Strahl and Allis, 2000; Turner, 2000).

For instance phosphorylation at serine 10 of H3 is correlated with different functions such as chromosome condensation prior to and during mitosis (Hendzel et al., 1997; in plants Houben et al., 1999), and transcription during interphase (Cheung et al., 2000b). H3S10-phosphorylation precedes and facilitates K14 acetylation during transcriptional activation (Cheung et al., 2000a; Lo et al., 2000), but negatively affects methylation at K9 (Rea et al, 2000). To enable K9 methylation during heterochromatin assembly in fission yeast, K14 and K9 have to be deacetylated first (Nakayama et al., 2001).

Modifications within one histone can influence those of other histones in a so-called 'trans-histone' regulatory pathway. This was first reported for *S.cerevisiae*, in which H2B ubiquitination controls methylation of H3K79 and H3K4 during gene silencing (Sun and Allis, 2002; Briggs et al., 2002).

The vast amount of data accumulated during recent years suggested it would be useful to distinguish between short- and long-term transcriptional effects of histone modifications (Turner, 2002). Whereas transcriptional activation (consisting of a cascade of transient events) of different genes might require distinct combinations of histone modifications (Agalioti et al., 2002; Daujat et al., 2002), long-term maintenance of transcriptional state appears to share common features even in evolutionarily distant organisms (Richards and Elgin, 2002). Because the setting of chromatin imprints involves also DNA methylation (Jones and Takai, 2001; Martienssen and Colot, 2001), and binding of silencing RNAs (Volpe et al., 2002), the histone code could represent a part of a comprehensive 'epigenetic code', which might be responsible in its entirety for the functionally organization of chromatin (Turner, 2002).

1.4. Interactions between histone modifications and DNA methylation in chromatin remodeling

Correct DNA methylation, mainly at cytosine residues, is essential for the normal development of mammals and plants. It is involved in epigenetic processes such as X chromosome inactivation, imprinting, and silencing of transposons (Jones and Takai, 2001; Martienssen and Colot, 2001).

How DNA methylation patterns are set up during development is not yet fully understood. The process of chromatin assembly behind the replication fork is very likely the crucial time point either for maintenance and/or *de novo* establishment of chromatin imprints. In addition to histone modifications, ATP-dependent chromatin remodeling factors are involved in changing the chromatin conformation (Tsukiyama, 2002). In Arabidopsis, a mutation of the gene encoding DDM1 (decrease in DNA methylation 1), homologous to yeast SWI2/SNF2 ATP-ases (Jeddeloh et al., 1999), results in a decrease of overall DNA methylation by about ~70% compared to the wild-type (Vongs et al., 1993). The activity of DNA methyltransferases remained unaffected in *ddm1* (Kakutani et al., 1995). Similar effects were found for the mutant *lsh*, affecting a mammalian homologue of DDM1 (Dennis et al., 2001), suggesting that these chromatin remodeling factors play a significant role in regulation of DNA methylation levels, possibly by regulating the access of methyltransferases to chromatin.

Histone modifications are tightly linked with cytosine methylation of DNA (Richards and Elgin, 2002). Methylated DNA associates with protein complexes that contain histone deacetylases and mediate transcriptional repression (Jones et al., 1998; Nan et al., 1998; Ng and Bird, 1999; Zhang et al., 1999). Thus, transcriptionally silent domains usually contain high levels of DNA methylation and low levels of histone acetylation. Shortly after replication DNA is hemimethylated and chromatin is enriched in acetylated histones. Maintenance DNA methyltransferase associated with histone deacetylases appear at replication foci of silent chromatin to ensure the retainment of high levels of DNA methylation and of histone hypoacetylation (Rountree et al., 2000).

The relationship between DNA methylation and histone methylation has been investigated in *Neurospora crassa*. A genetic screen for DNA hypomethylation mutants yielded the *dim-5* mutant, with the gene encoding a histone H3 methyltransferase being disrupted. This suggested that K9 methylation of H3 acts upstream of DNA methylation (Tamaru and Selker, 2001). In Arabidopsis, KRYPTONITE, a major H3K9-specific histone methyltransferase was found to direct CpNpG methylation at the *superman* locus (Jackson et al., 2002). However, CpG methylation at 180-bp centromeric repeats was not affected in the

absence of KRYPTONITE (Jackson et al., 2002; Johnson et al., 2002), suggesting a more complex relationship between H3K9-methylation and DNA methylation in Arabidopsis than in Neurospora.

1.5. Short characterization of the three investigated plant species

Microscopical studies on chromatin modifications within entire nuclei during the cell cycle and in relation to specific nuclear processes are anticipated to provide a more global view on nuclear chromatin organization and to complement molecular data on the chromatin modifications and on the functional role of genes involved. For this purpose, three plant species offering different advantages have been selected, allowing to uncover the features of general relevance.

Vicia faba (2n=12), due to easy handling of root tip meristems and large size of metaphase chromosomes, is historically one of the best cytologically characterized plant species. Reconstructed karyotypes which allowed to distinguish individual chromosomes, proved to be a powerful tool in characterization of chromosomal domains (for instance by various banding techniques, as well as by localization of repetitive sequences by FISH; reviewed in Fuchs et al., 1998). Faba bean was the first plant species for which chromosomal distribution patterns of acetylated histones were reported (Houben et al., 1996), indicating a modulation of histone H4 acetylation during interphase (Belyaev et al., 1997) and differences between the chromosomal acetylation patterns of H3 and H4 (Belyaev et al., 1998).

Hordeum vulgare (2n=14) is regarded as an important monocot model plant, with a high level of synteny to other grasses, many of which are important crop plants (e.g., wheat, oat, rice, maize). Barley mitotic chromosomes are also cytologically well characterized and can be individually distinguished by visualizing heterochromatin located around centromeres and proximal parts of chromosome arms (Kakeda et al., 1991; Pedersen and Linde-Laursen, 1994; Pedersen et al., 1996), while most of genes are located at distal parts of chromosome arms (Künzel et al., 2000; Sandhu and Gill, 2002). Barley nuclei show the polar Rabl orientation of interphase chromosomes (Anamthawat-Jonsson and Heslop-Harrison, 1990; Noguchi and Fukui, 1995; Dong and Jiang, 1998). Structural chromosome mutants resulting in repression of one of the NORs are described (Schubert and Künzel, 1990).

Arabidopsis thaliana (2n=10) is considered as a model organism for many aspects of plant research. Its small genome (~125Mb) is nearly completely sequenced (The Arabidopsis

Genome Initiative, 2000). The small size of its metaphase chromosomes, uncomfortable for cytogenetic studies, is compensated by largely extended (~25-fold, compared to mitotic metaphase) meiotic pachytene chromosomes (Fransz et al., 1998). Heterochromatin makes up only a small fraction of the entire genome and in interphase nuclei is compartmentalized around the centromeres as distinct chromocenters (Fransz et al., 2002). The potential for cytogenetical research of Arabidopsis is enormous due to the high (and steadily increasing) number of molecularly and phenotypically characterized mutants, which influence nuclear architecture and chromatin organization.

1.6. Aims of the work

In 1999, at the beginning of the experimental work for this thesis, chromatin modifications were mostly studied at the level of genes and studies at the level of entire nuclei and distinct chromatin domains therein to provide more global insight were still lacking for plants. One reason for this was the difficulty to combine immunolabeling of chromosomal components and fluorescent *in situ* hybridisation (FISH) to visualize DNA sequences of interest, together with the determination of the cell cycle on the same nucleus.

Therefore, the first task was to establish an approach for large-scale isolation of nuclei with maintained *in vivo*-structure from meristematic tissues (containing naturally cycling cells) and their sorting by flow-cytometry according to the DNA content into different cell cycle fractions. The nuclear fractions had to be tested as to their suitability for immunolabeling of histone isoforms and other chromatin components in combination with FISH to identify chromatin domains such as NORs/nucleoli, euchromatin and heterochromatin fractions.
After establishing this approach for faba bean root meristems, it had to be adapted for other plant species (barley, Arabidopsis), and other tissues, e.g. from leaves and flower buds.

This should elucidate chromatin modification dynamics and chromatin organization in plants in correlation to nuclear processes such as replication, transcription and heterochromatin formation and help to answer the following questions:

In *Vicia faba* and barley:

a) What are the histone H3 and H4 acetylation patterns of specific chromatin domains (NORs/nucleoli, euchromatin, heterochromatin)? Are these patterns stable or dynamic throughout the cell cycle? If dynamic, to what processes can the different levels of histone modifications be correlated?

For instance: What is the relation of histone acetylation and on-going DNA replication?
Is histone acetylation correlated with the transcriptional activity of chromatin domains?

b) Are histone acetylation patterns of chromatin domains and their dynamics similar in monocot and dicot plants?
c) Do active and inactive NORs in barley differ in histone acetylation levels?

In barley additionally:

a) Is the polar chromatin organization (so-called Rabl orientation) of barley chromosomes maintained along interphase?
b) What is the sequence of replicating domains in S-phase? At which point of S-phase do centromeres replicate?

In Arabidopsis:

a) Are the distribution patterns of acetylated histone H3 and H4 and their cell cycle-dependent dynamics comparable to that of observed for plants with large genomes (faba bean, barley)?
b) What is the chromosomal distribution and dynamics of methylated H3 isoforms (H3 dimethylK9 vs. H3 dimethylK4) in interphase and dividing nuclei?
c) What is the relationship between DNA methylation, histone acetylation and methylation during assembly of constitutive heterochromatin? (To be studied in mutants with defects in DNA methylation (*ddm1, met1*) or H3K9 methylation (*kyp*) in comparison to wild-type plants.)

2. Summary of results

1 An approach to combine immunodetection of chromatin modifications with FISH for identification of chromatin domains, run-on transcription (to detect transcriptional activity of chromatin domains), and BrdU incorporation (to reveal replicating chromatin) on isolated nuclei flow-sorted into different cell cycle fractions (G1, earlyS, midS, lateS, G2) was established for faba bean [1] and barley [2] root meristems, as well as for various Arabidopsis tissues [3; 4].

2 Histone acetylation in *Vicia faba* and barley

a) *Histone H4 (but not H3) acetylation of euchromatin and heterochromatin is cell cycle dependent and correlated with replication rather than with transcription.*

The chromosomal acetylation patterns of histones H3 and H4 differed in *V.faba*. H3 acetylation (at lysines 9/18 and 14) was more intense at NORs/nucleoli and within the proximal DAPI-positive heterochromatin fraction lacking 59 bp Fok-tandem repeats, while interstitial Fok-element-containing heterochromatin (comprising ~70% of cytologically defined heterochromatin in faba bean) showed less intense acetylation than euchromatin. This labeling pattern was detected in the majority of nuclei of all cell cycle stages indicating that H3 acetylation is fairly constant along interphase.

In contrast, H4 acetylation at lysines 5, 8, 12 and 16 showed pronounced cell cycle-dependent modulation at NORs/nucleoli, euchromatin, and heterochromatin. NORs and nucleoli showed the highest levels of acetylated H4 during mitosis, G1 and G2 phases, but they lacked acetylated H4 (and even unmodified H4) during S-phase. Euchromatin revealed the most intense acetylation in midS. All heterochromatin fractions were weakly acetylated during mitosis, G1, and most of S-phase, but revealed strong acetylation at lysines 5, 12 and 16 of H4 in lateS/earlyG2. Toward mitosis, heterochromatin became underacetylated below the level detected at euchromatin. Deacetylation of lysine 16 was delayed until mitosis, and occasionally even until next G1 [1].

Experiments using a short BrdU-pulse to visualize replicating DNA combined with histone immunodetection revealed in *V.faba* nuclei increased H4 acetylation levels at euchromatin during early- and midS and at heterochromatin during lateS/earlyG2 indicating a close correlation of a deposition-related acetylation of H4 and DNA replication [1].

Experiments combining BrUTP incorporation (to label on-going transcriptional activity of chromatin in unfixed isolated nuclei) and histone H4 acetylation in *V.faba* nuclei did

not support a tight correlation between transcription and histone acetylation at the level of chromatin domains. Nucleoli showed intense transcription irrespective of histone H4 acetylation level. Euchromatin revealed less intense BrUTP-immunosignals, and a variable overlap of immunosignals for BrUTP and H4 acetylated at lysine 5 (besides complete and partial colocalization of immunosignals, frequently non-overlapping signals were detected). Heterochromatin domains generally lacked BrUTP-immunosignals, as expected. The well-documented fine-tuned acetylation at nucleosomes containing gene promoters is thus masked by the pronounced replication-linked H4 acetylation of larger chromatin domains [1].

b) *Histone H3 and H4 acetylation patterns of the monocot H.vulgare and dicot V.faba are largely similar but not identical.*

The monocot barley in general revealed many similarities with the dicot *Vicia faba* as to the preservation of histone acetylation patterns.

H3 acetylation remained constant along cell cycle. While acetylation of K14 was more intense at the telomeric pole harbouring most gene-rich chromatin, acetylation of K9/18 was uniformly distributed in interphase nuclei.

H4 acetylation at lysines 5, 12, and in addition at lysine 8 (but not at lysine 16 as in *V.faba*), showed a cell cycle-dependent modulation which was correlated with the replication timing of euchromatin and heterochromatin. Increased levels of H4 acetylation were detected also at centromeres around their replication time. Lysine 16 of H4 was more acetylated at the telomeric pole along the entire interphase [2].

c) *High histone acetylation levels at plant rDNA-containing chromatin do not correspond with its transcriptional activity.*

The active NOR6, as well as the inactive NOR7 of the translocation line T2052 carrying both barley NORs at the opposite arms of chromosome 6, showed high acetylation levels at lysines 5 and 12 of H4 during mitosis. Moreover, in wild-type, condensed (and transcriptionally inactive) rDNA-containing ‘knobs’ located outside nucleoli revealed strong H4 acetylation at lysines 5 and 8 in a considerable fraction of interphase nuclei indicating that H4 acetylation is not tightly linked to the transcriptional activity of rDNA-containing chromatin [2]. Thus it is unlikely that H4 deacetylation represents a general way of silencing the NORs as suggested by Chen and Pikaard (1997).

3 Interphase chromatin organization in barley

a) *The polar nuclear organization is preserved throughout interphase.*

Barley interphase nuclei showed strong polar arrangement of chromosomes with telomeres and centromeres located at the opposite nuclear poles (Rabl-orientation), as shown by two-colour FISH experiments using the barley subtelomeric 118 bp repeat HvT01 (Schubert et al., 1998) and a BAC containing centromere-specific retroelements and satellite sequences (Hudakova et al., 2001). In addition, also heterochromatin (mainly located within the proximal regions of all 7 chromosomes and detected by FISH with $(GAA)_{10}$ oligonucleotides, Pedersen et al., 1996) was found to be clustered at the centromeric poles of barley nuclei. This chromosomal orientation was preserved throughout the entire interphase. In addition, centromeres (probably of homologous chromosomes) were found to associate most frequently (in 56-62% of nuclei) during midS/lateS [2].

b) *Replication of chromatin domains is temporally ordered and reflects the polar nuclear organization.*

The sequence of replication of chromatin domains was determined by BrdU pulse-labeling *in vivo*, followed by immediate fixation and flow-sorting into defined S-phase fractions (early, middle, and lateS, respectively). Immunodetection of replicating domains at the time of fixation was combined with FISH using the barley centromere-specific BAC probe. The domains were replicating in the following order [2]:

NORs/nucleoli (earlyS) $\rightarrow$ euchromatin (first at the telomeric pole, earlyS/midS) $\rightarrow$ centromeres (midS/lateS) $\rightarrow$ heterochromatin (lateS)

This showed that plant centromeres apparently replicate before heterochromatin, similar as in mammals (Bartholdi, 1991, O'Keefe et al., 1992) and Drosophila (Ahmad and Henikoff, 2001; Sullivan and Karpen, 2001).

4 Histone modifications and heterochromatin formation in *Arabidopsis thaliana*

a) *Nuclear histone acetylation patterns in Arabidopsis differ from those of other plants.*

Euchromatin was persistently enriched in H4 acetylated at lysines 5, 8 and 12, while nucleoli and heterochromatic chromocentes of Arabidopsis were not labeled in root and leaf nuclei. Similar patterns were detected also in endopolyploid 8C nuclei of these tissues. A pronounced cell cycle-dependent modulation was found only for H4 acetylated at lysine 16, with most pronounced acetylated of euchromatin and heterochromatic chromocenters around their replication. This is in contrast to faba bean (which showed cell cycle-dependent acetylation at lysines 5, 12, 16, and also at lysine 8 in euchromatin and nucleoli) and barley (cell cycle-dependent modulation at

lysines 5, 8, 12). Additionally, both faba bean and barley revealed intense H4 acetylation within nucleoli in G1 and G2 phases, which was not the case in Arabidopsis. Unlike faba bean and barley, Arabidopsis showed cell cycle-dependent and apparently replication-linked modulation of acetylation of lysine 18 (but not lysine 9) of H3 within euchromatin and heterochromatin [4].

b) *Heterochromatic chromocenters are enriched in methylated H3K9, whereas euchromatin is enriched in methylated H3K4.*

The labeling patterns of methylated H3 were found to be rather constant throughout the cell cycle. Immunosignals of H3-dimethylK9 occurred preferentially at the chromocenters in Arabidopsis root, leaf, and flower bud nuclei. Heterochromatin remained labeled also during mitotic and meiotic division stages. Euchromatin showed only barely detectable immunosignals, and nucleoli were generally unlabeled.

High levels of H3-dimethylK4 occurred in euchromatin of interphase nuclei, as well as of mitotic and meiotic chromosomes, while NORs/nucleoli and heterochromatic chromocenters did not yield detectable immunosignals [3; 4].

The general enrichment of methylated H3K9 in heterochromatin and of methylated H3K4 in euchromatin of Arabidopsis is comparable to the distribution of methylated histones found for fission yeast (Noma et al., 2001). However, plants with large genomes (>1pg/2C) have euchromatin enriched in H3-dimethylK4 and additionally levels of H3-dimethylK9 as high as in heterochromatin (Houben et al., *in press*).

c) *Maintenance DNA methylation precedes H3K9 methylation during assembly of constitutive heterochromatin; both modifications are epigenetically inherited; assembly of constitutive heterochromatin does not require high levels of H3-dimethylK9.*

The Arabidopsis mutants *ddm1* (ddm1 encodes a SWI2/SNF2-like chromatin remodeling factor) and *met1* (*MET1* represents a major DNA maintenance methyltransferase), with overall DNA methylation decreased by ~70%, enabled to investigate the relationship between chromatin organization, DNA methylation and histone modifications (acetylation, methylation) in comparison to wild-type plants. Both mutants showed a reduction of chromocenter size caused by dispersion of pericentromeric low-copy sequences away from heterochromatin [3]. The intensity of immunosignals for methylated DNA and methylated H3K9 at the chromocenters was decreased in both *met1* and *ddm1*, implying that maintenance DNA methylation at CpG directs H3K9 methylation during heterochromatin assembly. Furthermore, F1-

hybrids between wild-type and DNA hypomethylation mutants revealed that DNA methylation (at CpG sites) and subsequent H3K9 methylation are epigenetically inherited and represent the genomic imprint required for maintenance of wild-type pericentromeric heterochromatin [3].

These findings seem to contradict those reported for Neurospora (Tamaru and Selker, 2001) and Arabidopsis (Jackson et al., 2002) which suggest that DNA methylation is directed by H3K9 methylation, and also that the loss of DNA methylation in *ddm1* might be due to reduced H3K9 methylation at heterochromatin (Gendrel et al., 2002). This apparent contradiction finds its explanation by the fact that only chromomethylase (CMT3) of Arabidopsis, specifically methylating non-CpG sites (Lindroth et al., 2001), is H3K9 methylation-dependent (Jackson et al., 2002) while only CpG methylation (maintained by MET1) is quantitatively relevant for Arabidopsis chromocenters. During post-replicative heterochromatin assembly CpG maintenance DNA methylation induces H3K9 methylation, and this in turn regulates CpNpG methylation. The positive feedback might then lead to spreading of heterochromatic features from chromatin containing high-copy repeats to that containing low copy pericentromeric sequences.

In addition, the chromatin remodeling factor DDM1 was found to be required for deacetylation of lysine 16 of histone H4 after DNA replication. Based on these data, a molecular definition for constitutive heterochromatin and a model for its reassembling after replication was proposed, according which H3K9 methylation is performed simultaneously, or shortly after maintenance CpG DNA methylation, and before deacetylation of H4K16. When in the absence of DDM1 or MET1 DNA methylation falls below a certain level, pericentromeric regions may acquire euchromatic features (elevated H3K4 methylation and H4 acetylation), and disperse from heterochromatic chromocenters [3]. An even more pronounced size reduction of chromocenters, with dispersion of centromeric pAL1 repeats was found in plants containing a stronger mutant allele of ddm1 (Probst et al., 2003).

High levels of H3-dimethylK9 were found to be not necessary for formation of heterochromatic chromocenters, because in *kryptonite* nuclei (lacking a major H3K9-specific histone methyltransferase) the chromocenter appearance and other chromatin modifications (DNA methylation at CpG sites, H3K4 methylation, H3K9 acetylation) were not affected [4]. Heterochromatic chromocenters are still present also in Drosophila lacking SU(VAR)3-9 (H3K9-specific histone methyltransferase) and mouse double null mutants for *Suv39h1* and *Suv39h2* (Schotta et al., 2002; Peters et al., 2001), supporting the idea that a high ratio between methylation of H3K9 and H3K4, rather than a high absolute level of H3K9 methylation, is crucial for assembly

and identity of constitutive heterochromatin [3]. This assumption is supported by the ratios of methylated H3K9 and H3K4 reported for plants with larger genomes (Houben et al., *in press*).

3. Conclusions

- Histone acetylation patterns revealed evolutionary highly conserved as well as more variable features. Contrary to animals, in plants the chromosomal acetylation patterns of histones H4 and H3 may differ. Cell cycle-dependent modulation of acetylation was detected in all three plant species (faba bean, barley, Arabidopsis) but for different lysine residues.

- The global acetylation of histone H4 within eu- and heterochromatin is correlated with replication rather than with transcription, masking the fine-tuning of acetylation at nucleosomes of active promoters. Deposition-related strong acetylation of histone H4 at freshly replicated chromatin seems to be highly conserved (detected in protozoa, insects, mammals and plants), although it might concern different lysine residues, even among dicot plants. It is probably required for post-replicational repair (Bird et al., 2002) and for DNA maintenance methylation.

- Constitutive heterochromatin in Arabidopsis wild-type plants is characterized by tandem and dispersed repeats and high levels of DNA and H3K9 methylation. Both DNA methylation and H3-dimethylK9 imprints are epigenetically inherited. During post-replicative heterochromatin assembly, maintenance (CpG) DNA methylation precedes methylation of H3K9. In *kyp*, lacking the major H3K9-specific histone methyltransferase, heterochromatic chromocenters were of wild-type appearance, suggesting that the ratio of H3K9 *vs.* H3K4 methylation is more relevant for the identity of heterochromatin (similar as in plants with large genomes) than high absolute levels of H3K9 methylation.

4. Zusammenfassung der wichtigsten Ergebnisse und Schlussfolgerungen

Vergleichende Untersuchungen an der einkeimblättrigen Gerste (*Hordeum vulgare*) und den zweikeimblättrigen Arten Ackerbohne (*Vicia faba*) und Ackerschmalwand (*Arabidopsis thaliana*) über die potentielle Dynamik von Histonmodifikationen in spezifisch definierten Chromatindomänen während des Zellzyklus, in Relation zu Replikations- und Transkriptionsvorgängen, und bei der postreplikativen Neuformierung von konstitutivem Heterochromatin auf cytologischer Ebene führten zu folgenden Ergebnissen:

1 Ein Ansatz zum gleichzeitigen Nachweis von Chromatinmodifikationen, Replikations- und Transkriptionsvorgängen über Immunfärbung, und zum Ansprechen spezifischer Chromatindomänen (NOR/Nukleoli, Eu- und Heterochromatinfraktionen) über Fluoreszenz-in situ Hybridisierung in nach DNA-Gehalt flow-sortierten pflanzlichen Zellkernen unterschiedlicher Zellzyklus- und Ploidiestadien wurde etabliert.

2 Die Acetylierung von Histon H4 (aber nicht H3) ist in *V. faba* zellzyklusabhängig moduliert. Die Lysinreste (K) 9/18 und 14 von H3 sind relativ konstant am stärksten in proximalem (Fok-element-freien) Heterochromatin, in NORs und Nucleoli, schwächer im Euchromatin und am schwächsten in Fok-element-haltigen Heterochromatinregionen acetyliert.
Die Acetylierung der Lysinreste 5, 8, 12 und 16 von H4 ist zellzyklusabhängig moduliert. Eu- und Heterochromatin weisen die stärkste Acetylierung während ihrer Replikation auf. Bis zur Mitose erfolgt im Heterochromatin eine Deacetylierung unter das Niveau der Acetylierung im Euchromatin; Nukleoli bzw. NORs sind in G1, G2 bzw. Mitose stark acetyliert, nicht jedoch während der S-Phase.

3 Die globale zellzyklusabhängige Acetylierung von H4 ist mit der Replikation (BrdU-Einbau) korreliert, nicht jedoch mit Transkriptionsaktivität (BrUTP-Einbau) der jeweiligen Chromatindomänen.

4 Histon H3- und H4-Acetylierungsmuster der monokotylen Gerste und der dikotylen Ackerbohne sind ähnlich aber nicht identisch. Die H3-Acetylierung bleibt auch in Gerste relativ konstant entlang des Zellzyklus. K14 ist im telomerischen (euchromatischen) Kernpolbereich am stärksten acetyliert; K9/18-Acetylierung ist ± uniform über den Zellkern verteilt.

Die H4-Acetylierung an K5, 12 und zusätzlich an K8 (aber nicht an K16 wie in V. faba) ist zellzyklusabhängig moduliert. K16 ist konstant am stärksten im telomerischen Polbereich acetyliert. Auch für Gerste ist die höchste H4-Acetylierungsintensität an Eu- und Heterochromatin mit der Replikation nicht aber mit der Transkriptionsaktivität korreliert. Aktive und inaktive Nukleolusorganisatoren (NOR6 versus NOR7) der Translokationslinie T2052 (mit beiden NORs an gegenüberliegenden Armen von Chromosom 6) wie auch transkriptionsinaktive rDNA-'knobs' in Interphasekernen weisen einen gleichmäßig hohen Acetylierungsgrad auf.

5 Nukleäre Acetylierungsmuster in Arabidopsis weisen Unterschiede zu denen in Ackerbohne und Gerste auf. Im Euchromatin sind K5, 8 und 12 von H4 gleichbleibend stark acetyliert, während Nukleoli und Heterochromatin durchgängig kaum Acetylierung aufweisen. Nur K16 von H4 ist in Eu- und Heterochromatin während der jeweiligen Replikation am stärksten acetyliert. Dagegen liegt im Unterschied zur Ackerbohne und Gerste zusätzlich eine replikationsgekoppelte Acetylierung des Lysinrestes 18 von H3 in Eu- und Heterochromatin vor.

6 Die heterochromatischen Chromozentren von Arabidopsis weisen Zellzyklus-unabhängig eine starke Methylierung von H3K9, und das Euchromatin eine starke Methylierung von H3K4 auf. In NORs/Nukleoli sind beide Lysinreste kaum methyliert.

7 DNA-'maintenance'-Methylierung kontrolliert die H3K9-Methylierung während der Assemblierung des konstitutiven Heterochromatins von Arabidopsis. Die DNA-Methylierungsmutanten *ddm1* (ddm1 kodiert für einen SWI2/SNF2-ähnlichen Chromatin-Remodellierungsfaktor) und *met 1* (met1 kodiert eine ‚major maintenance'-Methyltransferase) mit einer 70%-igen Reduktion der DNA-Methylierung weisen eine Größenreduktion der Chromozentren (um ~30%) auf, die mit einer Dispersion pericentromerischer 'low copy' Sequenzen aus dem Heterochromatin in das Euchromatin einhergeht. Die Methylierung von DNA als auch von H3K9 ist in den Chromozentren beider Mutanten im Vergleich zum Wildtyp stark vermindert. Das weist daraufhin, dass CpG-'maintenance'-Methylierung die H3K9-Methylierung während der postreplikativen Reasssemblierung von konstitutivem Heterochromatin steuert. H3K9-Methylierung ihrerseits reguliert die Methylierung an CpNpG-Sequenzen (Jackson et al. 2002).

Der erhöhte Anteil von *ddm1*-Kernen mit starker Acetylierung von H4K16 in den Chromozentren weist auf eine zusätzliche Funktion von DDM1 bei der postreplikativen Deacetylierung hin.

8 DNA- und H3K9-Methylierung werden epigenetisch vererbt (wie für Chromozentren der Mutanteneltern in Zellkernen von F1-Hybriden zwischen Wildtyp und DNA-Hypomethylierungsmutanten gezeigt wurde) und dienen als Imprint für die Bildung von konstitutivem Heterochromatin in Wildtyp-Pflanzen.

9 Die Ausbildung von konstitutivem Heterochromatin erfordert keine überdurchschnittlich starke Methylierung von H3K9 wie in Kernen der *kryptonite*-Mutante gefunden wurde, der eine H3K9-spezifische Histonmethyltransferase fehlt, die aber in der DNA-Methylierung an CpG-Orten, der H3K4-Methylierung, der H3K9-Acetylierung und der Chromozentrenstruktur dem Wildtyp gleicht.

Aus diesen Daten können folgende Schlüsse gezogen werden:

i) Chromosomale und nukleäre Histonacetylierungsmuster weisen evolutionär konservierte wie auch variable Merkmale auf. Eine zellzyklusabhängige Modulation der Acetylierungsintensität tritt bei allen untersuchten Pflanzenarten auf. Diese kann jedoch artspezifisch unterschiedliche Lysinreste betreffen.

ii) Die global hohe Acetylierungsintensität von Histonen in Eu- und Heterochromatin korreliert mit der Replikation nicht aber mit der Transkriptionsaktivität und überlagert die fein-abgestimmte Acetylierung an Nukleosomen aktiver Promotersequenzen. Die replikationsgekoppelte Acetylierung ist wahrscheinlich für den Ablauf der ubiquitären Postreplikationsreparatur (Bird et al. 2002) und die ‚maintenance'-Methylierung der DNA erforderlich.

iii) Konstitutives Heterochromatin von Arabidopsis ist durch einen hohen Gehalt an Tandem- und anderen Repeat-Sequenzen mit starker DNA-Methylierung (CpG) charakterisiert. Während der postreplikativen Reassemblierung des Heterochromatins geht die DNA-Methylierung der H3K9-Methylierung voran. Ein weites Verhältnis von H3K9- zu H3K4-Methylierung ist jedoch eher von Bedeutung für die Heterochromatinausprägung als eine überdurchschnittlich hohe Methylierung von H3K9. Dies betätigen auch an anderen Organismen erhobene Befunde (Peters et al. 2001, Schotta et al. 2002, Houben et al. in press).

5. References

ABRANCHES R, BEVEN AF, ARAGON-ALCAIDE L, SHAW PJ (1998) Transcription sites are not correlated with chromosome territories in wheat nuclei. J Cell Biol 143:5-12

AGALIOTI T, CHEN G, THANOS D (2002) Deciphering the transcriptional histone acetylation code for a human gene. Cell 111:381-392

AHMAD K, HENIKOFF S (2001) Centromeres are specialized replication domains in heterochromatin. J Cell Biol 153:101-109

AHMAD K, HENIKOFF S (2002) The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. Mol Cell 9:1191-1200

ALLFREY V, FAULKNER RM, MIRSKY AE (1964) Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. Proc Natl Acad Sci USA 51:786-794

ANAMTHAWAT-JONSSON K, HESLOP-HARRISON JS (1990) Centromeres, telomeres and chromatin in the interphase nucleus of cereals. Caryologia 43:205-213

BANNISTER AJ, ZEGERMAN P, PARTRIDGE JF, MISKA EA, THOMAS JO, ALLSHIRE RC, KOUZARIDES T (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromodomain. Nature 410:120-124

BANNISTER AJ, SCHNEIDER R, KOUZARIDES T (2002) Histone methylation: Dynamic or static? Cell 109:801-806

BARTHOLDI MF (1991) Nuclear distribution of centromeres during the cell cycle of human diplod fibroblasts. J Cell Sci 99:255-263

BELMONT AS, DIETZEL S, NYE AC, STRUKOV YG, TUMBAR T (1999) Large-scale chromatin structure and function. Curr Opin Cell Biol 11:307-311

BELYAEV ND, HOUBEN A, BARANCZEWSKI P, SCHUBERT I (1997) Histone H4 acetylation in plant heterochromatin is altered during the cell cycle. Chromosoma 106:193-197

BELYAEV ND, HOUBEN A, BARANCZEWSKI P, SCHUBERT I (1998) The acetylation patterns of histones H3 and H4 along plant chromosomes are different. Chromosome Res 6:59-63

BERNARD P, MAURE JF, PARTRIDGE JF, GENIER S, JAVERZAT JP, ALLSHIRE RC (2001) Requirement of heterochromatin for cohesion at centromeres. Science 294:2539-2542

BERNSTEIN BE, HUMPHREY EL, ERLICH RL, SCHNEIDER R, BOUMAN P, LIU JS, KOUZARIDES T, SCHREIBER SL (2002) Methylation of histone H3 Lys4 in coding regions of active genes. Proc Natl Acad Sci USA 99:8695-8700

BIRD AW, YU DY, PRAY-GRANT MG, QIU Q, HARMON KE, MEGEE PC, GRANT PA, MITCHELL SMITH M, CHRISTMAN MF (2002) Acetylation of histone H4 by Esa1 is required for DNA double-strand break repair. Nature 419:411-415

BRIGGS SD, BRYK M, STRAHL BD, CHEUNG WL, DAVIE JK, DENT SYR, WINSTON F, ALLIS CD (2001) Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in *Saccharomyces cerevisiae*. Genes Dev 15:3286-3295

BRIGGS SD, XIAO T, SUN Z-W, CALDWELL JA, SHABANOWITZ J, HUNT DF, ALLIS CD, STRAHL BD (2002) *Trans*-histone regulatory pathway in chromatin. Nature 418:498

BROWN DT (2001) Histone variants: are they functionally heterogeneous? Genome Biol 2:reviews0006.1-0006.6

BROWNELL JE, ZHOU J, RANALLI T, KOBAYASHI R, ROTH SY, ALLIS CD (1996) Tetrahymena histone acetyltransfearse A: a homolog to yeast GCN5 linking histone acetylation to gene activation. Cell 84:843-851

CHEN ZJ, PIKAARD CS (1997) Epigenetic silencing of RNA polymerase I transcription: a role for DNA methylation and histone modification in nucleolar dominance. Genes Dev 11:2124-2136

CHEUNG P, TANNER KG, CHEUNG WL, SASSONE-CORSI P, DENU JM, ALLIS CD (2000a) Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation. Mol Cell 5 :905-915

CHEUNG P, ALLIS CD, SASSONE-CORSI P (2000b) Signalling to chromatin through histone modifications. Cell 103:263-271

CONSTANZI C, PEHRSON JR (1998) Histone macroH2A1 is concentrated in the inactive X chromosome of female mammals. Nature 393:599-601

CREMER T, KURZ A, ZIRBEL R, DIETZEL S, RINKE B, SCHRÖCK E, SPEICHER MR, MATHIEU U, JAUCH A, EMMERICH P, SCHERTHAN H, RIED T, CREMER C, LICHTER P (1993) Role of chromosome territories in

the functional compartmentalization of the cell nucleus. Cold Spring Harbor Symp Quant Biol 58:777-792

CREMER T, CREMER C (2001) Chromosome territories, nuclear architecture and gene regulation in mammalian cells. Nat Rev Genet 2:292-301

DAUJAT S, BAUER U-M, SHAH V, TURNER B, BERGER S, KOUZARIDES T (2002) Crosstalk between CARM1 methylation and CBP acetylation on histone H3. Curr Biol 12:2090-2097

DELANGE RJ, FAMBROUGH DM, SMITH EL, BONNER J (1969) Calf and pea histone IV. 3. Complete amino acid sequence of pea seedling histone IV: comparison with the homologous calf thymus histone. J Biol Chem 244:5669-5679

DENNIS K, FAN T, GEIMAN TM, YAN Q, MUEGGE K (2001) Lsh, a member of the SNF2 family, is required for genome wide methylation. Genes Dev 15:2940-2944

DONG F, JIANG J (1998) Non-Rabl patterns of centromere and telomere distribution in the interphase nuclei of plant cells. Chromosome Res 6:551-558

EISSENBERG JC, ELGIN SC (2000) The HP1 protein family: getting grip on chromatin. Curr Opin Genet Dev 10:204-210

EKWALL K, JAVERZAT JP, LORENTZ A, SCHMIDT H, CRANSTON G, ALLSHIRE R (1995) The chromodomain protein Swi6: a key component at fission yeast centromeres. Science 269:1429-1431

FAAST R, THONGLAIROAM V, SCHULZ TC, BEALL J, WELLS JRE, TAYLOR H, MATTHAEI K, RATHJEN PD, TREMETHICK DJ, LYONS I (2001) Histone variant H2A.Z is required for early mammalian development. Curr Biol 11:1183-1187

FENG Q, WANG H, NG HH, ERDJUMENT-BROMAGE H, TEMPST P, STRUHL K, ZHANG Y (2002) Methylation of H3-lysine 79 is mediated by a new HMTases without a SET domain. Curr Biol 12:1052-1058

FOUREL G, LEBRUN E, GILSON E (2002) Protosilencers as building blocks for heterochromatin. BioEssays 24:828-835

FRANSZ P, ARMSTRONG S, ALONSO-BLANCO C, FISCHER TC, TORRES-RUIZ RA, JONES G (1998) Cytogenetics for the model system *Arabidopsis thaliana*. Plant J 13:867-876

FRANSZ P, DE JONG JH, LYSAK M, RUFFINI CASTIGLIONE M, SCHUBERT I (2002) Interphase chromosomes in Arabidopsis are organized as well defined chromocenters from which euchromatin emanate. Proc Natl Acad Sci USA 99:14584-14589

FUCHS J, STREHL S, BRANDES A, SCHWEIZER D, SCHUBERT I (1998) Molecular-cytogenetic characterization of the Vicia faba genome – heterochromatin differentiation, replication patterns and sequence localization. Chromosome Res 6:219-230

GENDREL AV, LIPPMAN Z, YORDAN C, COLOT V, MARTIENSSEN R (2002) Dependence of heterochromatic histone H3 methylation patterns on the *Arabidopsis* gene *DDM1*. Science 297:1871-1873

GRUNSTEIN M (1997) Histone acetylation in chromatin structure and transcription. Nature 389:349-352

GRUNSTEIN M (1998) Yeast heterochromatin: regulation of its assembly and inheritance by histones. Cell 93:325-328

HEBBES TR, THORNE AW, CRANE-ROBINSON C (1988) A direct link between core histone acetylation and transcriptionally active chromatin. EMBO J 7:1395-1402

HEITZ E (1928) Das Heterochromatin der Moose. Jahrb Wiss Botanik 69:762-818

HENDZEL MJ, WEI Y, MANCINI MA, VAN HOOSER A, RANALLI T, BRINKLEY BR, BAZETT-JONES DP, ALLIS CD (1997) Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma 106:348-360

HENIKOFF S (2000a) Heterochromatin function in complex genomes. Biochim Biophys Acta 1470:1-8

HENIKOFF S, AHMAD K, PLATERO JS, VAN STEENSEL B (2000b) Heterochromatic deposition of centromeric histone H3-like proteins. Proc Natl Acad Sci USA 97:716-21

HORN PJ, PETERSON CL (2002) Chromatin higher order folding: Wrapping up transcription. Science 297:1824-1827

HOUBEN A, WAKO T, FURUSHIMA-SHIMOGAWARA R, PRESTING G, KÜNZEL G, SCHUBERT I, FUKUI K (1999) The cell cycle dependent phosphorylation of histone H3 is correlated with the condensation of plant mitotic chromosomes. Plant J 18:675-679

HOUBEN A, BELYAEV ND, TURNER BM, SCHUBERT I (1996) Differential immunostaining of plant chromosomes by antibodies recognizing acetylated histone H4 variants. Chromosome Res 4:191-194

HOUBEN A, DEMIDOV D, GERNAND D, MEISTER A, LEACH CR, SCHUBERT I (2003) Methylation of histone H3 in euchromatin of plant chromosomes depends on basic nuclear DNA content. Plant J, *in press*

HUDAKOVA S, MICHALEK W, PRESTING GG, TEN HOOPEN R, DOS SANTOS K, JASENCAKOVA Z, SCHUBERT I (2001) Sequence organization of barley centromeres. Nucleic Acids Res 29:5029-5035

IKURA T, OGRYZKO VV, GRIGORIEV M, GROISMAN R, WANG J, HORIKOSHI M, SCULLY R, QIN J, NAKATANI Y (2000) Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. Cell 102:463-473

JACKSON JP, LINDROTH AM, CAO X, JACOBSEN SE (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature 416:556-560

JEDDELOH JA, STOKES TL, RICHARDS EJ (1999) Maintenance of genomic methylation requires a SWI2/SNF2-like protein. Nat Genet 22:94-97

JEPPESEN P, TURNER BM (1993) The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. Cell 74:281-289

JEPPESEN P (1997) Histone acetylation: A possible mechanism for the inheritance of cell memory at mitosis. BioEssays 19:67-74

JOHNSON LM, CAO X, JACOBSEN S (2002) Interplay between two epigenetic marks: DNA methylation and histone H3 lysine 9 methylation. Curr Biol 12:1360-1367

JONES PA, TAKAI D (2001) The role of DNA methylation in mammalian epigenetics. Science 293:1068-1070

JONES PL, VEENSTRA GJ, WADE PA, VERMAAK D, KASS SU, LANDSBERGER N, STROUBOULIS J, WOLFFE AP (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet 19:187-191

KAKEDA K, FUKUI K, YAMAGATA H (1991) Heterochromatic differentiation in barley chromosomes revealed by C- and N-banding techniques. Theor Appl Genet 81:144-150

KAKUTANI T, JEDDELOH JA, RICHARDS EJ (1995) Characterization of an *Arabidopsis thaliana* DNA hypomethylation mutant. Nucleic Acids Res 23:130-137

KELLUM R, ALBERTS BM (1995) Heterochromatin protein 1 is required for correct chromosome segregation in *Drosophila* embryos. J Cell Sci 108:1419-1431

KÜNZEL G, KORZUN L, MEISTER A (2000) Cytologically integrated physical restriction fragment length polymorphism maps for the barley genome based on translocation breakpoints. Genetics 154:397-412

LACHNER M, O'CARROLL D, REA S, MECHTLER K, JENUWEIN T (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature 410:116-120

LACHNER M, JENUWEIN T (2002) The many faces of histone lysine methylation. Curr Opin Cell Biol 14:286-298

LAMOND AI, EARNSHAW WC (1998) Structure and function in the nucleus. Science 280:547-553

LINDROTH AM, CAO X, JACKSON JP, ZILBERMAN D, MCCALLUM CM, HENIKOFF S, JACOBSEN SE (2001) Requirement of *CHROMOMETHYLASE3* for maintenance of CpXpG methylation. Science 292:2077-2080

LITT MD, SIMPSON M, GASZNER M, ALLIS CD, FELSENFELD G (2001) Correlation between histone lysine methylation and developmental changes at the chicken β–globin locus. Science 293:2453-2455

LO WS, TRIEVEL RC, ROJAS JR, DUGGAN L, HSU JY, ALLIS CD, MARMORSTEIN R, BERGER SL (2000) Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14. Mol Cell 5 :917-926

LYSAK MA, FRANSZ PF, ALI HBM, SCHUBERT I (2001) Chromosome painting in Arabidopsis thaliana. Plant J 28:689-697

LYSAK MA, PECINKA A, SCHUBERT I (2003) recent progress in chromosome painting of Arabidopsis and related species. Chromosome Res, *in press*

LU BY, EMTAGE PCR, DUYF BJ, HILLIKER AJ, EISSENBERG JC (2000) Heterochromatin protein 1 is required for the normal expression of two heterochromatin genes in Drosophila. Genetics 155:699-708

LUGER K, MÄDER AW, RICHMOND RK, SARGENT DF, RICHMOND TJ (1997) Crystal structure of te nucleosome core particle at 2.8 Å resolution. Nature 389 251:251-260

MARTIENSSEN RA, COLOT A (2001) DNA methylation and epigenetic inheritance in plants and filamentous fungi. Science 293:1070-1074

MCBLANE F, BOYES J (2000) Stimulation of V(D)J recombination by histone acetylation. Curr Biol 10:483-486

MCMURRY MT, KRANGEL MS (2000) A role for histone acetylation in the developmental regulation of V(D)J recombination. Science 287:495-498

MELUH PB, YANG P, GLOWCZEWSKI L, KOSHLAND D, SMITH MM (1998) Cse4p is a component of the core centromere of Saccharomyces cerevisiae. Cell 94:607-613

MOAZED D (2001) Common themes in mechanisms of gene silencing. Mol Cell 8:489-498

MURRAY K (1964) The occurrence of ε-methyl lysine in histones. Biochemistry 3:10-15

NAKAYAMA J, RICE JC, STRAHL BD, ALLIS CD, GREWAL SIS (2001) Role of histone H3 lysine methylation in epigenetic control of heterochromatin assembly. Science 292:110-113

NAN X, NG H-H, JOHNSON CA, LAHERTY CD, TURNER BM, EISENMAN RN, BIRD A (1998) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 393:386-389

NG H-H, ZHANG Y, HENDRICH B, JOHNSON CA, TURNER BM, ERDJUMENT-BROMAGE H, TEMPST P, REINBERG D, BIRD A (1999) MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. Nature Gen 23:58-61

NG H-H, BIRD A (1999) DNA methylation and chromatin modification. Curr Opin Gen Dev 9:158-163

NIELSEN SJ, SCHNEIDER R, BAUER U-M, BANNISTER AJ, MORRISON A, O'CARROLL D, FIRESTEIN R, CLEARY M, JENUWEIN T, HERRERA RE, KOUZARIDES T (2001) Rb targets histone H3 methylation and HP1 to promoters. Nature 412:561-565

NOGUCHI J, FUKUI K (1995) Chromatin arrangements in intact interphase nuclei examined by laser confocal microscopy. J Plant Res 108:209-216

NOMA K, ALLIS CD, GREWAL SIS (2001) Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. Science 293:1150-1155

NONAKA K, KITAJIMA T, YOKOBAYASHI S, XIAO G, YAMAMOTO M, GREWAL SIS, WATANABE Y (2001) Recruitment of cohesin to heterochromatic regions by Swi6/HP1 in fission yeast. Nat Cell Biol 4:89-93

O'KEEFE RT, HENDERSON SC, SPECTOR DL (1992) Dynamic organization of DNA replication in mammalian cell nuclei: spatially and temporally defined replication of chromosome-specific α-satellite DNA sequences. J Cell Biol 116:1095-1110

PAULL TT, ROGAKOU EP, YAMAZAKI V, KIRCHGESSNER CU, GELLERT M, BONNER WM (2002) A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol 10:886-895

PEDERSEN C, LINDE-LAURSEN I (1994) Chromosomal locations of four minor rDNA loci and a marker microsatellite sequence in barley. Chromosome Res 2:65-71

PEDERSEN C, RASMUSSEN SK, LINDE-LAURSEN I (1996) Genome and chromosome identification in cultivated barley and related species of the Triticeae (Poaceae) by in situ hybridization with the GAA-satellite sequence. Genome 39:93-104

PETERS AHFM, O'CARROL D, SCHERTHAN H, MECHTLER K, SAUER S, SCHÖFER C, WEIPOLTSHAMMER K, PAGANI M, LACHNER M, KOHLMAIER A, OPRAVIL S, DOYLE M, SIBILIA M, JENUWEIN T (2001) Loss of the *Suv39h* histone methyltransferases impairs mammalian heterochromatin and genome stability. Cell 107:323-337

PHILIPS DMP (1963) The presence of acetyl groups in histones. Biochem J 87:258-263

PROBST A, FRANSZ PF, PASZKOWSKI J, MITTELSTEN-SCHEID O (2003) Two means of transcriptional reactivation within heterochromatin. Plant J 33:743-749

RABL C (1885) Über Zelltheilung. Morphol Jahrb 10:214-330

REA S, EISENHABER F, O'CARROLL D, STRAHL BD, SUN Z, SCHMID M, OPRAVIL S, MECHTLER K, PONTING CP, ALLIS CD, JENUWEIN T (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 406:593-599

RICHARDS EJ, ELGIN SCR (2002) Epigentic codes for heterochromatin formation and silencing: Rounding up the usual suspects. Cell 108:489-500

ROUNTREE MR, BACHMAN KE, BAYLIN SB (2000) DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. Nat Genet 25:269-277

SANDHU D, GILL KS (2002) Gene-containing regions of wheat and the other grass genomes. Plant Physiol 128:803-811

SCHOTTA G, EBERT A, KRAUSS V, FISCHER A, HOFFMANN J, REA S, JENUWEIN T, DORN R, REUTER G (2002) Central role of *Drosophila* SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. EMBO J 21:1121-1131

SCHÜBERT I, KÜNZEL G (1990) Position-dependent NOR activity in barley. Chromosoma 99:352-359

SCHUBERT I, SHI F, FUCHS J, ENDO TR (1998) An efficient screening for terminal deletions and translocations of barley chromosomes added to common wheat. Plant J 14:489-495

SMITH JG, HILL RS, BALDWIN JP (1995) Plant chromatin structure and post-translational modifications. Crit Rev Plant Sci 14:299-328

SOBEL RE, COOK RG, PERRY CA, ANNUNZIATO AT, ALLIS CD (1995) Conservation of deposition-related acetylation sites in newly synthesized histones H3 and H4. Proc Natl Acad Sci USA 92:1237-1241

SPENCER VA, DAVIE JR (1999) Role of covalent modifications of histones in regulating gene expression. Gene 240:1-12

STRAHL BD, OHBA R, COOK RG, ALLIS CD (1999) Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in *Tetrahymena*. Poc Natl Acad Sci USA 96:14967-14972

STRAHL BD, ALLIS CD (2000) The language of covalent histone modifications. Nature 413:41-45

STRUHL K (1998) Histone acetylation and transcriptional regulatory mechanisms. Genes Dev 12:599-606

SULLIVAN B, KARPEN G (2001) Centromere identity in *Drosophila* is not determined in vivo by replication timing. J Cell Biol 154:683-690

SULLIVAN KF, HECHENBERGER M, MASRI K (1994) Human CENP-A contains a histone H3 related histone fold that is required for targeting to the centromere. J Cell Biol 127:581–592.

SUN Z-W, ALLIS CD (2002) Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. Nature 418:104-108

TACHIBANA M, SUGIMOTO K, NOZAKI M, UEDA J, OHTA T, OHKI M, FUKUDA M, TAKEDA N, NIIDA H, KATO H, SHINKAI Y (2002) G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. Genes Dev 16:1779-1791

TAKAHASHI K, CHEN ES, YANAGIDA M (2000) Requirement of Mis6 centromere connector for localizing a CENP-A-like protein in fission yeast. Science 288:2215-2219

TALBERT PB, MASUELLI R, TYAGI AP, COMAI L, HENIKOFF S (2002) Centromeric localization and adaptive evolution of an Arabidopsis histone H3 variant. Plant Cell 14:1053-1066

TAMARU H, SELKER EU (2001) A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. Science 414:277-283

THATCHER TH, GOROVSKY MA (1994) Phylogenetic analysis of the core histones H2A, H2B, H3, and H4. Nucleic Acids Res 22:174-179

THE ARABIDOPSIS GENOME INITIATIVE (2000) Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature 408:796-815

TSUKIYAMA T (2002) The *in vivo* functions of ATP-dependent chromatin-remodelling factors. Nat Rev Mol Cell Biol 3:422-429

TURNER BM, BIRLEY AJ, LAVENDER JS (1992) Histone H4 isoforms at specific lysine residues define individual chromosomes and chromatin domains in Drosophila polytene nuclei. Cell 69:375-384

TURNER BM (1993) Decoding the nucleosome. Cell 75:5-8

TURNER BM (1998) Histone acetylation as an epigenetic determinant of long-term transcriptional competence. Cell Mol Life Sci 54:21-31

TURNER BM (2000) Histone acetylation and an epigenetic code. BioEssays 22:936-845

TURNER BM (2002) Cellular memory and the histone code. Cell 111:285-291

VAN HOLDE KE (1989) Chromatin. Springer-Verlag

VAN LEEUWEN F, GAFKEN PR, GOTTSCHLING DE (2002) Dot1p modulates silencing in yeast by methylation of the nucleosome. Cell 109:745-756

VOLPE TA, KIDNER C, HALL IM, TENG G, GREWAL SIS, MARTIENSSEN R (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science 297:1833-1837

VONGS A, KAKUTANI T, MARTIENSSEN RA, RICHARDS EJ (1993) *Arabidopsis thaliana* DNA methylation mutants. Science 260:1926-1928

WALDEYER W (1888) Ueber Karyokinese und ihre Beziehungen zu den Befruchtungsvorgängen. Arch Mikr Anat 32:1-122

WATERBORG JH (1992) Identification of five sites of acetylation in alfalfa histone H4. Biochemistry 31:6211-6219

WOLFFE AP, HAYES JJ (1999) Chromatin disruption and modification. Nucleic Acids Res 27:711-720

ZHANG Y, NG H-H, ERDJUMENT-BROMAGE H, TEMPST P, BIRD A, REINBERG D (1999) Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev 13:1924-1935

ZHANG Y, REINBERG D (2001) Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes Dev 15:2343-2360

6. Print-outs of the publications on which this thesis is based and declaration on the contribution to these publications

[1] JASENCAKOVA Z, MEISTER A, WALTER J, TURNER BM, SCHUBERT I (2000) Histone H4 acetylation of euchromatin and heterochromatin is cell cycle dependent and correlated with replication rather than with transcription. Plant Cell 12:2087-2100

Most of experimental work (immunodetection of histones, FISH, data evaluation) was done by myself. Flow-cytometry was performed by Dr. A. Meister, and confocal microscopy by J. Walter.

[2] JASENCAKOVA Z, MEISTER A, SCHUBERT I (2001) Chromatin organization and its relation to replication and histone acetylation during the cell cycle in barley. Chromosoma 110:83-92

Experimental work, except flow-cytometry (which was performed by Dr. A. Meister) was done by myself.

[3] SOPPE WJJ, JASENCAKOVA Z, HOUBEN A, KAKUTANI T, HUANG MS, MEISTER A, JACOBSEN S, SCHUBERT I, FRANSZ PF (2002) DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in *Arabidopsis*. EMBO J 23:6549-6559

Experimental work was shared between me (histone immunodetection, combined with FISH) and Dr. W. Soppe (FISH, detection of DNA methylation). Flow-cytometry was performed by Dr. A. Meister.

[4] JASENCAKOVA Z, SOPPE WJJ, MEISTER A, GERNAND D, TURNER BM, SCHUBERT I (2003) Histone modifications in *Arabidopsis* – high methylation of H3 lysine 9 is dispensable for constitutive heterochromatin. Plant J 33:471-480

Experimental work was shared between me (histone immunodetection, combined with FISH), Dr. W. Soppe (detection of DNA methylation), and Dr. D. Gernand (histone immunodetection on Arabidopsis pachytene chromosomes). Flow-cytometry was performed by Dr. A. Meister.

6.1. JASENCAKOVA Z, MEISTER A, WALTER J, TURNER BM, SCHUBERT I

Histone H4 acetylation of euchromatin and heterochromatin is cell cycle dependent and correlated with replication rather than with transcription.

Plant Cell 12:2087-2100, 2000

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Histone H4 Acetylation of Euchromatin and Heterochromatin Is Cell Cycle Dependent and Correlated with Replication Rather Than with Transcription

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Reversible acetylation of nucleosomal histones H3 and H4 generally is believed to be correlated with potential transcriptional activity of eukaryotic chromatin domains. Here, we report that the extent of H4 acetylation within euchromatin and heterochromatic domains is linked with DNA replication rather than with transcriptional activity, whereas H3 acetylation remains fairly constant throughout the cell cycle. Compared with euchromatin, plant nucleolus organizers were more strongly acetylated at H4 during mitosis but less acetylated during S phase, when the nucleolus appeared to be (at least transiently) devoid of nucleosomes. Deposition-related acetylation of lysines 5 and 12 of H4 seems to be conserved in animals and plants and extended to K16 in plants. A possibly species-specific above-average acetylation at lysines 9/18 and 14 of H3 appeared in 4′,6-diamidino-2-phenylindole (DAPI)–stained heterochromatin fractions. These results were obtained by combining immunodetection of all acetylatable isoforms of H3 and H4 on mitotic chromosomes and nuclei in G1, early S, mid-S, late S, and G2 phases of the field bean with identification of specific chromatin domains by fluorescence in situ hybridization or DAPI staining. In addition, the histone acetylation patterns of distinct domains were compared with their replication and transcription patterns.

INTRODUCTION

The histone proteins H2A, H2B, H3, and H4 form octamers that constitute the nucleosome core particles in all eukaryotes. Their N-terminal tails are subject to post-translational modifications such as acetylation, phosphorylation, methylation, ubiquitination, glycosylation, and ADP ribosylation (reviewed in Smith et al., 1995; Spencer and Davie, 1999).

The reversible acetylation of N-terminal lysine residues at positions 5, 8, 12, and 16 of H4 and 9, 14, 18, and 23 of H3 mediates decondensation of the nucleosome structure (Loidl, 1988, 1994; Garcia-Ramirez et al., 1995), alters histone–DNA interactions (Hong et al., 1993), and facilitates access and binding of transcription factors to genes transcribed by RNA polymerases II or III (Lee et al., 1993; Vettese-Dadey et al., 1996).

A correlation between histone acetylation and potential transcriptional activity, initially proposed by Allfrey et al. (1964), has been proved in several cases (reviewed in Csordas, 1990; Turner, 1991, 1993; Loidl, 1994; Grunstein, 1997; Struhl, 1998). According to one attractive recent hypothesis, histone modifications may constitute a concerted code to "specify unique downstream functions" (Strahl and Allis, 2000; Turner, 2000).

After indirect immunolabeling with antibodies raised against acetylated isoforms of histone H4 (Turner and Fellows, 1989; Turner et al., 1989), mammalian metaphase chromosomes show intense acetylation of euchromatic R-bands and less intense acetylation of constitutive and facultative heterochromatin (Jeppesen and Turner, 1993). The patterns of histone H4 acetylation described for plant chromosomes (Houben et al., 1996, 1997; Belyaev et al., 1997; Vyskot et al., 1999) also reveal a below-average acetylation of late-replicating heterochromatin. However, whereas the most conserved histones H3 and H4 showed similar acetylation patterns along the mammalian chromosomes (Belyaev et al., 1996), the patterns for H3 and H4 differed conspicuously in field bean chromosomes (Belyaev et al., 1998).

Although H4 acetylation of mammalian nuclei appears to be confined to early replicating and actively transcribing euchromatin (Sadoni et al., 1999), and facultative heterochromatin is less acetylated than euchromatin in endosperm nuclei of *Gagea lutea* (Buzek et al., 1998), little is known about histone acetylation of specific chromosomal domains during defined interphase stages.

Treatment with trichostatin A, a specific inhibitor of histone deacetylase (Yoshida et al., 1990), several hours

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before mitosis mediated a switch to extensive acetylation of H4 (at lysines 5, 12, and 16) within the heterochromatin of field bean metaphase chromosomes, but H3 acetylation remained unchanged (Belyaev et al., 1997, 1998). This indicated that histone H4 acetylation of specific chromosomal domains may vary during interphase. Such alterations might be correlated with replication because newly replicated chromatin contains acetylated histones (Ruiz-Carrillo et al., 1975), which become deacetylated shortly after incorporation into chromatin (Jackson et al., 1976). Deposition-related acetylation of lysines 5 and 12 of H4, that is, incorporation of these acetylated isoforms into newly replicated chromatin, appears to be a highly conserved phenomenon (Sobel et al., 1995). Moreover, Idei et al. (1996) reported different histone H4 acetylation patterns of plant interphase nuclei; however, they were unable to relate the different patterns with either defined cell cycle stages or specific chromatin domains (except for the nucleolus).

Transcriptionally active rDNA genes were shown to be devoid of nucleosomes (Sogo et al., 1984; Conconi et al., 1989, 1992; Dammann et al., 1993), but the presence of histones within the nucleolus and their degree of acetylation during the course of interphase is still an open question (Derenzini et al., 1985; Thiry and Muller, 1989; González-Melendi et al., 1998).

To learn whether the extent of acetylation at all acetylatable positions of the core histones H3 and H4 remains constant along the cell cycle for specific chromatin domains (nucleolus organizers, euchromatin, and two fractions of heterochromatin of the field bean), we developed a new approach. After immunodetection of histone isoforms on isolated meristematic nuclei sorted on the basis of their DNA content into G1, early S, mid-S, late S, and G2 fractions, defined chromatin domains of individual chromosomes are identified by fluorescence in situ hybridization (FISH) with specific probes. This approach has revealed distinct types of immunolabeling of specific chromatin domains depending on the isoform addressed and the cell cycle stage. We also compared the H4 acetylation patterns of nucleoli, euchromatin, and heterochromatin domains with the replication pattern and the potential transcriptional activity in these domains.

RESULTS

Acetylation Patterns of Histone H4 at Specific Chromatin Domains Are Modulated during the Cell Cycle

Mitosis

In accordance with our previous data (Houben et al., 1996; Belyaev et al., 1997), antisera recognizing histone H4 that was acetylated at lysines 5 (Figure 1D), 8, and 12 labeled the NOR of metaphase chromosomes of the field bean more intensely, and the interstitial heterochromatin less intensely, than they did the euchromatic regions.

Antibodies against H4Ac16 were previously shown to label chromosomes uniformly, except for the NOR, which was

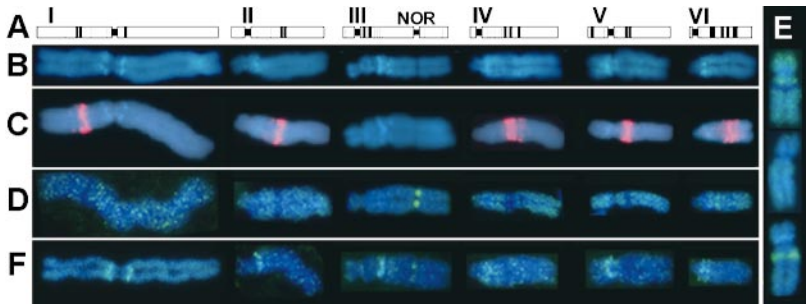

Figure 1. The Six Chromosomes of the Field Bean Karyotype ACB.

(A) Scheme of Giemsa banding pattern, representing the heterochromatic regions.
(B) Fluorescence bands after staining with DAPI.
(C) FISH with tandemly repeated Fok elements (59 bp, red).
(D) Immunostaining of H4Ac5. Note that the acetylation is stronger at the NOR and weaker at the interstitial heterochromatin than at the euchromatin. The same pattern was obtained with antibodies against H4Ac8 and H4Ac12.
(E) Immunostaining of H4Ac16. Chromosome V is used to illustrate the three types of labeling during mitosis: 30% of the chromosomes showed an acetylation pattern identical to that obtained for H4Ac5 (top); 30% showed a uniform acetylation (middle), as described by Belyaev et al. (1997); and 40% revealed more strongly acetylated interstitial heterochromatin (bottom).
(F) Immunostaining of H3Ac14. Note the decreased acetylation of Fok element–containing **(C)** and the increased acetylation of Fok element–free, DAPI-positive **(B)** interstitial heterochromatic regions in comparison with euchromatin. The same pattern was obtained also for H3Ac9/18.

more strongly labeled. Inspecting a higher number of chromosomes, we observed two additional patterns. Either the heterochromatin was more weakly labeled than euchromatin (30% of chromosomes), as seen for H4Ac5, 8, and 12, or it was more strongly labeled than euchromatin (40% of chromosomes; Figure 1E).

Incubation with the deacetylase inhibitor trichostatin A for 2 to 10 hr before mitosis resulted in highly intense acetylation of heterochromatin for the lysines K5, K12, and K16 (but not K8) of H4 (Belyaev et al., 1997). This agrees with the idea that deposition-related acetylation of K5 and K12 indeed might be highly conserved (Sobel et al., 1995) and that K16 in plants also might be acetylated in a deposition-related manner.

Interphase

Immunodetection of H4Ac5 in interphase nuclei of the field bean revealed four distinct types and two subtypes of labeling patterns, which are shown in Figure 2 and described here:

Type I shows the most intense signals within the nucleolus. The remaining chromatin is more weakly labeled by dispersed signals, and several unlabeled regions ("empty spots") are visible. This type corresponds to the metaphase labeling pattern.

Subtype Ia differs from type I by weaker labeling of the nucleolus, the signals locating preferentially at the nucleolar periphery.

Type II shows nearly no signals within the nucleolus, stronger labeling of the average chromatin than in type I, and again, clear empty spots.

Type III shows unlabeled nucleoli; the chromatin on average is weakly labeled but contains several bright signal spots instead of empty spots.

Subtype IIIa differs from III by additional signals within the nucleolus, often forming a ring at the nucleolar periphery.

Type IV represents an intermediate between types I and II. It has empty spots but shows no difference in labeling intensity of nucleoli and the remaining chromatin.

Comparable labeling patterns were observed after immunodetection of H4Ac12, H4Ac16, and tetraacetylated H4 (not shown), though we noted that H4Ac16-labeled nuclei with bright spots always contained labeled nucleoli (subtype IIIa). Nuclei of types III and IIIa (that is, those with bright signal spots outside the nucleoli) were not seen when labeled with antibodies to H4Ac8.

Type II and III nuclei (those with unlabeled nucleoli) appeared to have less acetylated histone H4 inside the nucleolus than in the extranucleolar chromatin. To determine whether this reflects a lower overall amount of H4 in such nucleoli, we sequentially labeled the nuclei with antibodies recognizing acetylated H4 and with antibodies recognizing histone H4 regardless of its acetylation status (R213). The nucleoli of types II and III remained less intensely labeled

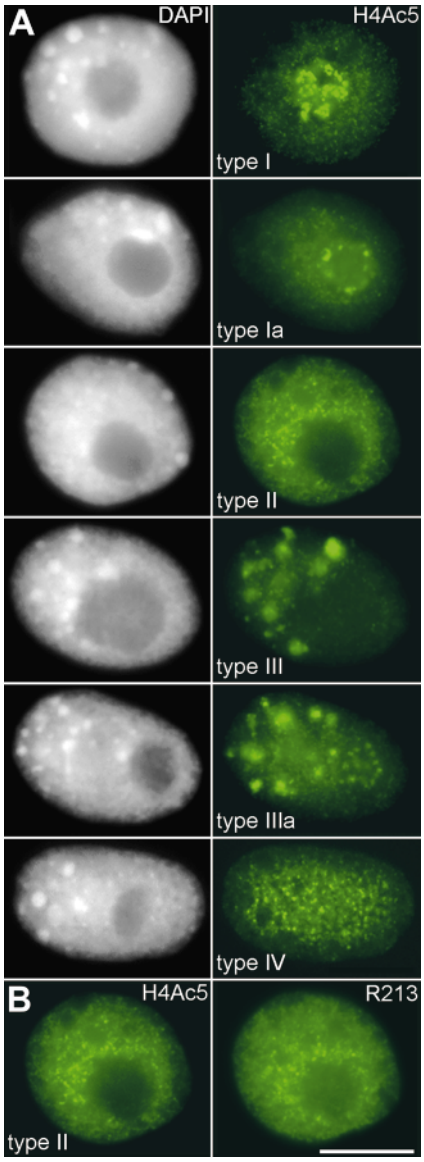

Figure 2. Types of Immunolabeling Pattern of Field Bean Interphase Nuclei Obtained with Antiserum against Histone H4Ac5.

(A) Nuclei counterstained with DAPI (left) and after immunolabeling of H4Ac5 (right). Note the different labeling intensities of nucleoli (strong in types I, Ia, and IIIa but absent in types II and III) and the additional "empty" spots (types I, Ia, II, and IV) or "bright" signal spots (types III and IIIa) in chromatin.

(B) Type II nucleus with nucleolus free of H4Ac5 (left) and also nearly free of immunosignals after subsequent labeling with antiserum R213 (right), which recognizes H4 regardless of acetylation. The absence of this label indicates depletion of H4 and therefore the absence of complete nucleosomes within nucleoli of these types of nuclei; the same was true for nucleoli of type III nuclei. Bar = 10 μm.

than the surrounding chromatin, even after labeling with R213 (Figure 2B). This result was independent of the order in which the antisera were added. If the H4 tail in the nucleoli is not inaccessible for R213, then this observation suggests that nucleoli in type II and III nuclei have less H4 than in other types of nuclei.

Variable Frequency of Histone H4 Labeling Patterns during the Cell Cycle

To ascertain whether the different histone H4 labeling patterns appeared with a constant frequency throughout the cell cycle, we flow-sorted formaldehyde-fixed field bean nuclei from unsynchronized root tip meristems according to their DNA content into fractions covering G1, early S, mid-S, late S, and G2 cell cycle phases (Figure 3). Several hundred nuclei from each fraction were immunolabeled with the specific antibodies. After immunodetection of H4Ac5, only nuclei of the intermediate types Ia and IV showed a similar, low frequency in all fractions (Table 1). Many of the nuclei in G1 (63%) and G2 (44%) contained both strongly labeled nucleoli and unlabeled regions (empty spots) within weakly labeled chromatin (type I), whereas in mid-S phase, the majority (74%) of the nuclei showed unlabeled nucleoli and strongly labeled chromatin (type II). Type III and IIIa nuclei with bright instead of empty spots in weakly labeled chromatin were observed only in late S and (early) G2 (Table 1 and Figure 4A). That not all nuclei of a fraction show the same pattern typical for the corresponding cell cycle stage might result primarily from nuclei showing an intermediate type such as Ia, IIIa, or IV. Missorting or "contamination" of G1 (or G2) fractions by nuclei from differentiated cells surrounding meristems cannot be totally excluded. However, the shape of the histogram (high and narrow G1 peak, separated clearly by S phase from a somewhat lower and broader G2 peak, and an absence of peaks for higher ploidy levels) (Figure 3) and the distribution of labeling types indicate that missorted and nonmeristematic nuclei should account for only a minor proportion within the sorted fractions.

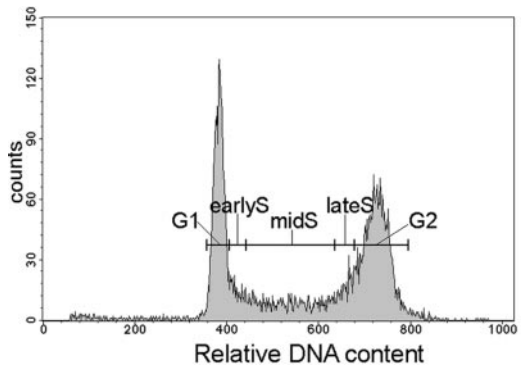

Figure 3. Histogram of Relative DNA Content of Unsynchronized Field Bean Root Tip Nuclei after DAPI Staining and Flow-Cytometric Analysis.

The gates (representing G1, early S, mid-S, late S, and G2 phases) used for sorting are as indicated.

Table 1. Histone H4Ac5 Labeling Patterns of Field Bean Nuclei during Interphase

	Cell Cycle Stage									
	G1		Early S		Mid-S		Late S		G2	
Labeling Type[a]	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*
I	63	258	30	112	9	35	27	120	44	217
Ia	14	56	12	43	11	43	8	38	5	24
II	18	75	50	184	74	284	46	207	12	58
III	0	0	1	3	1	2	10	45	24	118
IIIa	0	0	1	2	0	0	4	17	10	48
IV	5	23	6	23	5	19	5	25	5	25
Σ	100	412	100	367	100	383	100	452	100	490

[a] For description of labeling types see Figure 2A and text.

A similar but less pronounced decrease of nucleolus labeling during S phase was observed after use of antibodies against H4Ac8 (minimum in mid-S), H4Ac12, and H4Ac16 (both with a minimum in early S; see Figure 4B). However, after immunostaining of H4Ac16, the proportion of type IIIa nuclei was greater in G2 (almost 50%), and some (15%) were found even in G1.

The temporal acetylation pattern of histone H4 of euchromatin was opposite that of the nucleolus organizers or nucleoli. Euchromatin was most intensely labeled (type II) for all acetylatable lysines, particularly during early and mid-S phase. To compare directly histone acetylation and DNA replication patterns, the cells were pulse-treated for 30 min with 5-bromo-2′-deoxyuridine (BrdUrd) before fixation and isolation of the nuclei. Immunodetection of BrdUrd and subsequently of H4Ac5 yielded similar labeling patterns. The high degree of colocalized signals (except for the nucleolus) shown in Figure 5 indicates that the most intense H4 acetylation occurs during or shortly after replication.

Acetylation of Histone H4 of Heterochromatic Regions: Strongest during Replication

FISH with the tandem repetitive Fok element (contained within ~75% of the Giemsa-banded interstitial heterochromatic regions of the field bean; see Figures 1A and 1C, and Fuchs et al., 1998) after immunodetection of H4Ac5 revealed that the 10 large Fok element sites exclusively colocalize with less intensely acetylated chromatin regions (empty spots) of labeling types I, Ia, II, and IV (see Figure

6A). The empty spots without Fok signals probably correspond to the interstitial heterochromatic regions, which contain repeats other than Fok elements. In type III and IIIa nuclei, bright spots (indicating strongly acetylated chromatin) were observed instead of empty spots. As Figure 6B shows, all major Fok element positions colocalize with the most strongly acetylated regions of type III and IIIa nuclei.

To be sure that neither bright FISH signals nor acetylation signals escaped detection by epifluorescence microscopy, we also performed optical sectioning of type III nuclei by using a confocal laser microscope after FISH with Fok elements and immunostaining for H4Ac5. As seen from Figure 7, all bright FISH and acetylation signals proved to be detectable by both techniques in individual field bean nuclei.

Because bright spots for H4 acetylation appeared only in late S and G2 nuclei and because the interstitial heterochromatin of the field bean was found to replicate latest in S phase (Döbel et al., 1978; Fuchs et al., 1998), presumably the H4 of the prominent interstitial heterochromatic domains becomes strongly acetylated at K5 during or shortly after replication. This agrees with our data from experiments combining H4Ac5 immunodetection and BrdUrd pulse labeling of early, mid, and late S-phase nuclei (Figure 5) and with the current view of deposition-related acetylation (Sobel et al., 1995).

During G2, H4Ac5 in heterochromatic domains is deacetylated to an extent clearly less than that in euchromatin. This process is finished at least 2 hr before mitosis (Belyaev et al., 1997); the deacetylated state then lasts until the next replication.

A similar temporal pattern of acetylation was observed for K12 of H4, but K8 in heterochromatin was never acetylated as strongly as or more strongly than euchromatin. Because strongly acetylated heterochromatin at K16 was found in 50% of G2 nuclei, in 40% of mitotic chromosomes (Figure 1E), and in 15% of G1 nuclei but in only 2% of early S and mid-S nuclei, deacetylation of K16 presumably is delayed in comparison with K5 and K12, both of which were highly acetylated within the heterochromatin only in late S and part of G2 but not during mitosis and G1.

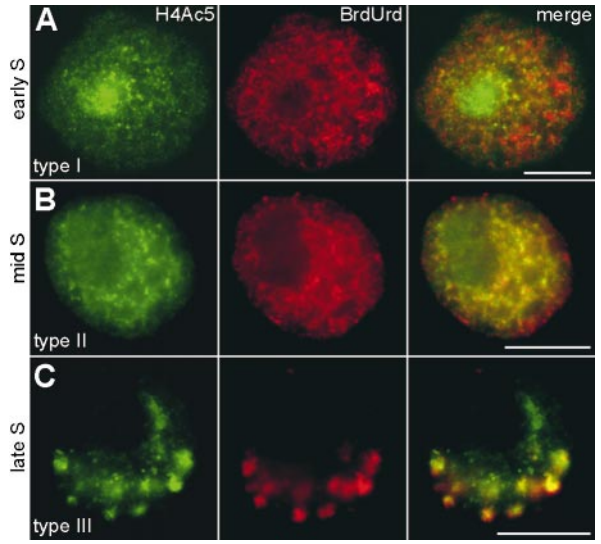

Figure 5. Correlation of Histone Acetylation (H4Ac5) and DNA Replication during S Phase.

After 30 min of BrdUrd pulse, the nuclei were isolated, flow-sorted, and double-immunolabeled for H4Ac5 (green, left) and BrdUrd (red, middle).

(A) Early S phase.

(B) Mid-S phase.

(C) Late S phase.

Note the large degree of colocalization of both immunosignals in early S (except for the NOR), mid-S, and late S nuclei. The bright spots in **(C)** represent late-replicating heterochromatin. Bars = 10 μm.

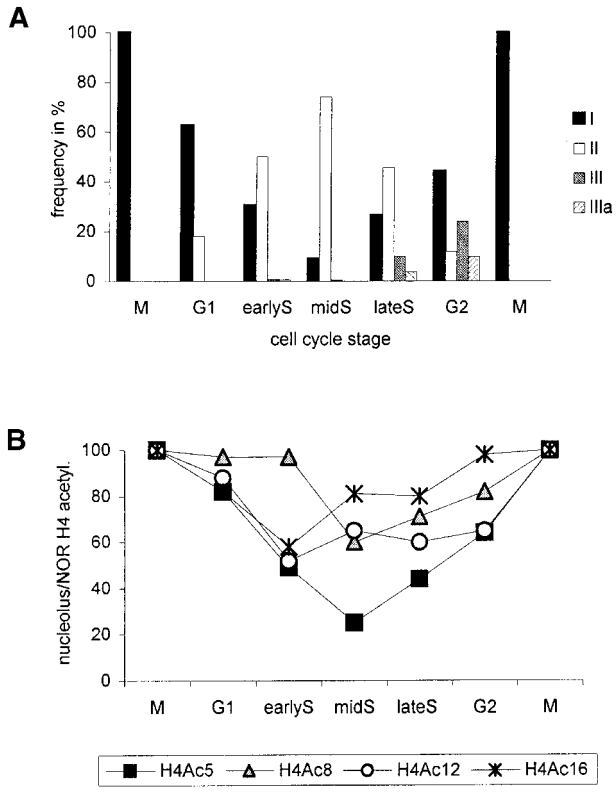

Figure 4. Variation of Histone H4 Acetylation Patterns during the Cell Cycle.

(A) Proportion of nuclei of labeling types I, II, III, and IIIa after immunodetection of H4Ac5 in different cell cycle stages. Because types Ia and IV revealed a nearly constant frequency, ranging from 5 to 14% and 5 to 6%, respectively, they therefore were omitted (cf. with Table 1).

(B) Relative frequency of nuclei showing acetylation of lysines 5, 8, 12, and 16 of H4 inside nucleoli during the cell cycle.

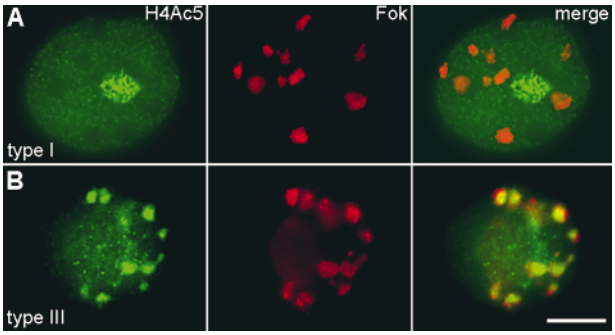

Figure 6. Histone H4 Acetylation of Interstitial Heterochromatin Changes during the Cell Cycle.

(A) FISH with Fok elements (red, middle) performed after H4Ac5 immunolabeling (green, left) shows that heterochromatin domains coincide with empty spots representing underacetylation in type I nuclei, that is, during G1 and G2 (cf. with Table 1); the same is true for nuclei of types Ia, II, and IV.
(B) In nuclei of type III (and IIIa), which appear during late S and early G2 (cf. with Table 1 and Figure 4A), late-replicating heterochromatin domains colocalize with bright spots of H4Ac5 labeling. Bar = 10 μm.

Histone H4 Acetylation: Nearly Absent from rDNA during S Phase and Not Directly Correlated with Transcriptional Activity

Only chromosome pair III of the field bean karyotype ACB harbors the genes for the 5.8, 18, and 25S rRNAs. Interphase nuclei therefore contain one or two nucleoli. In G1, 60 to 70% of the nuclei have only one nucleolus, compared with 85 to 90% in G2. These results suggest the nucleoli have a tendency to fuse as the cell cycle progresses.

After FISH with labeled rDNA, what we observed most frequently in isolated nuclei were two perinucleolar signal clusters. Signals inside the nucleoli appeared as intensely fluorescing small dots or faint threadlike or diffuse signals (Figure 8A).

During mitosis, the NOR is more strongly acetylated than the euchromatin (Figure 1D; Belyaev et al., 1997). After H4Ac5 immunolabeling of type I nuclei (with strongly immunolabeled nucleoli), FISH with rDNA revealed that at least part of the rDNA inside the nucleoli, but not the perinucleolar rDNA, was colocalized with H4Ac5 immunosignals, as seen in Figure 8A. However, intense signals for rDNA as well as for H4Ac5 often were found in separate positions inside the nucleoli of type I nuclei.

To compare histone H4 acetylation with transcriptional activity, we labeled nascent RNA transcripts with 5-bromouridine-5′-triphosphate (BrUTP). As demonstrated in Figure 8B, after 4 min of BrUTP incorporation into isolated nuclei, all nuclei revealed intensely labeled nucleoli, regardless of the degree of H4 acetylation within the nucleoli. Type I nuclei showed a partial colocalization of BrUTP and acetylation signals inside nucleoli. The remaining chromatin in the field bean cells—unlike that in the observations made with mammalian cells (Jackson et al., 1993; Wansink et al., 1993; Sadoni et al., 1999)—was less densely labeled in all types of nuclei. Types II and III nuclei (representative of most of the S-phase cells) showed no H4 acetylation signals within nucleoli; that is, there was no association of acetylated H4 and intranucleolar rDNA (Figures 2A, 4A, 4B, and 8B). Heterochromatin domains were free of BrUTP signals. This became clear from overlaying BrUTP and H4Ac5 signals in type III nuclei (see bright spots in Figure 8B) but also was true for the empty spots of types I and II nuclei. This confirmed the transcriptional inactivity of the interstitial field bean heterochromatin (Houben et al., 1994) .

Histone H3 Acetylation Patterns in Interphase Nuclei Differ from Those of H4 and Are Nearly Invariant during Cell Cycle Progression

Labeling field bean chromosomes with antisera recognizing histone H3 acetylated at lysine positions 14 (H3Ac14; Figure 1F) and 9/18 (H3Ac9/18) looked different from the pattern obtained after labeling of histone H4Ac5 (Figure 1D). Besides the NOR, the Fok element–free interstitial heterochromatin also was more strongly acetylated, whereas Fok

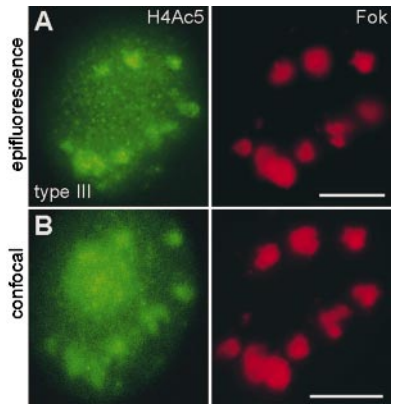

Figure 7. Images of a Type III Nucleus of Late S to Early G2 Phase after Immunodetection and FISH.

(A) and **(B)** Immunodetection of H4Ac5 (green, left) followed by FISH with Fok elements (red, right) as observed under **(A)** epifluorescence and **(B)** confocal microscopy overlaying 13 optical sections through the nucleus. H4Ac5 immunosignals were captured.
(A) Before FISH.
(B) After FISH.
The major immuno- and FISH signals are identical within both images. The green signal covering the nucleolus in **(B)** is autofluorescence, which frequently appeared when images were taken after FISH, although it was absent from the same nuclei when checked before FISH, as in **(A)**. Bars = 10 μm.

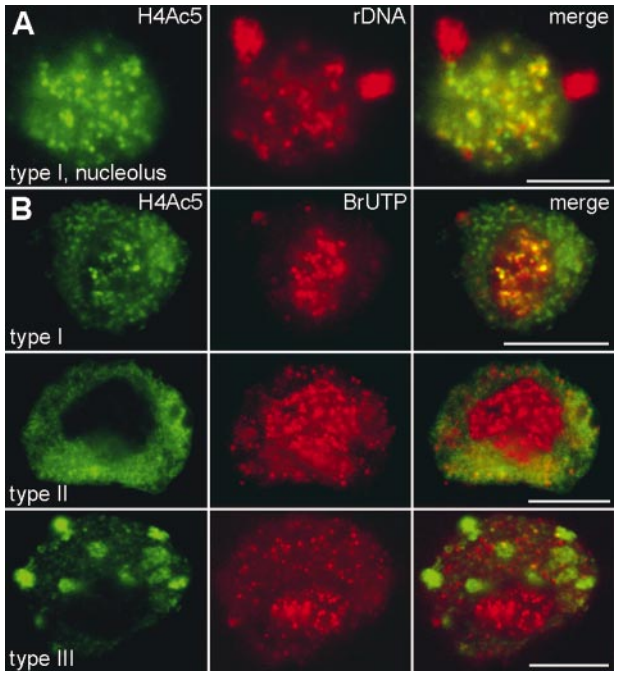

Figure 8. Histone Acetylation and Transcriptional Activity of rDNA during Interphase.

(A) FISH with rDNA (red, middle) after immunolabeling of H4Ac5 (green, left) from a type I nucleus (only the nucleolus is shown) characteristic for G1 and G2 stages. Perinucleolar knobs containing inactive rDNA and some foci of condensed rDNA inside nucleoli are free of H4Ac5, as shown after merging of both signals. Most of H4Ac5 immunosignals are confined to faint, diffuse rDNA signals.
(B) Immunostaining of H4Ac5 (left), BrUTP incorporation (middle), and merging of both signals (right) for type I (G1), II (mid-S), and III (late S) nuclei. The transcriptional activity of rDNA is not correlated with H4 acetylation. Nucleoli are heavily labeled already after 4 min of BrUTP incorporation (red), irrespective of their acetylation status (green). Although BrUTP signals outside nucleoli are much weaker, no transcription signals were detected within heterochromatin domains (neither within empty spots in type II nuclei nor within bright spots in type III nuclei).
Bar in **(A)** = 5 μm; bars in **(B)** = 10 μm.

element–containing heterochromatin (Figure 1C) was again less acetylated than euchromatin. H3Ac23 immunolabeling was uniform along the chromosomes, except for the NOR, which was less strongly labeled (Belyaev et al., 1998).

Immunodetection of H3Ac14 in interphase nuclei revealed two main patterns of labeling that differed by the presence or absence of intensely labeled spots in chromatin (Figure 9). These spots, mostly colocalizing with regions stained brightly by 4′,6-diamidino-2-phenylindole (DAPI), were not uniformly distributed throughout the nucleus but instead were clustered at one nuclear pole. Because most of the Fok element–free but DAPI-positive heterochromatin is located close to the centromeres (Figure 1B; Fuchs et al., 1998), this corresponds to the so-called Rabl orientation (Rabl, 1885). The number of these spots varied from five to 18, with a median number of eight. Empty spots were present in (almost) all nuclei. In many nuclei, however, they were not easily recognizable because of weak average chromatin labeling. In nuclei without bright spots, the average chromatin labeling was stronger, and empty spots were more easily detectable (Figures 9C and 9D). Nucleoli were either labeled or unlabeled, whether bright spots were present or not. The labeling of the nucleolus, if present, was generally less intense than in type I nuclei after H4Ac5 labeling. The signals of the nucleoli usually appeared as small dots in the center or as a ring at the periphery of the nucleolus.

Comparable labeling patterns of interphase nuclei were obtained with the antiserum that recognized H3Ac9/18. After immunodetection of H3Ac23, interphase chromatin was more or less homogeneously labeled and the nucleoli were slightly less labeled. Neither bright spots nor empty spots were found.

H3Ac14 labeling on sorted nuclei revealed that the pattern with bright spots in chromatin and labeled nucleoli was the one seen most frequently (63 to 74%) in all cell cycle stages. Between 72 and 85% of nuclei (with either labeled or unlabeled nucleoli) showed bright spots; only between 10 and 18% of the nuclei revealed unlabeled nucleoli during all cell cycle stages (Table 2). Together these data show that, unlike histone H4 acetylation, the variability of H3 acetylation patterns was much less pronounced and not clearly dependent on the cell cycle stage.

To determine whether the H3Ac14 labeling of metaphase chromosomes (with highly acetylated Fok element–free and nonacetylated Fok element–containing heterochromatin) persists or alters during interphase and whether Fok element–containing heterochromatin is subject also to histone H3 acetylation during interphase, we performed FISH with Fok elements after H3Ac14 immunolabeling (Figure 9E). Fok elements were mostly colocalizing with empty spots (lacking detectable H3Ac14), regardless of the presence or absence of bright H3Ac14 spots in the respective nuclei. Strongly immunolabeled spots did not contain Fok elements. Usually, Fok elements occurred in less polar positions than the brightest immunosignals. This is reasonable given that, in most cases, Fok elements are located more distantly from the centromeres than are the Fok element–free heterochromatic regions (Figures 1B, 1C, and 9E).

These studies gave the following results: (1) strong histone H3 acetylation is excluded from Fok element–containing heterochromatin, (2) Fok element–containing and Fok element–free heterochromatin occupy separate compartments within interphase nuclei, (3) only Fok element–free heterochromatin and nucleolus organizers are targets for very strong acetylation of histone H3, (4) H3 acetylation is not clearly related to the replication of euchromatin and heterochromatin domains, and (5) H3 acetylation shows no

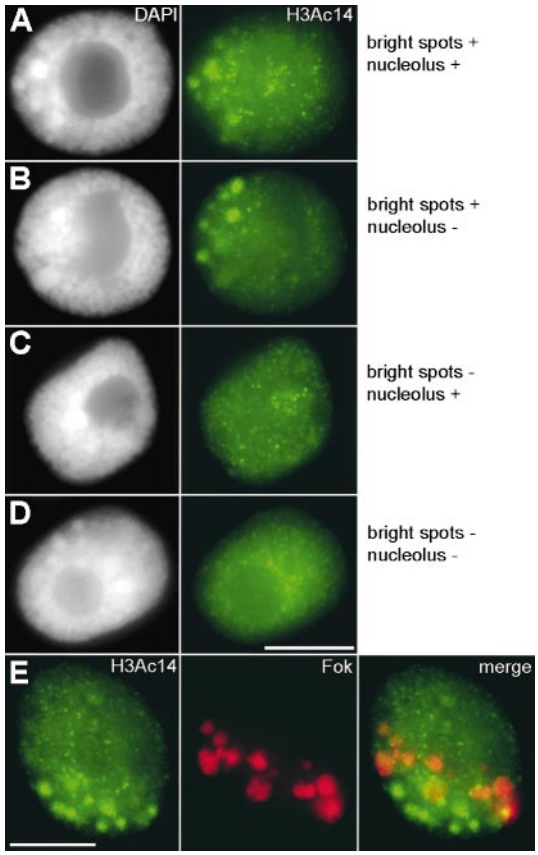

Figure 9. H3Ac14 Labeling Patterns of Field Bean Interphase Nuclei and Their Correlation with Heterochromatic Domains.

(A) Nucleus with bright signal spots for H3Ac14 and labeled nucleolus. This type represents the majority (63 to 74%) of nuclei in all interphase stages.
(B) Nucleus with bright signal spots but without intense labeling of the nucleolus.
(C) Nucleus without bright signal spots; the nucleolus is somewhat more strongly labeled than the remaining chromatin.
(D) Nucleus with neither bright signal spots nor intensely labeled nucleolus.
DAPI staining (left) and immunodetection (right). Note the presence of more weakly labeled areas in all nuclei and the correlation of bright signal spots with areas of positive DAPI fluorescence. The frequencies of these types in the course of interphase are given in Table 2.
(E) Same type of nucleus as in **(B)** after immunodetection of H3Ac14 (left), FISH with Fok elements (middle), and merging of both (right). Fok element sites (red) occupy the less acetylated areas and do not colocalize with the bright signal spots for H3Ac14, which represent Fok element–free heterochromatin.
Bars = 10 μm.

clear correlation with the transcriptional status of the investigated chromatin domains.

DISCUSSION

Overall Acetylation of Large Chromatin Domains Correlates with Replication Rather Than with Transcription

We have shown by FISH with Fok elements that the interstitial heterochromatin domains of individual field bean chromosomes form distinct compartments during interphase. Immunostaining of acetylated isoforms of histones H3 and H4 and subsequent identification of chromatin domains by FISH on meristematic nuclei, sorted according to their DNA content into five cell cycle fractions, allowed us for the first time to follow histone acetylation/deacetylation of defined chromatin domains of individual chromosomes through the cell cycle. BrdUrd pulse labeling allowed comparison of the acetylation intensity with replicational activity of the corresponding domains and showed that acetylation of H4 in euchromatin of the field bean is most pronounced during replication and is weaker from late S through M to G1. In contrast, H4 acetylation in early replicating rDNA is most intense during mitosis, decreases in G1 toward a minimum in early S (K12, K16) or mid-S (K5, K8), and increases again from late S onward (Figures 4A, 4B, and 5). The prominent interstitial heterochromatin domains replicate late, are transcriptionally silent, and represent hot spots of mutagen-induced chromosomal aberrations (reviewed in Fuchs et al., 1998). They become strongly acetylated at all acetylatable lysines of H4 (except K8) during late S phase, are deacetylated in G2 (>2 hr before mitosis; Belyaev et al., 1997), and remain deacetylated until the next replication. Only deacetylation of K16 of H4 is not always completed before mitosis, and acetylation of this residue may persist until the next G1 (for a summary, see Figure 10). A narrow time window of H4 acetylation at K5 and K12, correlating with replication, recently was reported also for mammalian heterochromatin (Taddei et al., 1999). In the field bean, H4 of euchromatin (as well as of centromeres and telomeres) and of all prominent interstitial heterochromatin thus shows most pronounced acetylation during and shortly after replication. This is in accordance with a phylogenetically conserved deposition-related acetylation at lysines 5 and 12 (Sobel et al., 1995). We predict that in plants lysine 16 also might be acetylated in a deposition-related manner.

Still undetermined is the reason for the apparently futile strong postreplicative hyperacetylation within the heterochromatin. Because recombinative assembly of immunoglobulin genes in mammals has been found to be stimulated by histone acetylation (McBlane and Boyes, 2000; McMurry and Krangel, 2000), perhaps acetylation of histones (especially H4 in plants) is supportive also for recombination re-

Table 2. Histone H3Ac14 Labeling Patterns of Field Bean Nuclei during Interphase

	Cell Cycle Stage									
	G1		Early S		Mid-S		Late S		G2	
Labeling Type[a]	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*
Bright spots + nucleolus +	71	267	63	236	66	259	67	262	74	298
Bright spots + nucleolus −	14	54	9	33	8	33	7	28	6	25
Bright spots − nucleolus +	8	28	14	52	15	59	11	43	10	38
Bright spots − nucleolus −	2	9	9	35	4	15	7	25	4	16
Bright spots − uniform	5	18	5	17	7	25	8	32	6	23
Σ	100	376	100	373	100	391	100	390	100	400
Total nucleolus +	79	295	77	288	81	318	78	305	84	336
Total bright spots +	85	321	72	269	75	292	74	290	80	323

[a] Empty spots occur in most nuclei of all labeling types.

pair of DNA damage, which preferentially occurs during or shortly after replication. This might be particularly important with regard to heterochromatin, which is less acetylated in other cell cycle stages.

Although histone acetylation at the genic level may well be correlated with transcriptional activity (see Introduction), the overall acetylation of large chromatin domains is correlated with replication rather than with transcription (Figures 5 and 8B). However, there are two exceptions to this. One is the acetylation of H3, which is more or less persistent throughout the cell cycle, including the apparently sequence-specific above-average acetylation at lysines 9, 14, and 18 within Fok element–free heterochromatic regions. Perhaps H3 acetylation is required for maintenance of some heterochromatin domains, as is the case, for instance, in yeast (Thompson et al., 1994; Hecht et al., 1995; Braunstein et al., 1996; Grunstein, 1998). The other exception is the NOR.

H4 Is More Strongly Acetylated at the NOR during Mitosis, Whereas Nucleoli in S Phase Appear to Be Less Acetylated Than Euchromatin and May Be Transiently Free of Nucleosomes

The overall H4 acetylation patterns of field bean rDNA genes apparently are not strictly correlated with their transcriptional activity but are inversely correlated with the replication of rDNA, which takes place very early in S phase (Schubert and Rieger, 1979; Fuchs et al., 1998). This became evident from the finding that the strongest acetylation of H4 at the NOR occurred during mitosis, when rDNA is being neither replicated nor transcribed. The same was observed for barley (Idei et al., 1996) and onion (L. Malysheva and I. Schubert, unpublished findings) but not for mammals (Jeppesen and Turner, 1993) except for the NOR of the inactive X chromosome of female marsupials (Keohane et al., 1998). The presence of essential components of the rDNA transcriptional machinery at the NOR during mitosis (Scheer et al., 1993), which enables early, efficient initiation of transcription already in telophase/early G1 (Roussel et al., 1996; Gébrane-Younès et al., 1997; Klein and Grummt, 1999; Scheer and Hock, 1999), and our data on BrUTP incorporation indicate that rDNA is intensely transcribed during the entire interphase, regardless of H4 acetylation within the nucleolus. Strong H4 acetylation also appears frequently within nucleoli of G1 and G2 nuclei but only rarely during S phase, when replication occurs. Moreover, the results obtained after labeling of type II nuclei with antiserum R213, which recognizes histone H4 regardless of acetylation, show that in most nucleoli during S phase too little H4 is present for detection by immunolabeling. This indicates the absence of complete nucleosome core particles (the presence of acetylated H3 could be attributable to free histone molecules).

According to Lucchini and Sogo (1995), the coding regions of newly replicated rDNA genes usually are organized in nucleosomes, and transcriptional activation of rDNA requires disruption of preformed nucleosomes. This agrees with the apparent depletion of nucleosomes from field bean nucleoli shortly after rDNA replication in early S phase, which is not reversed until late S/G2, and invites the speculation that not only active rDNA genes but also the entire nucleolus is devoid of nucleosomes during most of S phase; consequently, histone acetylation cannot be detected.

Thus, the question arises as to the degree of acetylated nucleosomal histones associated with transcriptionally active versus inactive rDNA during G1 and G2. Only a subset of rRNA genes is actively transcribed during interphase (Shaw et al., 1995), even in yeast with comparatively few rRNA genes (Dammann et al., 1993), and heavily transcribed cistrons are not organized in nucleosomal structures (e.g., Sogo et al., 1984; Conconi et al., 1992; Dammann et al., 1993). Apparently, transcriptional activity within nucleoli is upregulated by increased activity of already active cistrons that are free of nucleosomes (reflected by "Christmas tree"–like structures of nascent transcripts in electron microscopic images; Miller and Beatty, 1969) rather than by activation of

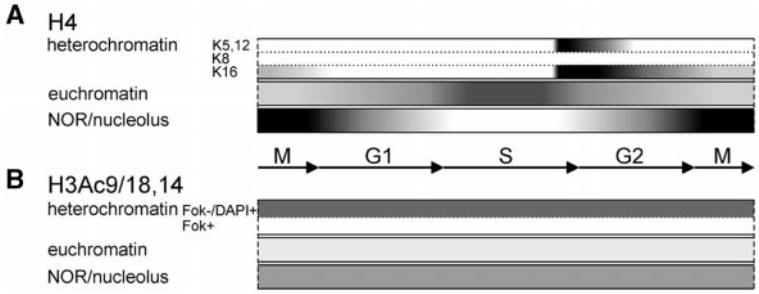

Figure 10. Acetylation of Nucleosomal Histones at the NOR, Euchromatic, and Heterochromatic Domains of the Field Bean during the Cell Cycle.

(A) Histone H4. A strong cell cycle–dependent histone H4 acetylation occurs at the level of distinct chromatin domains. Heterochromatin contains acetylated H4 (except H4Ac8) during and (shortly) after replication; euchromatin, too, is most strongly acetylated during replication; the NOR contains acetylated H4 during mitosis, as do nucleoli in G1 and G2, but during S phase the histone H4 acetylation within nucleoli is considerably decreased.

(B) Histone H3. The intensity of H3 acetylation differs between Fok element–free heterochromatin, Fok element–containing heterochromatin, euchromatin, and the NOR/nucleolus but, unlike H4 acetylation, remains fairly constant throughout the cell cycle.

(all) silent genes (Banditt et al., 1999). Transcriptionally silent rDNA copies forming condensed perinucleolar knobs are free of acetylated H4, as are some condensed rDNA blocks inside nucleoli during G1 and G2, whereas acetylation signals were found in nucleolar areas exhibiting diffuse or very faint signals after FISH with rDNA (Figure 8A). Arrays of inactive rDNA genes might become associated, forming intranucleolar condensed chromatin, whereas active rDNA genes loop out. These loops of less condensed rDNA might be still acetylated in G1 (type I and Ia nuclei) and already in G2 (type IIIa and I nuclei). When rDNA transcription increases during G1, at least some of these loops might become heavily covered by RNA polymerase I molecules, while at the same time being free of nucleosomes and histone H4 molecules (type II and III nuclei). Closely adjacent highly active and less active genes could explain the occasional proximity and partial overlap of acetylated H4 and condensed intranucleolar rDNA in G1 and G2 nuclei. Highly active rDNA genes are assumed to have a decondensed structure close to the limit of resolution of optical microscopy (Thompson et al., 1997). Such genes might be responsible for the faint diffuse signals observed after FISH with rDNA, as compared with the bright signals at condensed rDNA repeats. When the rate of rDNA transcription decreases in the course of G2, the fewer RNA polymerase I molecules per transcribed gene allow (acetylated) nucleosomes to bind to DNA, resulting in increased acetylation within the nucleoli. This acetylation pattern is then maintained at the NOR during mitosis and becomes reversed during the course of G1.

According to the hypothesis proposed by Jeppesen (1997), histone acetylation can provide a mechanism for propagating "cell memory." He suggests that "genes in chromatin domains active before mitosis are marked by histone acetylation, and hence have the potential for being preferentially reactivated in the following G1 phase. Acetyl groups then serve as 'tags' for recognition by other proteins involved in regulating transcription." This hypothesis could explain the temporal pattern of H4 acetylation at the NOR chromatin observed in the present study, which correlates with neither replication nor transcriptional activity.

Conclusions

The extent of H4 acetylation is greatest during or shortly after replication within eu- and heterochromatin of the field bean—except for rDNA chromatin, which was most highly acetylated at and around mitosis and was apparently free of H4 during S phase. Because heterochromatin is transcriptionally silent, but rDNA and large parts of euchromatin are transcribed throughout interphase, the overall H4 acetylation of large chromatin domains (except rDNA) apparently is linked to replication (and possibly postreplicative recombination repair) rather than to transcriptional activity.

The amount of H3 acetylation did not show a clear cell cycle dependence. Therefore, no clear correlation with replication or transcription could be stated with regard to the large chromatin domains in this species. Contrary to the situation in mammals, chromatin fractions of the field bean showed deviations between the acetylation patterns of H4 and H3; thus, the requirements for acetylation of these two histones may be different in plants.

The replication-associated stronger acetylation of K5 and K12 of H4 in late-replicating heterochromatin (Taddei et al., 1999) and the degree of acetylation of euchromatin (which is most intense during early to mid-S phase) probably are conserved for plants and animals, whereas the increased acetylation of K16 at heterochromatic domains—which occurs

during late S and disappears between G2 and G1—might be plant specific.

Stronger acetylation of H4 at nucleolus organizers during mitosis (cf. with the situation for euchromatin) as well as the very high transcriptional activity within nucleoli (cf. with mammalian cells) (Jackson et al., 1993; Wansink et al., 1993; Sadoni et al., 1999) seems to be typical for several plant species.

Further investigation will show whether the apparently sequence-dependent strong acetylation of K9, K14, and K18 of H3 represents a particular feature of the DAPI-positive heterochromatin fraction of the field bean or is more widespread in other (plant) species.

METHODS

Plant Material, Preparation of Slides, and Isolation and Sorting of Nuclei

Root tip meristems of the field bean (*Vicia faba*) karyotype ACB with individually distinguishable chromosome pairs (Fuchs et al., 1998) were used in all experiments. Suspensions of nuclei from unsynchronized root tip meristems and chromosomes from synchronized meristems (fixed in 4% formaldehyde, 10 mM Tris-HCl, 10 mM Na_2 EDTA, 100 mM NaCl, and 0.1% Triton X-100, pH 7.5, for 20 min under vacuum) were prepared as described (Schubert et al., 1993). Isolated nuclei and chromosomes were centrifuged onto a microscopic slide by using a Cytospin3 (Shandon, Frankfurt, a.M., Germany) cytological centrifuge at 18*g* for 5 min; the loaded slides then were stored in glycerol at 4°C until use. Nuclei isolated from unsynchronized meristems (the first 2 mm of the root tips) and stained with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) were sorted into G1, early S, mid-S, late S, and G2 fractions with a FACStar^{Plus} (Becton Dickinson) flow cytometer. The gates for sorting were determined according to the histogram for nuclear suspensions (Figure 3). Approximately 1000 nuclei of each fraction were sorted onto a microscopic slide into a 15-μL drop of buffer consisting of 100 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.05% Tween 20, and 5% sucrose (Kubaláková et al., 1997). The drops with nuclei were nearly air-dried (sucrose prevents complete drying), and unless used immediately for immunolabeling or fluorescence in situ hybridization (FISH), the slides were stored at −20°C.

Indirect Immunodetection of Histone Isoforms

Polyclonal antisera against histones H3 and H4 acetylated at defined lysine residues were raised by immunization of rabbits with ovalbumin-conjugated synthetic peptides, as previously described (Turner and Fellows, 1989; White et al., 1999). The antisera used, and their specificities, were as follows: R41 (H4Ac5), R232 (H4Ac8), R101 (H4Ac12), R252 (H4Ac16), R243 (preferentially tri- and tetraacetylated H4), R213 (preferentially nonacetylated H4), R47 (H3Ac9, H3Ac18, or both), R224 (H3Ac14), and R222 (H3Ac23); see Turner et al. (1989), Belyaev et al. (1996), Stein et al. (1997), and White et al. (1999) for further details. The specificity of these sera to the same histone isoforms of plants was shown by protein gel blot analysis (Buzek et al., 1998). Preimmune sera reacted with neither the nuclear proteins (Buzek et al., 1998) nor chromosomes of the tested plants (Vyskot et al., 1999).

The nuclei were postfixed in 4% (w/v) paraformaldehyde in PBS for 20 min, washed three times in PBS, and blocked for 1 hr at 37°C in PBS containing 3% BSA and 10% horse serum. Slides then were incubated for 1 hr at room temperature (RT) in primary sera diluted 1:200 or 1:100 in AK (antibody) buffer (PBS containing 1% BSA, 10% horse serum, and 0.1% Tween 20; see ten Hoopen et al., 2000). After three washes in PBS, fluorescein isothiocyanate (FITC)–conjugated goat anti–rabbit secondary antibodies (Sigma) diluted 1:80 in AK buffer were applied for 1 hr at RT. The slides then were washed in PBS, and the DNA was counterstained with DAPI (1 μg/mL in mounting medium [Vectashield; Vector Labs, Burlingame, CA]). Secondary antibodies alone did not stain chromosomes or nuclei of the field bean.

Nascent RNA Labeling

5-Bromouridine-5′-triphosphate (BrUTP) was incorporated into isolated nuclei essentially as described (Thompson et al., 1997). In brief, unfixed nuclei from root tip meristems were released into MPB (modified physiological buffer: 100 mM potassium acetate, 20 mM KCl, 20 mM Hepes, 1 mM $MgCl_2$, 1 mM ATP, and 1 mM DTT, pH 7.4) containing 1 M hexylene glycol (2-methyl-2,4-pentanediol), centrifuged onto a slide, washed in MPB, permeabilized in MPB plus 0.05% Tween 20 for 10 sec (Abranches et al., 1998), and incubated for 3 to 10 min at RT with the following transcription mix: 50 μM CTP, 50 μM GTP, and 25 μM BrUTP (all nucleotides purchased from Sigma), 0.5 mM phenylmethylsulfonyl fluoride in MPB, and 100 U/mL RNase inhibitor (RNA Guard; Pharmacia). After being washed in MPB, nuclei were fixed for 40 min in 4% paraformaldehyde in PBS, washed three times in PBS, and blocked for 1 hr at 37°C. Incorporation of BrUTP was detected by incubation for 1 hr at RT with mouse anti-BrdU monoclonal antibody (Becton Dickinson) diluted 1:10 in AK buffer, followed by three washes in PBS and incubation with the secondary FITC-conjugated sheep anti–mouse (Boehringer Mannheim) antibody diluted 1:30, or when combined with histone immunolabeling, in Alexa^{594}-conjugated goat anti–mouse (Molecular Probes, Eugene, OR) antibody diluted 1:500 to 1:1000 in AK for 1 hr at RT.

Replication Labeling

Main roots of 4-day-old seedlings were incubated in 5-bromo-2′-deoxyuridine (BrdUrd; 100 μM), fluorodeoxyuridine (0.1 μM), and uridine (5 μM), for 30 min in the dark. After a short rinse, the roots were immediately fixed in 4% formaldehyde/Tris-HCl buffer. After further washes in Tris-HCl buffer, nuclei were isolated and sorted as described above. Before immunodetection of BrdUrd, the nuclei were postfixed in 4% formaldehyde/PBS for 20 min and washed in PBS. DNA was denatured by treating the slides at 80°C for 1 min in 50% formamide/PBS. The slides then were immediately transferred into ice-cold PBS for 5 min and blocked. BrdUrd immunodetection was as described for BrUTP.

In Situ Hybridization

The following probes were used: Fok elements (59-bp tandem repeats, cleavable by the restriction endonuclease *FokI* [Kato et al.,

1984], characteristic for ~75% of the heterochromatic Giemsa bands of the field bean [Fuchs et al., 1994, 1998]) and pVER17 (with a 3.7-kb insert consisting of part of 18S, 5.8S, and most of the coding region of 25S rRNA genes of the field bean [Yakura and Tanifuji, 1983]). pVER17 was directly labeled with tetramethylrhodamine-5-dUTP (Boehringer Mannheim) by using a nick translation kit (Boehringer Mannheim) according to the manufacturer's instructions; FokI elements were amplified from genomic field bean DNA and labeled with tetramethylrhodamine-5-dUTP or digoxigenin-11-dUTP (Boehringer Mannheim) by polymerase chain reaction with sequence-specific primers.

When FISH was performed after immunolabeling, the slides first were evaluated for immunosignals, washed in 4 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) plus 0.1% Tween 20 to remove the cover slip, and then washed briefly in 2 × SSC. The nuclei were again postfixed in 4% paraformaldehyde/2 × SSC, washed in 2 × SSC, dehydrated in 70 and 96% ethanol, and air-dried. Before FISH with pVER17, slides were incubated with RNase (50 μg/mL) for 15 min at 37°C. The hybridization mixture containing probe, 50% formamide, 10% dextran sulfate, and 2 × SSC was denatured at 80°C for 10 min and cooled on ice. The target DNA was denatured together with the probe on slides at 80°C for 2 min. When using directly labeled probes (tetramethylrhodamine-5-dUTP), after posthybridization washes (3 × 5 min in 50% formamide in 2 × SSC at 42°C and 5 min in 2 × SSC at RT), the nuclei were counterstained with DAPI. Digoxigenin-11-dUTP–labeled probes were detected with FITC- or rhodamine-conjugated anti-digoxigenin antibodies (Boehringer Mannheim).

Microscopy, Image Processing, and Evaluation of Data

The preparations were inspected with an Axiophot 2 (Zeiss, Thornwood, NY) epifluorescence microscope equipped with a cooled charge-coupled device camera (Photometrics, Tucson, AZ). Images were taken with use of IPLab Spectrum software, pseudocolored, merged, and processed in Adobe Photoshop.

To determine the frequency of distinct immunolabeling patterns for each acetylated isoform of histone H3 and H4, we evaluated at least 100 nuclei in G1, early S, mid-S, late S, and G2 phases, respectively.

Confocal Microscopy

Confocal microscopy was performed with a Zeiss (Jena, Germany) LSM 410. FITC (H4Ac5 immunosignals) and rhodamine (Fok element FISH signals) signals were recorded separately with excitation wavelengths of 488 and 543 nm and bandpass filters at 510 to 525 nm and 575 to 640 nm, respectively. Optical sections of the whole nucleus were obtained at a step width of 500 nm with the pinhole adjusted to yield an axial resolution (full width at half maximum) of 3.1 μm. Image stacks of details in the rhodamine signal were recorded with a step width of 250 nm and an axial resolution of 1.1 μm. The lateral pixel size was 50 nm in all images.

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REFERENCES

Abranches, R., Beven, A.F., Aragón-Alcaide, L., and Shaw, P.J. (1998). Transcription sites are not correlated with chromosome territories in wheat nuclei. J. Cell Biol. **143,** 5–12.

Allfrey, V., Faulkner, R.M., and Mirsky, A.E. (1964). Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. Proc. Natl. Acad. Sci. USA **51,** 786–794.

Banditt, M., Koller, T., and Sogo, J.M. (1999). Transcriptional activity and chromatin structure of enhancer-deleted rRNA genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **19,** 4953–4960.

Belyaev, N.D., Keohane, A.M., and Turner, B.M. (1996). Differential underacetylation of histones H2A, H3 and H4 on the inactive X chromosome in human female cells. Hum. Genet. **97,** 573–578.

Belyaev, N.D., Houben, A., Baranczewski, P., and Schubert, I. (1997). Histone H4 acetylation in plant heterochromatin is altered during the cell cycle. Chromosoma **106,** 193–197.

Belyaev, N.D., Houben, A., Baranczewski, P., and Schubert, I. (1998). The acetylation patterns of histones H3 and H4 along *Vicia faba* chromosomes are different. Chromosome Res. **6,** 59–63.

Braunstein, M., Sobel, R.E., Allis, C.D., Turner, B.M., and Broach, J.R. (1996). Efficient transcriptional silencing in *Saccharomyces cerevisiae* requires a heterochromatin histone acetylation pattern. Mol. Cell. Biol. **16,** 4349–4356.

Buzek, J., Riha, K., Siroky, J., Ebert, I., Greilhuber, J., and Vyskot, B. (1998). Histone H4 underacetylation in plant facultative heterochromatin. Biol. Chem. **379,** 1235–1241.

Conconi, A., Widmer, R.M., Koller, T., and Sogo, J.M. (1989). Two different chromatin structures coexist in ribosomal RNA genes throughout the cell cycle. Cell **57,** 753–761.

Conconi, A., Sogo, J.M., and Ryan, C.A. (1992). Ribosomal gene clusters are uniquely proportioned between open and closed chromatin structures in both tomato leaf cells and exponentially growing suspension cultures. Proc. Natl. Acad. Sci. USA **89,** 5256–5260.

Csordas, A. (1990). On the biological role of histone acetylation. Biochem. J. **265,** 23–28.

Dammann, R., Lucchini, R., Koller, T., and Sogo, J.M. (1993). Chromatin structures and transcription of rDNA in yeast *Saccharomyces cerevisiae*. Nucleic Acids Res. **21,** 2331–2338.

Derenzini, M., Pession, A., Licastro, F., and Novello, F. (1985). Electronmicroscopical evidence that ribosomal chromatin of human circulating lymphocytes is devoid of histones. Exp. Cell Res. **157,** 50–62.

Döbel, P., Schubert, I., and Rieger, R. (1978). Distribution of heterochromatin in a reconstructed karyotype of *Vicia faba* as identified by banding- and DNA-late replication patterns. Chromosoma **69,** 193–209.

Fuchs, J., Pich, U., Meister, A., and Schubert, I. (1994). Differentiation of field bean heterochromatin by *in situ* hybridization with a repeated *Fok*I sequence. Chromosome Res. **2,** 25–28.

Fuchs, J., Strehl, S., Brandes, A., Schweizer, D., and Schubert, I. (1998). Molecular-cytogenetic characterization of the *Vicia faba* genome—Heterochromatin differentiation, replication patterns and sequence localization. Chromosome Res. **6,** 219–230.

Garcia-Ramirez, M., Rocchini, C., and Ausio, J. (1995). Modulation of chromatin folding by histone acetylation. J. Biol. Chem. **270,** 17923–17928.

Gébrane-Younès, J., Fomproix, N., and Hernandez-Verdun, D. (1997). When rDNA transcription is arrested during mitosis, UBF is still associated with non-condensed rDNA. J. Cell Sci. **110,** 2429–2440.

González-Melendi, P., Testillano, P.S., Mena, C.G., Muller, S., Raska, I., and Risueño, M.C. (1998). Histones and DNA ultrastructural distribution in plant cell nucleus: A combination of immunogold and cytochemical methods. Exp. Cell Res. **242,** 45–59.

Grunstein, M. (1997). Histone acetylation in chromatin structure and transcription. Nature **389,** 349–352.

Grunstein, M. (1998). Yeast heterochromatin: Regulation of its assembly and inheritance by histones. Cell **93,** 325–328.

Hecht, A., Laroche, T., Strahl-Bolsinger, S., Gasser, S.M., and Grunstein, M. (1995). Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: A molecular model for the formation of heterochromatin in yeast. Cell **80,** 583–592.

Hong, L., Schroth, G.P., Matthews, H.R., Yau, P., and Bradbury, E.M. (1993). Studies of the DNA binding properties of histone H4 amino terminus. J. Biol. Chem. **268,** 305–314.

Houben, A., Brandes, A., and Schubert, I. (1994). The distribution of cDNA sequences on field bean chromosomes. Genome **37,** 1065–1067.

Houben, A., Belyaev, N.D., Turner, B.M., and Schubert, I. (1996). Differential immunostaining of plant chromosomes by antibodies recognizing acetylated histone H4 variants. Chromosome Res. **4,** 191–194.

Houben, A., Belyaev, N.D., Leach, C.R., and Timmis, J.N. (1997). Differences of histone H4 acetylation and replication timing between A and B chromosomes of *Brachycome dichromosomatica*. Chromosome Res. **5,** 233–237.

Idei, S., Kondo, K., Turner, B.M., and Fukui, K. (1996). Tomographic distribution of acetylated histone H4 in plant chromosomes, nuclei and nucleoli. Chromosoma **105,** 293–302.

Jackson, D.A., Hassan, A.B., Errington, R.J., and Cook, P.R. (1993). Visualization of focal sites of transcription within human nuclei. EMBO J. **12,** 1059–1065.

Jackson, V., Shires, A., Tanphaichitr, N., and Chalkley, R. (1976). Modifications of histones immediately after synthesis. J. Mol. Biol. **104,** 471–483.

Jeppesen, P. (1997). Histone acetylation: A possible mechanism for the inheritance of cell memory at mitosis. Bioessays **19,** 67–74.

Jeppesen, P., and Turner, B.M. (1993). The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. Cell **74,** 281–289.

Kato, A., Yakura, K., and Tanifuji, S. (1984). Sequence analysis of *Vicia faba* repeated DNA, the *Fok*I repeat element. Nucleic Acids Res. **12,** 6415–6426.

Keohane, A.M., Lavender, J.S., O'Neill, L.P., and Turner, B.M. (1998). Histone acetylation and X inactivation. Dev. Genet. **22,** 65–73.

Klein, J., and Grummt, I. (1999). Cell cycle–dependent regulation of RNA polymerase I transcription: The nucleolar transcription factor UBF is inactive in mitosis and early G1. Proc. Natl. Acad. Sci. USA **96,** 6096–6101.

Kubaláková, M., Macas, J., and Dolezel, J. (1997). Mapping of repeated DNA sequences in plant chromosomes by PRINS and C-PRINS. Theor. Appl. Genet. **94,** 758–763.

Lee, D.Y., Hayes, J.J., Pruss, D., and Wolffe, A.P. (1993). A positive role for histone acetylation in transcription factor access to nucleosomal DNA. Cell **72,** 73–84.

Loidl, P. (1988). Towards an understanding of the biological function of histone acetylation. FEBS Lett. **227,** 91–95.

Loidl, P. (1994). Histone acetylation: Facts and questions. Chromosoma **103,** 441–449.

Lucchini, R., and Sogo, J.M. (1995). Replication of transcriptionally active chromatin. Nature **374,** 276–280.

McBlane, F., and Boyes, J. (2000). Stimulation of V(D)J recombination by histone acetylation. Curr. Biol. **10,** 483–486.

McMurry, M.T., and Krangel, M.S. (2000). A role for histone acetylation in the developmental regulation of V(D)J recombination. Science **287,** 495–498.

Miller, O.L., and Beatty, R.R. (1969). Visualization of nucleolar genes. Science **164,** 955–957.

Rabl, C. (1885). Über Zelltheilung. Gegenbaurs Morphol. Jahrb. **10,** 214–330.

Roussel, P., André, C., Comai, L., and Hernandez-Verdun, D. (1996). The rDNA transcription machinery is assembled during mitosis in active NORs and absent in inactive NORs. J. Cell Biol. **133,** 235–246.

Ruiz-Carrillo, A., Wangh, L., and Allfrey, V. (1975). Assembly of newly replicated chromatin. Science **190,** 117–128.

Sadoni, N., Langer, S., Fauth, C., Bernardi, G., Cremer, T., Turner, B.M., and Zink, D. (1999). Nuclear organization of mammalian genomes: Polar chromosome territories build up functionally distinct higher order compartments. J. Cell Biol. **146,** 1211–1226.

Scheer, U., and Hock, R. (1999). Structure and function of the nucleolus. Curr. Opin. Cell Biol. **11,** 385–390.

Scheer, U., Thiry, M., and Goessens, G. (1993). Structure, function and assembly of the nucleolus. Trends Cell Biol. **3,** 236–241.

Schubert, I., and Rieger, R. (1979). Asymmetric banding of *Vicia faba* chromosomes after BrdU incorporation. Chromosoma **70,** 385–391.

Schubert, I., Dolezel, J., Houben, A., Scherthan, H., and Wanner, G. (1993). Refined examination of plant metaphase chromosome structure at different levels made feasible by new isolation methods. Chromosoma **102,** 96–101.

Shaw, P.J., Highett, M.I., Beven, A.F., and Jordan, E.G. (1995). The nucleolar architecture of polymerase I transcription and processing. EMBO J. **14,** 2896–2906.

Smith, J.G., Hill, R.S., and Baldwin, J.P. (1995). Plant chromatin structure and post-translational modifications. Crit. Rev. Plant Sci. **14,** 299–328.

Sobel, R.E., Cook, R.G., Perry, C.A., Annunziato, A.T., and Allis, C.D. (1995). Conservation of deposition-related acetylation sites in newly synthesized histones H3 and H4. Proc. Natl. Acad. Sci. USA **92,** 1237–1241.

Sogo, J.M., Ness, P.J., Widmer, R.M., Parish, R.W., and Koller, T. (1984). Psoralen-crosslinking of DNA as a probe for the structure of active nucleolar chromatin. J. Mol. Biol. **178,** 897–928.

Spencer, V.A., and Davie, J.R. (1999). Role of covalent modifications of histones on regulating gene expression. Gene **240,** 1–12.

Stein, P., Worrad, D.M., Belyaev, N.D., Turner, B.M., and Schultz, R.M. (1997). Stage-dependent redistribution of acetylated histones in nuclei of the early preimplantation mouse embryo. Mol. Reprod. Dev. **47,** 421–429.

Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature **413,** 41–45.

Struhl, K. (1998). Histone acetylation and transcriptional regulatory mechanisms. Genes Dev. **12,** 599–606.

Taddei, A., Roche, D., Sibarita, J.-B., Turner, B.M., and Almouzni, G. (1999). Duplication and maintenance of heterochromatin domains. J. Cell Biol. **147,** 1153–1166.

ten Hoopen, R., Manteuffel, R., Dolezel, J., Malysheva, L., and Schubert, I. (2000). Evolutionary conservation of kinetochore protein sequences in plants. Chromosoma (in press).

Thiry, M., and Muller, S. (1989). Ultrastructural distribution of histones within Ehrlich tumour cell nucleoli: A cytochemical and immunocytochemical study. J. Histochem. Cytochem. **37,** 853–862.

Thompson, J.S., Ling, X., and Grunstein, M. (1994). Histone H3 terminus is required for telomeric and silent mating locus repression in yeast. Nature **369,** 245–247.

Thompson, W.F., Beven, A.F., Wells, B., and Shaw, P.J. (1997). Sites of rDNA transcription are widely dispersed through the nucleolus in *Pisum sativum* and can comprise single genes. Plant J. **12,** 571–581.

Turner, B.M. (1991). Histone acetylation and control of gene expression. J. Cell Sci. **99,** 13–20.

Turner, B.M. (1993). Decoding the nucleosome. Cell **75,** 5–8.

Turner, B.M. (2000). Histone acetylation and an epigenetic code. Bioessays **22,** 836–845.

Turner, B.M., and Fellows, G. (1989). Specific antibodies reveal ordered and cell-cycle-related use of histone-H4 acetylation sites in mammalian cells. Eur. J. Biochem. **179,** 131–139.

Turner, B.M. O'Neill, L.P., and Allan, I.M. (1989). Histone H4 acetylation in human cells. Frequency of acetylation at different sites defined by immunolabeling with site-specific antibodies. FEBS Lett. **253,** 141–145.

Vettese-Dadey, M., Grant, P.A., Hebbes, T.R., Crane-Robinson, C., Allis, C.D., and Workman, J.L. (1996). Acetylation of histone H4 plays a primary role in enhancing transcription factor binding to nucleosomal DNA *in vitro*. EMBO J. **15,** 2508–2518.

Vyskot, B., Siroky, J., Hladilova, R., Belyaev, N.D., and Turner, B.M. (1999). Euchromatic domains in plant chromosomes as revealed by H4 histone acetylation and early DNA replication. Genome **42,** 343–350.

Wansink, D.G., Schul, W., van der Kraan, I., van Steensel, B., van Driel, R., and de Jong, L. (1993). Fluorescent labeling of nascent RNA reveals transcription by RNA polymerase II in domains scattered throughout the nucleus. J. Cell Biol. **122,** 283–293.

White, D.A., Belyaev, N.D., and Turner, B.M. (1999). Preparation of site-specific antibodies to acetylated histones. Methods **19,** 417–421.

Yakura, K., and Tanifuji, S. (1983). Molecular cloning and restriction analysis of EcoRI-fragments of *Vicia faba* rDNA. Plant Cell Physiol. **24,** 1327–1330.

Yoshida, M., Kijima, M., Akita, M., and Beppu, T. (1990). Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by Trichostatin A. J. Biol. Chem. **265,** 17174–17179.

6.2. JASENCAKOVA Z, MEISTER A, SCHUBERT I

Chromatin organization and its relation to replication and histone acetylation during the cell cycle in barley.

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Chromatin organization and its relation to replication and histone acetylation during the cell cycle in barley

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Abstract We have studied the replication time, nuclear organization and histone acetylation patterns of distinct chromatin domains [nucleolus organizers (NORs), centromeres, euchromatin and heterochromatin] of barley during the cell cycle. The Rabl orientation of chromosomes, with centromeres and telomeres located at opposite nuclear poles, was found to be maintained throughout interphase. Replication started at the rDNA loci within nucleoli and then proceeded from the euchromatic distal chromosome regions toward the heterochromatic pole. Centromere association frequently occurred in mid- and late S-phase, i.e., during and after centromere replication. Euchromatin, centromeres and heterochromatin were found to be enriched in acetylated histone H4 (except for lysine 16) during their replication; then deacetylation occurred. The level of deacetylation of H4 in heterochromatin was more pronounced than in euchromatin. Deacetylation is finished in early G2-phase (lysine 8) or may last until mitosis or even the next G1-phase (lysines 5 and 12). The NORs were found to be most strongly acetylated at lysines 5 and 12 of H4 during mitosis, independently of their potential activity in nucleolus formation and rDNA transcription. The acetylation pattern of chromosomal histone H3 was characterized by low acetylation intensity at centromeres (lysines 9/18) and pericentromeric regions (lysine 14) and more intense uniform acetylation of the remaining chromatin; it remained fairly constant throughout the cell cycle. These results have been compared with the corresponding data published for mammals and for the dicot *Vicia faba*. This revealed conserved features as well as plant- or species-specific peculiarities. In particular, the connection of acetylation intensity of H4 at microscopically identifiable chromatin domains with replicational but not with transcriptional activity during the cell cycle seems to be conserved among eukaryotes.

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Introduction

The chromatin of interphase nuclei is highly organized. As early as 1885 Rabl (1885) proposed a model according to which the anaphase/telophase orientation of chromosomes is maintained in interphase nuclei, resulting in centromeres and telomeres being located at opposite nuclear poles. During the following century, cytogenetic investigation of chromosome structure and composition was mainly focused on mitotic/meiotic chromosomes because it was difficult to identify specific chromosome territories and individual chromatin domains in interphase nuclei, where essential processes such as replication, gene expression and DNA repair take place. During recent years, fluorescent in situ hybridization (FISH), immunostaining and flow-sorting of nuclei have been introduced and improved. In particular, the painting of entire chromosomes by FISH allowed the discovery and analysis of distinct chromosome territories within interphase nuclei in mammals (see, e.g., Eils et al. 1996; Dietzel et al. 1999; Nagele et al. 1999; Visser and Aten 1999; Tajbakhsh et al. 2000). These techniques enabled the development of complex approaches in combination with high resolution image processing for microscopic studies of the territories and structural composition of individual chromosomes and/or distinct chromatin domains in the course of the cell cycle. It thus became possible to examine directly structural/functional interrelations, i.e., to correlate spatial and temporal modification of chromatin structure with specific functions of chromatin domains.

One of the diverse chromatin modifications is the reversible acetylation of N-terminal lysine residues of the nucleosomal histones H3 (K9, 14, 18, 23) and H4 (K5, 8, 12, 16), which occurs in all eukaryotes studied so far. The degree of histone acetylation varies along the mitotic chromosomes of insects (Turner et al. 1992), mammals (Jeppesen and Turner 1993) and plants (Houben et al. 1996a; Belyaev et al. 1997, 1998; Vyskot et al. 1999) and is generally more intense in euchromatic than in heterochromatic domains. A high acetylation status

was found to be connected with transcription (reviewed, e.g., in Grunstein 1997; Struhl 1998), recombination (McBlane and Boyes 2000; McMurry and Krangel 2000) and DNA repair (Ikura et al. 2000) at the level of genes, providing, together with other chromatin modifications, in a concerted manner an epigenetic code used to specify unique downstream functions (for recent reviews see Strahl and Allis 2000; Turner 2000). Previously, studies were undertaken to elucidate at the microscopic level chromatin acetylation patterns during interphase in mammalian (Sadoni et al. 1999; Taddei et al. 1999) and plant nuclei (Buzek et al. 1998; Jasencakova et al. 2000).

Experiments on *Vicia faba* interphase nuclei have shown that acetylation of histone H4 (at lysine positions 5, 12 and 16) of eu- and heterochromatin domains (except for the nucleolus) is correlated with replication rather than with transcription, while histone H3 acetylation of chromatin domains did not change significantly during the cell cycle (Jasencakova et al. 2000).

Here we investigate the chromatin organization of mitotic and interphase chromosomes in barley. The arrangement of chromosomes during interphase stages, the sequential order of replication and the intensity of histone H3 and H4 acetylation of distinct chromatin domains such as nucleolus organizer regions (NORs), euchromatin, centromeres and heterochromatin during the mitotic cycle was studied. For this purpose, we combined FISH and immunolabeling techniques to identify chromosomal domains and their acetylation status in relation to the corresponding replicational and transcriptional activities in nuclei isolated from unsynchronized root-tip meristems and flow-sorted into G1-, early S-, mid S-, late S-, and G2-phase fractions. The results were compared with those obtained for *V. faba* and revealed common features as well as differences between monocot and dicot plants on the one hand and between animals and plants on the other. This led to conclusions as to the evolutionary conservation of the respective phenomena and to what degree histone acetylation of distinct chromatin domains is stable or cell cycle dependent and whether or not different intensities of histone acetylation reflect potential transcriptional activity of the corresponding domains or are temporally linked with replication processes.

Materials and methods

All experiments were performed on barley, *Hordeum vulgare* L. (2*n*=14), using the line MK14/2034, which is characterized by two homozygous translocations involving chromosomes 3S/4L (=T3-4ae) and 1S/7Sat (=T1-7an) see http://wheat.pw.usda.gov/ggpages/Barley_physical/Idiograms/. To study the correlation between histone acetylation and transcriptional activity of the NORs we additionally used the translocation line T2052 carrying NOR6 and NOR7 on the opposite arms of chromosome 6, and showing nucleolar dominance of NOR6 and nearly complete suppression of NOR7 (Schubert and Künzel 1990). Seeds were germinated on soaked paper at 24°C. Synchronization and fixation of root tip meristems and isolation of chromosomes were done according to Lysák et al. (1999).

Nuclei from unsynchronized root tips were fixed in 4% formaldehyde, TRIS buffer (10 mM TRIS, 10 mM Na_2EDTA, 100 mM Triton X-100, pH 7.5), washed in TRIS buffer and isolated from the meristematic regions as described previously (Schubert et al. 1993). After staining with 4′,6-diamidino-2-phenylindole (DAPI, 1 µg/ml) they were flow-sorted into G1-, early S-, mid S-, late S-, and G2-phase fractions using a FACStar^{Plus} flow cytometer and cell sorter (Becton Dickinson) with a Sort Enhancement Module (SEM) and an Argon-ion laser (INNOVA 90C-5) emitting UV light with 200 mW output power controlled by a Macintosh Computer with Cell Quest Software. The gates for sorting were set according to the histograms obtained for each suspension of nuclei. A representative histogram is given in Fig. 1. About 1000 nuclei of each fraction were sorted onto microscopic slides into a drop containing 100 mM TRIS, 50 mM KCl, 2 mM $MgCl_2$, 0.05% Tween 20 and 5% sucrose (Kubaláková et al. 1997), air-dried at room temperature for several hours and used immediately for immunolabeling and/or FISH, or stored at –20°C until use.

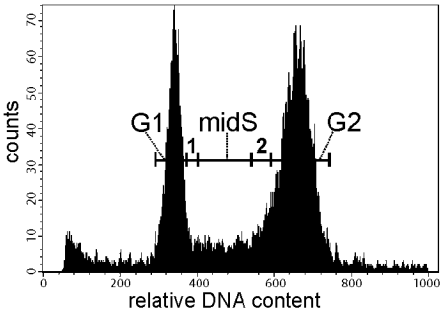

Fig. 1 Histogram of the relative DNA content of unsynchronized barley nuclei after 4′,6-diamidino-2-phenylindole (DAPI) staining and flow-cytometric analysis. The gates used for sorting are indicated (*1* early S-, *2* late S-phase)

Fluorescent in situ hybridization

The following probes were used: BAC7 containing barley centromere-specific retroelement sequences (Presting et al. 1998), pVER17 (with a 3.7 kb insert comprising 18S, 5.8S and most of the coding region of 25S rRNA genes of *V. faba*, Yakura and Tanifuji 1983), $(GAA)_{10}$ oligonucleotides (MWG-Biotech), which label the heterochromatin of barley (Pedersen and Linde-Laursen 1994; Pedersen et al. 1996), and HvT01, a 118 bp subtelomeric repeat of barley (Belostotsky and Ananiev 1990; Schubert et al. 1998).

Centromere- and NOR-specific probes were labeled with digoxigenin-11-dUTP, biotin-16-dUTP or tetramethylrhodamine-5-dUTP using a nick translation kit, and $(GAA)_{10}$ oligonucleotides using an end-labeling kit (both from Roche Biochemicals) according to the manufacturer's instructions. HvT01 repeats were amplified and labeled with digoxigenin-11-dUTP by the polymerase chain reaction with sequence-specific primers.

Treatment with RNase A (50 µg/ml in 2×SSC) for 30 min at 37°C was applied only prior to FISH with the rDNA probe. (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate.) Preparations on slides were postfixed in 4% paraformaldehyde, 2×SSC for 15 min, washed three times in 2×SSC, dehydrated through an ethanol series (70%, 96%) and air-dried. The hybridization mixture containing probe, 50% formamide, 10% dextran sulfate and 2×SSC was heated for 10 min at 80°C, cooled on ice and denatured again together with target DNA on slides for 1 min at 80°C. When double-color FISH was performed, one of the probes (BAC7) was directly labeled with rhodamine-5-dUTP. After overnight hybridization at 37°C, the slides were washed three times for 5 min each in 2×SSC at 37°C. Digoxigenin-labeled probes were detected with 1:50 anti-digoxigenin-fluorescein isothiocyanate (FITC; Roche Biochemicals). DNA was counterstained with DAPI (1 µg/ml in Vectashield, Vector).

5-Bromo-2′-deoxyuridine labeling combined with FISH

For pulse-labeling of replicating chromatin, the roots were incubated in 5-bromo-2′-deoxyuridine (BrdU, 100 µM), fluorodeoxyuridine (0.1 µM) and uridine (5 µM) for 30 min in the dark. After rinsing, the roots were fixed in 4% formaldehyde, TRIS buffer before isolation and flow-sorting of nuclei.

Detection of BrdU incorporation was combined with FISH with BAC7 to discriminate between the centromeric and telomeric poles. The slides were treated first as described above for FISH (postfixation, dehydration, air-drying, denaturation and overnight hybridization with probe). Incorporated BrdU was then detected together with the probe, using mouse anti-BrdU antibodies (Becton Dickinson, 1:20–1:50) applied together with anti-digoxigenin-FITC (Roche Biochemicals, 1:50) for digoxigenin-labeled BAC7, followed by anti-mouse-Alexa^{596} (Molecular Probes, 1:500–1:1000) and counterstaining with DAPI.

For detection of replicating centromeres, FISH with BAC7 was performed after BrdU labeling. In this experiment BrdU detection was as described (Jasencakova et al. 2000). Briefly, the slides were postfixed in 4% paraformaldehyde/PBS, washed in PBS, denatured in 50% formamide/PBS at 80°C for 1 min and cooled down in ice-cold PBS. After blocking, the slides were incubated with mouse anti-BrdU (Becton Dickinson, 1:20) followed by anti-mouse-Alexa^{596} (Molecular Probes, 1:500). After counterstaining with DAPI (1 µg/ml in Vectashield) the slides were checked and images of nuclei together with their coordinates were recorded. After washing in TNT (100 mM TRIS-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20), the slides were dehydrated through an ethanol series, air-dried, and baked at 60°C for 30 min. Fluorescent in situ hybridization with BAC7 was then performed as described above. Biotin-labeled BAC7 was detected using streptavidin-AMCA (7-amino-4 methylcoumarin-3-acetic acid,Vector, 1:50), followed by biotinylated anti-streptavidin (Vector, 1:200) and streptavidin-AMCA (1:50) for signal amplification. Slides were mounted in Vectashield. The BrdU labeling patterns of nuclei before FISH were then compared with FISH signals obtained with the BAC7 probe.

Histone immunolabeling

For histone immunolabeling the following rabbit polyclonal antisera recognizing histone H3 and H4 isoforms with acetylated lysine residues (given in parentheses) were used: R41 (H4Ac5), R232 (H4Ac8), R101 (H4Ac12), R252 (H4Ac16), R243 (binding preferentially to tri- and tetra-acetylated H4), R47 (H3Ac9 and/or 18) and R224 (H3Ac14) (Turner and Fellows 1989; Turner et al. 1989; Belyaev et al. 1996; Stein et al. 1997; White et al. 1999). The specificity of these antibodies to the corresponding isoforms of plants has been shown on immunoblots (Buzek et al. 1998). Preimmune sera neither reacted with nuclear proteins (Buzek et al. 1998) nor with chromosomes of the tested plants (Vyskot et al. 1999). The immunolabeling procedure was carried out as described (Jasencakova et al. 2000): after postfixation in 4% paraformaldehyde in PBS and washes in PBS, the slides were blocked for 1 h at 37°C, and then incubated with the primary antisera diluted 1:100–1:200 in PBS buffer containing 1% BSA, 10% horse serum, and 0.1% Tween 20. Antisera were detected by anti-rabbit-FITC (Sigma, 1:80), and nuclei were counterstained with DAPI (1 µg/ml, Vectashield). Secondary antibodies stained neither mitotic chromosomes nor nuclei of barley.

When histone immunolabeling and FISH were combined, immunolabeling was performed first. After checking the slides, coverslips were removed and dehydration, baking and FISH were done as described above. In this case, the probe was either directly labeled (tetramethylrhodamine-dUTP) or, when digoxigenin-labeled probe was used, it was detected with anti-digoxigenin-rhodamine (Roche Biochemicals, 1:50).

Usually, histone immunolabeling signals were preserved after FISH. In case their intensity decreased considerably, images of nuclei captured before FISH were compared with those obtained after FISH.

Nascent RNA labeling

5′-Bromo-5′-triphosphate (BrUTP) incorporation into isolated nuclei was done on slides according to Thompson et al. (1997) and Abranches et al. (1998). Preparations were incubated with the transcription mix consisting of 50 µM CTP, 50 µM GTP, 25 µM BrUTP (Sigma), 100 U/ml RNase inhibitor RNA Guard (Pharmacia) in MPB buffer (100 mM potassium acetate, 20 mM KCl, 20 mM HEPES, 1 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, pH 7.4) for 6 min at room temperature. Nuclei were fixed in 4% paraformaldehyde/PBS for 30 min, washed in PBS and blocked for 45 min at 37°C. BrUTP was then detected using the same antibodies as for BrdU (see above).

The slides were inspected using a Zeiss Axiophot 2 epifluorescence microscope equipped with a cooled CCD camera (Photometrics). Images were captured using IPLab Spectrum software, pseudocolored, and merged in Adobe Photoshop.

Results

Polar nuclear organization of barley nuclei is preserved throughout the entire interphase

A strong polar organization of nuclei with centromeres clustered at one pole and telomeres at the opposite nuclear pole (Rabl orientation, Rabl 1885), as observed for barley by Anamthawat-Jonsson and Heslop-Harrison (1990), Noguchi and Fukui (1995) and Dong and Jiang (1998), was confirmed by our two-color FISH with BAC7 (containing centromere-specific retroelement sequences, Presting et al. 1998) and HvT01 (a 118 bp subtelomeric tandem repeat, Belostotsky and Ananiev 1990), see Fig. 2A. This was maintained throughout all interphase stages. To identify heterochromatin domains in interphase nuclei, $(GAA)_{10}$ re-

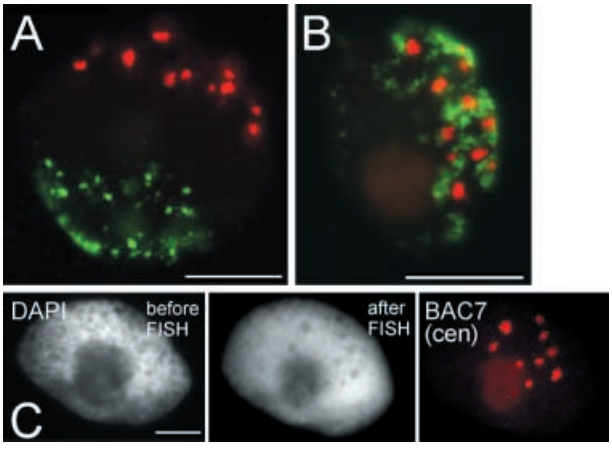

Fig. 2A–C Polar chromatin organization of barley interphase nuclei as revealed by fluorescent in situ hybridization (FISH). **A** Centromeres (BAC7, *red*) and telomeres (HvT01, *green*) are located at opposite nuclear poles. **B** Heterochromatin represented by $(GAA)_{10}$ oligonucleotide signals (*green*) is located exclusively at the centromeric pole (BAC7, *red*). *Bars* represent 10 µm. **C** The polar organization of barley nuclei is also reflected by DAPI staining (*left* and *middle*). The pole stained more extensively with DAPI contains centromeres (BAC7 signals, *right*). The DAPI-negative spots (*middle*) correspond to the centromeres (*right*). *Bar* represents 5 µm

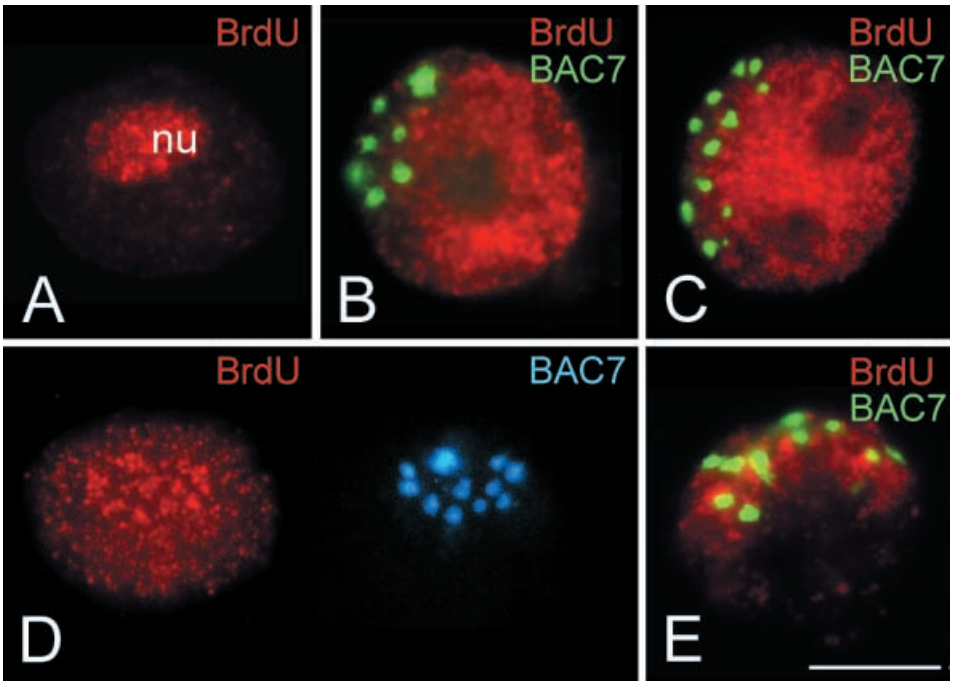

Fig. 3A–E Replication patterns of isolated and flow-sorted nuclei of barley root tip meristems during S-phase after a 30 min 5-bromo-2′-deoxyuridine (BrdU) pulse. Centromere-specific BAC7 was used as a probe to identify the nuclear poles. **A** Early S-phase with nucleoli (*nu*) exclusively labeled. **B** Early S-phase with stronger BrdU labeling at the telomeric pole. **C** Mid S-phase with BrdU signals distributed uniformly throughout the nucleus. Centromeres (*green* BAC7 signals) are not labeled with BrdU. **D** Mid/late S-phase with uniformly labeled chromatin and more intensely labeled spots (*left*) at the regions of centromeres [BAC7 detected by streptavidin-AMCA (7-amino-4 methylcoumarin-3-acetic acid), *blue*, *right*]. **E** Late S-phase with only the centromeric pole BrdU labeled. *Bar* represents 10 µm

peats locating to heterochromatic C-bands on barley chromosomes (Pedersen and Linde-Laursen 1994; Pedersen et al. 1996) were used. Most of the $(GAA)_{10}$ signals were found to be clustered at the centromeric pole close to the centromeres when $(GAA)_{10}$ was hybridized simultaneously with BAC7 (Fig. 2B). A polar orientation of chromatin was also observed after DAPI staining. The centromere-containing pole proved to be more intensely and the telomere-containing pole less intensely stained while centromeres themselves appeared as DAPI-negative spots. The polar DAPI staining intensity sometimes diminished or disappeared after the FISH procedure, whereas DAPI-negative spots indicating centromeres were more pronounced after FISH (Fig. 2C).

The majority of nuclei probed with BAC7 revealed 12 to 14 intense signal spots. This corresponds to the centromeric FISH signals on mitotic chromosomes with occasionally weaker signals at the centromeres of one of the seven chromosome pairs. Nuclei with fewer than 12, but larger and more intense signals indicate association of centromeres as reported for centromeres in field bean nuclei (Houben et al. 1995). To see whether or not centromere association in barley nuclei is correlated with a specific cell cycle stage, the BAC7 signals were counted in sorted G1-, early S-, mid S-, late S- and G2-phase nuclei. The results (Table 1) show that the proportion of nuclei with less than 12 BAC7 signals increased from ~41% in G1- and early S- to mid S-phase (55.9%), reached a maximum in late S-phase (62.5%) and then decreased slightly in G2-phase (59.4%). It cannot yet be proved, however, whether or not this association is confined to homologous centromeres as usually observed for *Arabidopsis* (Fransz et al. 2001).

Table 1 Number of FISH signals obtained with the BAC7 probe representing centromeres in flow-sorted nuclei of *Hordeum vulgare*

	<12 signals		12–14 signals[a]		*n*
	n	%	*n*	%	
G1-phase	40	41.7	54	56.2	96
Early S-phase	38	41.3	54	58.7	92
Mid S-phase	57	55.9	45	44.1	102
Late S-phase	55	62.5	29	32.9	88
G2-phase	57	59.4	39	40.6	96

[a] More than 14 signals were found in 2.1% of G1-phase and 4.5% of late S-phase nuclei; the additional signals probably represent background artifacts

Replication of the chromatin domains is temporally ordered and reflects the polar nuclear organization

Fluorescent in situ hybridization with BAC7 combined with detection of BrdU incorporation in flow-sorted nuclei allowed the temporal and spatial replication patterns of chromatin domains during S-phase to be followed. Almost 40% of nuclei in early S-phase showed labeled nucleoli after a 30 min BrdU pulse, while only 2% of nuclei showed this pattern in mid S-phase (see Fig. 3 and Table 2 for the frequency of the replication patterns during S-phase). The telomeric pole was strongly labeled in more than 40% of nuclei in early S-phase, in 15% of mid S-phase nuclei, and not at all in late S-phase. In contrast, the centromeric pole was found to be labeled much more strongly than the remaining chromatin in about 10% of mid S-phase and 40% of late S-phase nuclei. Occasionally occurring BrdU-labeled nuclei in G2-phase exclusive-

Table 2 Replication patterns of barley nuclei during S-phase

	n	Nucleolus labeled		Telomeric pole labeled		Uniformly labeled		Centromeres labeled		Centromeric pole labeled	
		n	%	*n*	%	*n*	%	*n*	%	*n*	%
Early S-phase	85	33	38.8	35	41.2	17	20.0	0	0.0	0	0.0
Mid S-phase	105	2	1.9	16	15.2	60	57.1	16	15.2	11	10.5
Late S-phase	86	0	0.0	0	0.0	17	19.8	34	39.5	35	40.7

Table 3 Histone H4Ac5 labeling patterns of barley nuclei during interphase

Labeling type	Cell cycle stage									
	G1-phase		Early S-phase		Mid S-phase		Late S-phase		G2-phase	
	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*
I+Ia	78.1	182	28.2	62	15.6	37	19.2	46	51.6	114
II	8.6	20	55.0	121	41.3	98	17.6	42	7.2	16
III	0.4	1	7.7	17	26.6	63	24.7	59	10.4	23
IIIa	5.6	13	5.5	12	11.0	26	32.2	77	26.7	59
IV	7.3	17	3.6	8	5.5	13	6.3	15	4.1	9
Total	100	233	100	220	100	237	100	239	100	221

ly showed this pattern. Uniform distribution of BrdU signals over the nuclei was found in 57% of mid S-phase nuclei.

To visualize BrdU-labeled centromeres it was necessary to perform FISH with BAC7 after BrdU detection (see Materials and methods). Intense incorporation of BrdU into centromeric regions was found in middle and late S-phase (15.2% and 39.5% of nuclei, respectively). The remaining chromatin in such nuclei was generally strongly labeled and the BrdU signals were uniformly distributed all over the nuclei (Fig. 3D).

From these data the following sequence of replication for defined chromatin domains in barley nuclei was derived: rDNA is replicated during early S-phase followed by replication of euchromatin, which starts from the telomeric pole and continues throughout mid S-phase, spreading all over the nucleus. In accordance with the data of Kakeda and Yamagata (1992) centromeres start replication in mid S-phase, when euchromatin replication is still ongoing. Heterochromatin (located at the centromeric pole) starts replication after centromeres, when euchromatin replication is mostly finished.

Histone acetylation of the monocot *H. vulgare* revealed similarities with as well as differences from the dicot *V. faba*

Interphase nuclei of *V. faba* revealed different H4Ac5 labeling patterns, altering in frequency during the cell cycle (Jasencakova et al. 2000). Comparable patterns were also observed for H4Ac5 in barley nuclei isolated from root-tip meristems (Fig. 4A–D): type I with strongly labeled nucleoli and weakly labeled chromatin occasionally including unlabeled spots; subtype Ia with less (often peripherally) labeled nucleoli (not shown); type II with unlabeled nucleoli and densely labeled chromatin; type III with unlabeled nucleoli, weak labeling of most chromatin and clusters of bright labeling at one pole; subtype IIIa differs from III by (strongly) labeled nucleoli; type IV (not shown) is characterized by more or less uniform labeling. The same patterns were also observed for barley nuclei after labeling of H4Ac12 and tetra-acetylated H4.

The frequency of the H4Ac5 labeling types during interphase is shown in Table 3 and Fig. 5. Similar to *V. faba*, strongly and moderately labeled nucleoli (types I and Ia) appeared most frequently in G1- and G2-phase, unlabeled nucleoli (type II) in early S-phase nuclei. Types III and IIIa with brightly labeled spots (at one pole) were frequently found in mid and late S- and with decreasing frequency in G2-phase nuclei. Uniformly labeled type IV nuclei occurred at a low frequency (3.6%–7.3%, see Table 3) within all cell cycle fractions.

In *V. faba* nuclei the strongly acetylated spots of type III and IIIa nuclei represented heterochromatin domains. To see whether the same is true also for barley, H4Ac5 immunolabeling was combined with FISH using $(GAA)_{10}$ oligonucleotides and BAC7 as probes. H4Ac5 bright spots were always found to be clustered at the centromeric pole of barley nuclei. In type III and IIIa nuclei the bright spots colocalized with BAC7 signals at the centromeres (Fig. 4E). Also nuclei with more H4Ac5 bright spots than BAC7 signals were found. To see whether or not the strongly acetylated chromatin domains, not identical with centromeres, might be heterochromatin domains, FISH with $(GAA)_{10}$ oligonucleotides was performed after H4Ac5 labeling. Mostly complete or at least partial colocalization of such H4Ac5 bright spots and $(GAA)_{10}$ was found (Fig. 4F), confirming that in addition to centromeres heterochromatin is also subject to H4 acetylation during late S-phase in barley. At a low

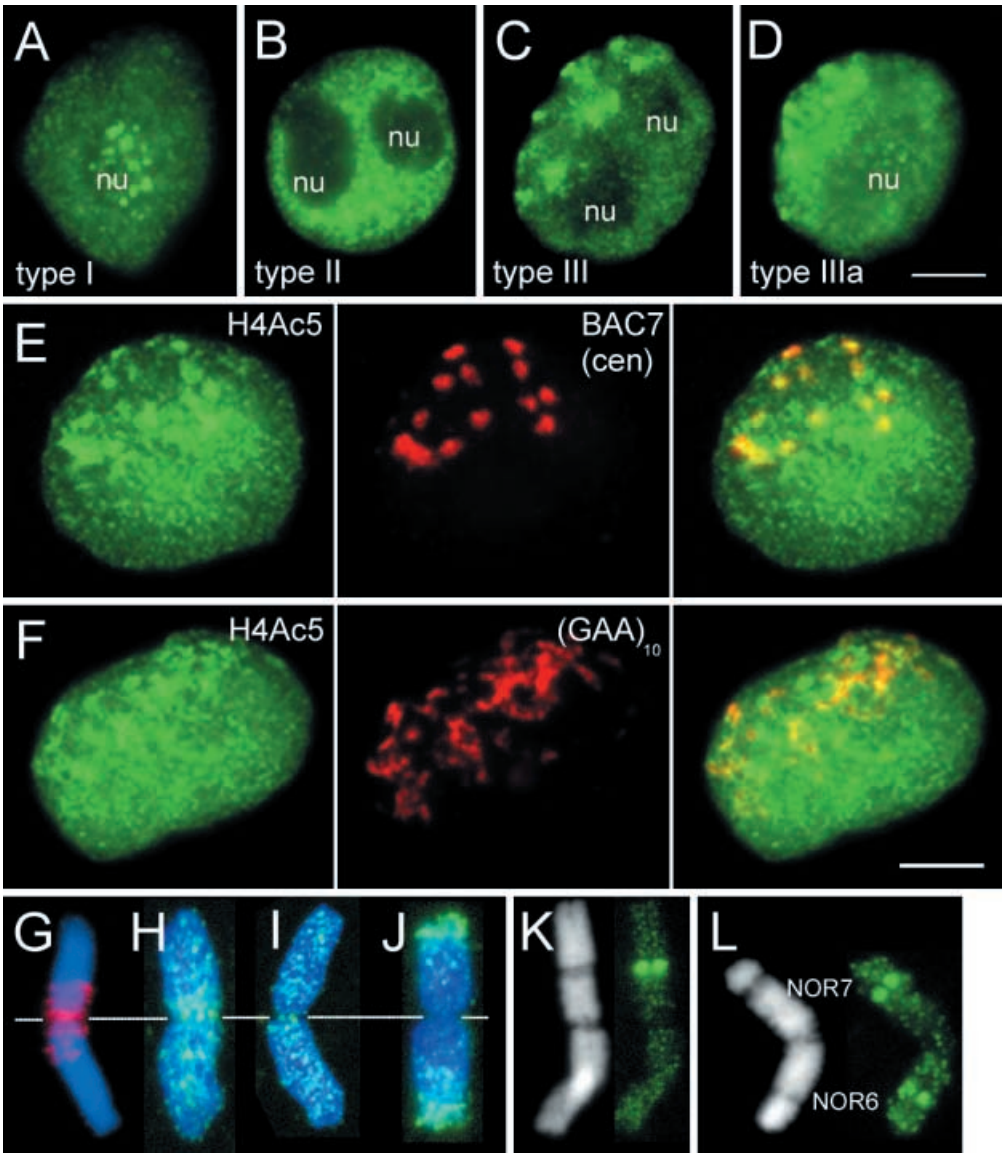

Fig. 4A–L Histone H4Ac5 labeling of barley interphase nuclei and metaphase chromosomes. **A–D** Different labeling patterns of interphase nuclei after immunostaining with antibodies against H4Ac5. **A** Type I. **B** Type II. **C** Type III. **D** Type IIIa. Note the differences in chromatin and nucleolus (*nu*) labeling. **E** Most of the brightly labeled spots in type III and IIIa nuclei (*left*) represent centromeres as shown after FISH (*red* BAC7 signals, *middle*). Signals for H4Ac5 and BAC7 colocalize to a great extent (*yellow*, *right*). In addition the nucleolus is strongly acetylated as is typical for type IIIa nuclei. **F** In addition to the centromeres heterochromatin is also subject to H4 acetylation in type III (mid to late S-phase) nuclei (*left*) as shown after FISH with $(GAA)_{10}$ (*red*, *middle*) and merging of both signals (*yellow*, *right*). **G** $(GAA)_{10}$ oligonucleotides localize to the pericentromeric heterochromatin (identical with C-bands) of barley chromosomes, as shown for chromosome 4. **H–J** Examples of representative metaphase chromosome labeling patterns after H4Ac5 detection. **H** The pericentromeric region is more strongly labeled than the remaining chromatin. **I** Uniform distribution of signals all over the chromosome. **J** Centromeric and pericentromeric heterochromatin is less labeled than the distal euchromatic regions (majority of chromosomes). **K** The nucleolus organizer region (NOR) reveals the most intense H4Ac5 labeling of the chromosome 7^1 of karyotype MK14/2034. **L** Chromosome 6 of the translocation line T2052 (carrying NOR6 and 7 on the opposite arms) with both NORs strongly labeled for H4Ac5 although only NOR6 is active in nucleolus formation (Schubert and Künzel 1990). *Bars* in **A–D** and **E, F** represent 5 µm

frequency prophases were also more intensely labeled at one pole. Occasionally, even metaphase chromosomes with strongly labeled pericentromeric regions were detected (Fig. 4H), and a few type IIIa nuclei were still present during G1-phase (Table 3), indicating delayed deacetylation of heterochromatic domains in these cases. In general, however, the centromeres and pericentromeric regions of mitotic chromosomes showed a similar or weaker acetylation of all acetylatable lysines of H4 than the distal euchromatic regions (Table 4; Fig. 4I, J).

While for heterochromatin of *V. faba*, K5, K12 and K16 of H4 appeared to be highly acetylated in a deposition-related manner during replication (Belyaev et al. 1997; Jasencakova et al. 2000), which indicates incorpo-

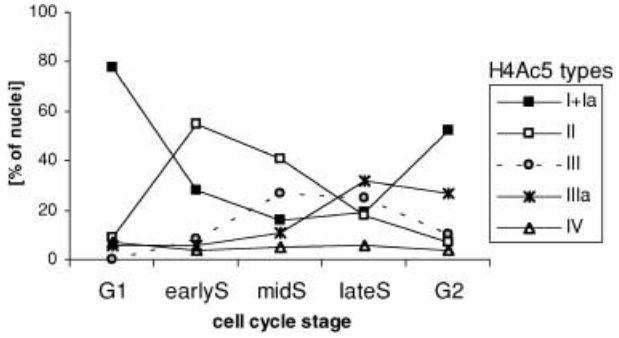

Fig. 5 Changes in frequency of the H4Ac5 labeling types during interphase (compare Fig. 4A–D and text)

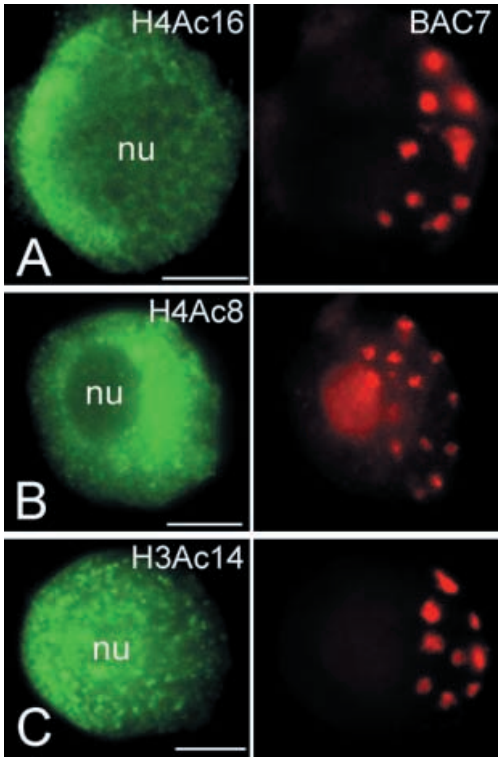

Fig. 6A–C Acetylated isoforms of histones H4 and H3 reveal a polar distribution in barley interphase nuclei. The positions of nucleoli (*nu*) are indicated. The centromere-specific BAC7 probe (*red* FISH signals, *right*) was used to identify the centromeric pole. **A** Intense H4Ac16 immunosignals (*left*) were found at the telomeric pole, while the centromeric pole remained almost unlabeled throughout interphase. **B** Stronger H4Ac8 immunolabeling signals were found at the centromeric pole in about 30% of mid S-phase nuclei. A strong autofluorescence of nucleoli does sometimes appear after FISH. **C** H3Ac14 immunosignals are more intense at the telomeric than at the centromeric pole. *Bars* represent 5 µm

ration of acetylated isoforms into newly replicated chromatin (Sobel et al. 1995), this was not the case for K16 in barley heterochromatin. Intense immunosignals for H4Ac16 were confined to the telomere-containing euchromatic pole (Fig. 6A) during all interphase stages. Instead, strong H4Ac8 labeling, which was not observed for *V. faba* heterochromatin, occurred at the centromeric

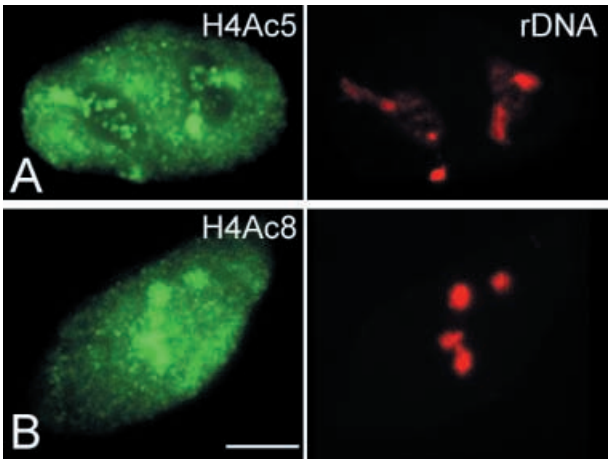

Fig. 7A, B Perinucleolar knobs of rDNA can be subject to strong histone H4 acetylation. **A** H4Ac5 signals (*left*) at perinucleolar knobs (*red* FISH signals for rDNA, *right*) were found in 10% of G1- and S-phase nuclei, and 15% of G2-phase nuclei. **B** Strong H4Ac8 immunosignals (*left*) at rDNA perinucleolar knobs (*right*) were detected in about 20% of nuclei during all cell cycle stages. *Bar* represents 5 µm

Table 4 Most frequent acetylation patterns of histones H4 and H3 in *H. vulgare* mitotic chromosomes. (+, –, and uni indicate a higher, lower or uniform level, respectively, of acetylation as compared with 'average' chromatin)

		Nucleolus organizer region	Heterochromatin[a] and centromeres
H4	K5	+	–
	K8	Uni	–
	K12	+	–
	K16	Uni	–
	TetraAcH4	+	–
H3	K14	Uni	–
	K9/18	Uni	Uni, cen$^-$

[a] Confined to pericentromeric regions

pole (Fig. 6B) in >30% of mid S-, 9% of late S- and 6% of G2-phase nuclei of barley.

As in *V. faba*, NORs turned out to be the most strongly labeled regions for H4Ac5 (Fig. 4K) in ~80% of NOR-bearing metaphase chromosomes of barley. The same was observed for barley NORs during mitosis (Idei et al. 1996). Within the barley translocation line T2052 carrying NOR6 and NOR7 on opposite arms of chromosome 6, both NORs showed similarly strong acetylation (Fig. 4L), albeit only NOR6 is active in nucleolus formation and NOR7 is nearly completely suppressed (Schubert and Künzel 1990). Both barley NORs were strongly labeled also for H4Ac12 and tetra-acetylated H4 but not for H4Ac8 and H4Ac16 (Table 4).

Previous experiments with *V. faba* have shown that perinucleolar knobs of rDNA generally do not contain acetylated H4 (Jasencakova et al. 2000) and are transcriptionally inactive (Shaw et al. 1995). Perinucleolar knobs of rDNA in barley nuclei, however, were found to contain H4Ac5 (Fig. 7A) in up to 10% of G1- and

S-phase and in ~15% of G2-phase nuclei (type I with strongly labeled nucleoli). The rDNA knobs also contained H4Ac8 (Fig. 7B) in about 20% of nuclei of all cell cycle stages while nucleolar labeling was in most cases not stronger than that of the remaining chromatin.

Two different heterochromatin fractions of *V. faba* were, depending on their base composition, either less or more heavily acetylated at K14 and K9/18 of H3 than the euchromatin throughout the cell cycle (Jasencakova et al. 2000). On barley mitotic chromosomes, centromeres were less labeled at K14 and K9/18 than the remaining chromatin, whereas pericentromeric heterochromatin showed weaker labeling with H3Ac14 but not with H3Ac9/18-specific antibodies (Table 4). In interphase nuclei, the pole opposite the centromeres was always more strongly labeled for H3Ac14 (Fig. 6C). Nucleoli were labeled similarly to or less than the remaining chromatin. No polar distribution of signals was observed after labeling of H3Ac9/18 in barley nuclei. As in *V. faba*, the interphase acetylation patterns of H3 were fairly constant and corresponded to those of mitotic chromosomes.

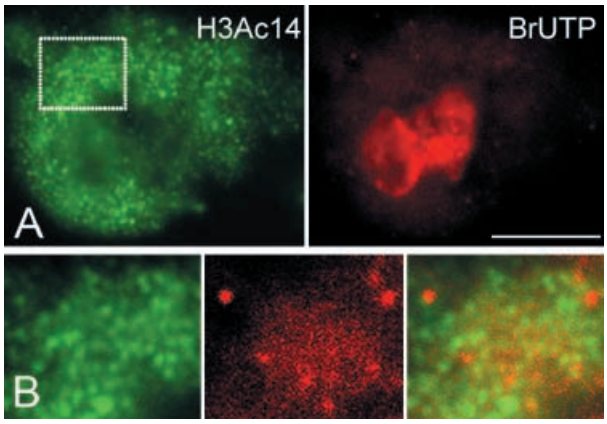

Fig. 8A, B Histone H3 acetylation and transcriptional activity. **A** Interphase nucleus after immunolabeling of H3Ac14 (*green*, *left*) and BrUTP incorporation (*red*, *right*). Most BrUTP signals occur within the nucleolus, which is not acetylated at K14 of H3; no gradient of signals appears over the remaining chromatin. To visualize chromatin labeling, BrUTP signals had to be enhanced; therefore signals over the nucleolus appear to be overexposed. **B** Detail of **A**; BrUTP signal foci (*middle*) do often not colocalize with H3Ac14 signal dots (*left*) when merged (*right*). *Bar* represents 10 µm

Histone acetylation does not reflect transcriptional activity of chromatin domains

Acetylation of H4 and H3 was reported to be linked with the transcriptional activity of genes (Kuo et al. 1996). In order to see whether such a correlation holds true at the level of larger chromatin domains, BrUTP incorporation into isolated, unfixed barley nuclei was tested. Despite the strong polar organization of barley nuclei into a heterochromatin-rich and a heterochromatin-free pole, and in contrast with the H3 acetylation patterns, no gradient of BrUTP signals was observed. This agrees with results obtained for wheat nuclei also showing a clear Rabl orientation of interphase chromosomes (Abranches et al. 1998). Most of the BrUTP signals appeared within the nucleolus independently of its acetylation status (Fig. 8A). Individual foci of BrUTP incorporation within the remaining chromatin frequently did not colocalize with the spots of strong H3Ac14 labeling (Fig. 8B). Thus, as observed for *V. faba* (Jasencakova et al. 2000), there is no obvious correlation between the biased H3 and/or H4 acetylation of distinct chromatin domains in interphase and their potential trancriptional activity. This suggests that the global acetylation patterns of large chromatin domains may deviate from and overwhelm the fine-tuning of acetylation at promoter-containing nucleosomes that is apparently correlated with gene expression. A lack of correlation between acetylation and trancriptional activity is also indicated by the strong acetylation of K5 and K8 of H4 at inactive rDNA knobs (Fig. 7) and of K5 and K12 of H4 at the inactive NOR7 within the translocation line T2052 (Fig. 4L). Although Chen and Pikaard (1997) found an at least transient de-repression of silent NORs in allopolyploid *Brassica* hybrids when hypermethylation and/or deacetylation of silenced NORs was inhibited, this is obviously not a general mechanism for reactivation of suppressed NORs. Our previous work on nucleolar dominance in barley has shown that the ability to form a nucleolus could be restored by inhibition of methylation in the case of rDNA silenced due to splitting of NOR6 via translocation, but not in cases of translocation of the entire NOR7 to the long arm of chromosome 6 (Schubert and Künzel 1990). In the latter cases neither reduced methylation nor high acetylation of histone H4 is sufficient to de-repress the silent NOR.

Discussion

We have investigated the chromatin organization within barley interphase nuclei and its relation to replication and histone acetylation. Barley interphase nuclei, flow-sorted into G1-, early S-, mid S-, late S- and G2-phase fractions, revealed a pronounced Rabl orientation (Rabl 1885) with centromeres and telomeres clustered at opposite nuclear poles and pericentromeric heterochromatin located at the centromeric pole (Fig. 2). This type of chromatin organization reflects the arrangement of chromatin domains in mitotic chromosomes and was stably maintained throughout the cell cycle.

Fluorescent in situ hybridization of BrdU-pulsed and flow-sorted meristematic nuclei has shown that replication starts at rDNA, followed by euchromatin and centromeres, and finishes at the pericentromeric heterochromatin. Initiation of centromere replication in mid S-phase has also been documentated for mammals (Bartholdi 1991; O'Keefe et al. 1992) but whereas immunologically detected human centromeres were found to be dispersed during replication (Bartholdi 1991), barley centromeres maintained their condensed character

throughout S-phase. Association of (homologous?) centromeres in barley was found to occur preferentially during their replication. Although association of homologous centromeres is usually not evident in mammals (Ferguson and Ward 1992; Vourc'h et al. 1993) it has been demonstrated for Indian muntjac centromeres (Hadlaczky et al. 1986).

The data obtained for histone H3 and H4 acetylation within distinct chromatin domains of barley during the cell cycle in comparison with those reported for the dicot *V. faba* lead to the following conclusions: (i) in contrast to H4, no significant alteration of H3 acetylation occurs during the cell cycle in either plant species. (ii) The sequence-dependent strong acetylation of K14 and K9/18 of H3 of a particular heterochromatin fraction is apparently a specific feature of *V. faba*. (iii) Deposition-related acetylation in barley seems to be restricted to K5 and K12 of H4, as in insects and mammals (Sobel et al. 1995). (iv) The stronger acetylation of the barley mitotic NORs is restricted to K5 and K12 of H4. (v) If, contrary to the situation in the field bean, K8 instead of K16 of H4 is additionally subjected to a high deposition-related acetylation in barley, this becomes reversed in heterochromatin during late S-/early G2-phase, while deacetylation of K5 and K12 can be delayed up to the next G1-phase in barley. (vi) Comparable to the situation in *V. faba*, increased acetylation of H4 is correlated with replication (except for the NOR) rather than with the transcriptional activity of large chromatin domains as indicated by the H4 acetylation patterns during interphase, by the BrUTP labeling pattern after 'run on transcription' as well as by the strong acetylation of rDNA knobs during interphase and of the inactive NOR7 of the line T2052. (vii) The below-average acetylation of H3/H4 at barley centromeres during mitosis is probably due to their high content of tandem repeats (Presting et al. 1998), while no centromere-specific tandem repeats (Houben et al. 1996b) and no underacetylation of H3 and H4 (Jasencakova et al. 2000) were detectable at *Vicia* centromeres.

Therefore, on the one hand, deposition-related acetylation of K5 and K12 of H4 (Sobel et al. 1995; Jasencakova et al. 2000) as well as the strong H4 acetylation within heterochromatic domains occurs around replication and becomes reversed later during the cell cycle to a level below that of euchromatin (Taddei et al. 1999; Jasencakova et al. 2000). This phenomenon seems to be conserved between animals and plants and may represent a common feature among eukaryotes. On the other hand, the stronger acetylation of (some) lysine positions at mitotic NORs, as compared with euchromatin (for review see Jasencakova et al. 2000), and the different acetylation patterns for H3 and H4 along specific chromatin domains seem to be typical for plants (Jasencakova et al. 2000) rather than for animals (Belyaev et al. 1996).

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References

Abranches R, Beven AF, Aragón-Alcaide L, Shaw PJ (1998) Transcription sites are not correlated with chromosome territories in wheat nuclei. J Cell Biol 143:5–12

Anamthawat-Jonsson K, Heslop-Harrison JS (1990) Centromeres, telomeres and chromatin in the interphase nucleus of cereals. Caryologia 43:205–213

Bartholdi MF (1991) Nuclear distribution of centromeres during the cell cycle of human diploid fibroblasts. J Cell Sci 99:255–263

Belostotsky DA, Ananiev EV (1990) Characterization of relic DNA from barley genome. Theor Appl Genet 80:374–380

Belyaev ND, Keohane AM, Turner BM (1996) Differential underacetylation of histones H2A, H3 and H4 on the inactive X chromosome in human female cells. Hum Genet 97:573–578

Belyaev ND, Houben A, Baranczewski P, Schubert I (1997) Histone H4 acetylation in plant heterochromatin is altered during the cell cycle. Chromosoma 106:193–197

Belyaev ND, Houben A, Baranczewski P, Schubert I (1998) The acetylation patterns of histones H3 and H4 along *Vicia faba* chromosomes are different. Chromosome Res 6:59–63

Buzek J, Riha K, Siroky J, Ebert I, Greilhuber J, Vyskot B (1998) Histone H4 underacetylation in plant facultative heterochromatin. Biol Chem 379:1235–1241

Chen ZF, Pikaard CS (1997) Epigenetic silencing of RNA polymerase I transcription: a role for DNA methylation and histone modification in nucleolar dominance. Genes Dev 11:2124–2136

Dietzel S, Schiebel K, Little G, Edelmann P, Rappold A, Eils R, Cremer C, Cremer T (1999) The 3D positioning of ANT2 and ANT3 genes within female X chromosome territories correlates with gene activity. Exp Cell Res 252:363–375

Dong F, Jiang J (1998) Non-Rabl patterns of centromere and telomere distribution in the interphase nuclei of plant cells. Chromosome Res 6:551–558

Eils R, Dietzel S, Bertin E, Schröck E, Speicher MR, Ried T, Robert-Nicoud M, Cremer C, Cremer T (1996) Three-dimensional reconstruction of painted human interphase chromosomes: active and inactive X chromosome territories have similar volumes but differ in shape and surface structure. J Cell Biol 135:1427–1440

Ferguson M, Ward DC (1992) Cell cycle dependent chromosomal movement in pre-mitotic human T-lymphocyte nuclei. Chromosoma 101:557–565

Fransz P, de Jong JH, Ruffini Castiglione M, Schubert I (2001) Chromosomes in interphase nuclei of *Arabidopsis thaliana* are organized as euchromatin loops around heterochromatin compartments. Nat Genet (in press)

Grunstein M (1997) Histone acetylation in chromatin structure and transcription. Nature 389:349–352

Hadlaczky G, Went M, Ringertz NR (1986) Direct evidence for the non-random localization of mammalian chromosomes in the interphase nucleus. Exp Cell Res 167:1–15

Houben A, Guttenbach M, Kress W, Pich U, Schubert I, Schid M (1995) Immunostaining and interphase arrangement of field bean kinetochores. Chromosome Res 3:27–31

Houben A, Belyaev ND, Turner BM, Schubert I (1996a) Differential immunostaining of plant chromosomes by antibodies recognizing acetylated histone H4 variants. Chromosome Res 4:191–194

Houben A, Brandes A, Pich U, Manteuffel R, Schubert I (1996b) Molecular-cytogenetic characterization of a higher plant centromere/kinetochore complex. Theor Appl Genet 93:477–484

Idei S, Kondo K, Turner BM, Fukui K (1996) Tomographic distribution of acetylated histone H4 in plant chromosomes, nuclei and nucleoli. Chromosoma 105:293–302

Ikura T, Ogryzko VV, Grigoriev M, Groisman R, Wang J, Horikoshi M, Scully R, Qin J, Nakatani Y (2000) Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. Cell 102:463–473

Jasencakova Z, Meister A, Walter J, Turner BM, Schubert I (2000) Histone acetylation of euchromatin and heterochromatin is correlated with replication rather than with transcription. Plant Cell 12:2087–2100

Jeppesen P, Turner BM (1993) The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. Cell 74:281–289

Kakeda K, Yamagata H (1992) Immunological analysis of chromosome replication in barley, rye, and durum wheat by using an anti-BrdU antibody. Hereditas 116:67–70

Kubalakova M, Macas J, Dolezel J (1997) Mapping of repeated DNA sequences in plant chromosomes by PRINS and C-PINS. Theor Appl Genet 94:758–763

Kuo M-H, Brownell JE, Sobel RE, Ranalli TA, Cook RG, Edmondson DG, Roth SY, Allis CD (1996) Transcription-linked acetylation by Gcn5p of histones H3 and H4 at specific lysines. Nature 383:269–272

Lysák M, Čihalíková J, Kubaláková M, Šimková H, Künzel G, Doležel J (1999) Flow karyotyping and sorting of mitotic chromosomes of barley (*Hordeum vulgare* L.). Chromosome Res 7:431–444

McBlane F, Boyes J (2000) Stimulation of V(D)J recombination by histone acetylation. Curr Biol 10:483–486

McMurry MT, Krangel MS (2000) A role for histone acetylation in the developmental regulation of V(D)J recombination. Science 287:495–498

Nagele RG, Freeman T, McMorrow L, Thompson Z, Kitson-Wind K, Lee H-Y (1999) Chromosomes exhibit preferential positioning in nuclei of quiescent human cells. J Cell Sci 112: 525–535

Noguchi J, Fukui K (1995) Chromatin arrangements in intact interphase nuclei examined by laser confocal microscopy. J Plant Res 108:209–216

O'Keefe RT, Henderson SC, Spector DL (1992) Dynamic organization of DNA replication in mammalian cell nuclei: spatially and temporally defined replication of chromosome-specific α-satellite DNA sequences. J Cell Biol 116:1095–1110

Pedersen C, Linde-Laursen I (1994) Chromosomal locations of four minor rDNA loci and a marker microsatellite sequence in barley. Chromosome Res 2:65–71

Pedersen C, Rasmussen SK, Linde-Laursen I (1996) Genome and chromosome identification in cultivated barley and related species of the Triticeae (Poaceae) by in situ hybridization with the GAA-satellite sequence. Genome 39:93–104

Presting GG, Malysheva L, Fuchs J, Schubert I (1998) A TY3/GYPSY retrotransposon-like sequence localizes to the centromeric regions of cereal chromosomes. Plant J 16:721–728

Rabl C (1885) Über Zelltheilung. Morphol Jahrbuch 10:14–330

Sadoni N, Langer S, Fauth C, Bernardi G, Cremer T, Turner BM, Zink D (1999) Nuclear organization of mammalian genomes: polar chromosome territories build up functionally distinct higher order compartments. J Cell Biol 146:1211–1226

Schubert I, Künzel G (1990) Position-dependent NOR activity in barley. Chromosoma 99:352–359

Schubert I, Dolezel J, Houben A, Scherthan H, Wanner G (1993) Refined examination of plant metaphase chromosome structure at different levels made feasible by new isolation methods. Chromosoma 102:96–101

Schubert I, Shi F, Fuchs J, Endo TR (1998) An efficient screening for terminal deletions and translocations of barley chromosomes added to common wheat. Plant J 14:489–495

Shaw PJ, Highett MI, Beven AF, Jordan EG (1995) The nucleolar architecture of polymerase I transcription and processing. EMBO J 14:2896–2906

Sobel RE, Cook RG, Perry CA, Annunziato AT, Allis CD (1995) Conservation of deposition-related acetylation sites in newly synthesized histones H3 and H4. Proc Natl Acad Sci USA 92:1237–1241

Stein P, Worrad DM, Belyaev ND, Turner BM, Schultz RM (1997) Stage-dependent redistribution of acetylated histones in nuclei of the early preimplantation mouse embryo. Mol Reprod Dev 47:421–429

Strahl BD, Allis CD (2000) The language of covalent histone modifications. Nature 413:41–45

Struhl K (1998) Histone acetylation and transcriptional regulatory mechanisms. Genes Dev 12:599–606

Taddei A, Roche D, Sibarita J-B, Turner BM, Almouzni G (1999) Duplication and maintenance of heterochromatin domains. J Cell Biol 147:1153–1166

Tajbakhsh J, Luz H, Bornfleth H, Lampel S, Cremer C, Lichter P (2000) Spatial distribution of GC- and AT-rich DNA sequences within human chromosome territories. Exp Cell Res 255: 229–237

Thompson WF, Beven AF, Wells B, Shaw PJ (1997) Sites of rDNA transcription are widely dispersed through the nucleolus in *Pisum sativum* and can comprise single genes. Plant J 12:571–581

Turner BM (2000) Histone acetylation and an epigenetic code. BioEssays 22:836–845

Turner BM, Fellows G (1989) Specific antibodies reveal ordered and cell-cycle-related use of histone-H4 acetylation sites in mammalian cells. Eur J Biochem 179:131–139

Turner BM, O'Neill LP, Allan IM (1989) Histone H4 acetylation in human cells: frequency of acetylation at different sites defined by immunolabelling with site-specific antibodies. FEBS Lett 253:141–145

Turner BM, Birley AJ, Lavender J (1992) Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in *Drosophila* polytene nuclei. Cell 69:375–384

Visser AE, Aten JA (1999) Chromosomes as well as chromosomal subdomains constitute distinct units in interphase nuclei. J Cell Sci 112:3353–3360

Vourc'h C, Taruscio D, Boyle AL, Ward DC (1993) Cell cycle-dependent distribution of telomeres, centromeres, and chromosome-specific subsatellite domains in the interphase nucleus of mouse lymphocytes. Exp Cell Res 205:142–151

Vyskot B, Siroky J, Hladilova R, Belyaev ND, Turner BM (1999) Euchromatic domains in plant chromosomes as revealed by H4 histone acetylation and early DNA replication. Genome 42:343–350

White DA, Belyaev ND, Turner BM (1999) Preparation of site-specific antibodies to acetylated histones. Methods 19:417–421

Yakura K, Tanifuji S (1983) Molecular cloning and restriction analysis of EcoRI fragments of *Vicia faba* rDNA. Plant Cell Physiol 24:1327–1330

6.3. SOPPE WJJ, JASENCAKOVA Z, HOUBEN A, KAKUTANI T, MEISTER A, HUANG MS, JACOBSEN SE, SCHUBERT I, FRANSZ PF

DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in *Arabidopsis*.

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DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in *Arabidopsis*

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We propose a model for heterochromatin assembly that links DNA methylation with histone methylation and DNA replication. The hypomethylated *Arabidopsis* mutants *ddm1* and *met1* were used to investigate the relationship between DNA methylation and chromatin organization. Both mutants show a reduction of heterochromatin due to dispersion of pericentromeric low-copy sequences away from heterochromatic chromocenters. DDM1 and MET1 control heterochromatin assembly at chromocenters by their influence on DNA maintenance (CpG) methylation and subsequent methylation of histone H3 lysine 9. In addition, DDM1 is required for deacetylation of histone H4 lysine 16. Analysis of F_1 hybrids between wild-type and hypomethylated mutants revealed that DNA methylation is epigenetically inherited and represents the genomic imprint that is required to maintain pericentromeric heterochromatin.

Keywords: *Arabidopsis*/DDM1/heterochromatin/histone methylation/MET1

Introduction

DNA methylation is essential for normal development of most higher eukaryotes and is involved in genomic imprinting, regulation of gene expression and defense against foreign DNA (Jost and Saluz, 1993; Finnegan *et al*., 1998). In concert with histone modifications, it contributes to chromatin remodeling (reviewed by Richards and Elgin, 2002). From fission yeast to mammals, methylation of histone H3 at lysine 9 (H3K9) is considered to be crucial for heterochromatin assembly, whereas methylation of H3 at lysine 4 (H3K4) occurs preferentially within transcriptionally competent chromatin (except for yeast rDNA, Briggs *et al*., 2001) (recently reviewed in Rice and Allis, 2001; Lachner and Jenuwein, 2002; Richards and Elgin, 2002). The interactions between DNA methylation, histone modifications and chromatin structure have mainly been studied at the molecular level for specific DNA sequences. Integrated genetic, molecular and cytological approaches can provide new insights into chromatin remodeling. For example, genome-wide H4 acetylation appeared to be tightly linked to DNA replication and possibly with post-replicative processes rather than with transcriptional activity (Jasencakova *et al*., 2000, 2001). Studies on DNA methylation and histone modifications at the nuclear level using DNA methylation mutants may elucidate the process of heterochromatin formation.

Several mutants with reduced DNA methylation levels have been isolated in *Arabidopsis*. The strongest effects on DNA methylation were found in the recessive mutants *decrease in DNA methylation1* (*ddm1*; Vongs *et al*., 1993) and *methyltransferase1* (*met1*; Finnegan *et al*., 1996; Ronemus *et al*., 1996). *DDM1* encodes a SWI/SNF-like protein, presumably a chromatin remodeling factor (Jeddeloh *et al*., 1999), while *MET1* encodes a maintenance methyltransferase (Finnegan and Kovac, 2000). They are the plant homologs of the mammalian Lsh and Dnmt1 genes, respectively (Finnegan *et al*., 1996; Dennis *et al*., 2001). In both *ddm1* and *met1*, repetitive and single-copy sequences become hypomethylated, causing a reduction in methylation level by ~70% (Vongs *et al*., 1993; Ronemus *et al*., 1996). Remethylation of hypomethylated sequences is extremely slow or absent when *ddm1* is backcrossed to the wild type (Kakutani *et al*., 1999). The mutants are further characterized by release of transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) (Mittelsten Scheid *et al*., 1998; Morel *et al*., 2000) and by reactivation of some transposons (Hirochika *et al*., 2000; Singer *et al*., 2001). Morphological phenotypes of *ddm1* and *met1* include altered flower morphology and leaf shape, sterility and late flowering, and appear in the first homozygous mutant generation in *met1*, but only after several generations of inbreeding in *ddm1* (Finnegan *et al*., 1996; Kakutani *et al*., 1996; Ronemus *et al*., 1996).

The *ddm1* and *met1* mutants have been analyzed at the molecular and morphological level, but not in relation to histone modifications and heterochromatin formation. Heterochromatin in *Arabidopsis* nuclei is concentrated at DAPI-bright chromocenters that contain major tandem repeats (the centromeric 180 bp repeat and rDNA genes) and dispersed pericentromeric repeats (Maluszynska and Heslop-Harrison, 1991; Heslop-Harrison *et al*., 1999; Fransz *et al*., 2002). The latter consist mainly of transposable elements and low-copy sequences (Fransz *et al*., 2000; The Arabidopsis Genome Initiative, 2000). All repeats are strongly methylated in wild-type plants but

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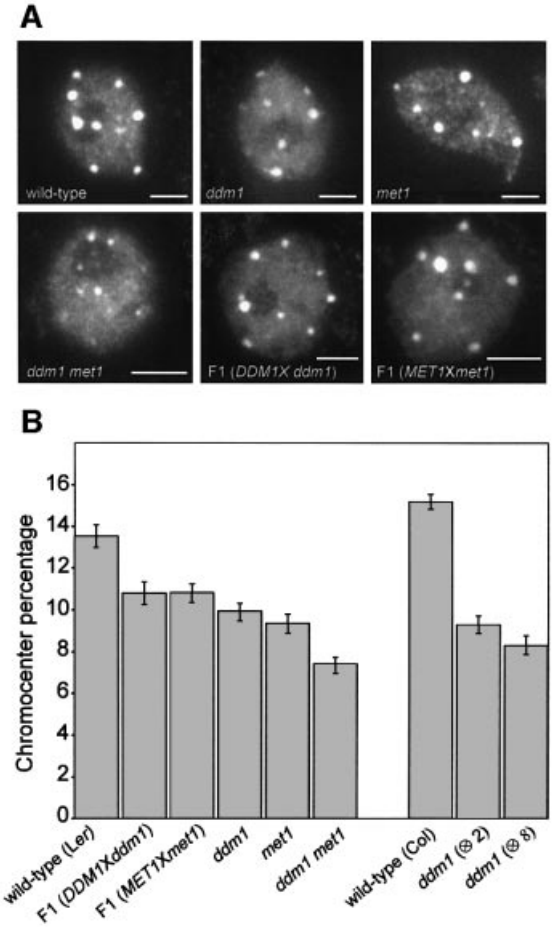


Fig. 1. Reduction of chromocenter size in hypomethylated mutants. (**A**) Phenotypes of representative DAPI-stained leaf interphase nuclei in a L*er* background. Chromocenters are smaller and weaker stained in *ddm1* and *met1* nuclei than in wild-type nuclei, chromocenters in the *ddm1 met1* double mutant show the weakest staining. Heterozygous *DDM1* × *ddm1* and *MET1* × *met1* F_1 plants show an intermediate nuclear phenotype between wild type and mutants. Bar = 5 μm. (**B**) Chromocenter fractions are shown as the percentage of area and staining intensity of chromocenters in relation to the entire nucleus. This histogram quantifies the observations shown in (A). Furthermore, it is shown that chromocenters in *ddm1* (in Col background) do not significantly reduce in size after two and eight selfing generations since the induction of the mutation. Percentages are derived from measurements of 50 nuclei each and the standard error of the mean is indicated on each bar.

weakly in *ddm1* and *met1* single mutants (Vongs *et al*., 1993; Ronemus *et al*., 1996; Kakutani *et al*., 1999).

To investigate the relationship between DNA methylation and genome-wide chromatin organization, and to elucidate the hierarchy of processes that control heterochromatin formation, we compared the location of (peri-) centromeric sequences, the nuclear patterns of DNA methylation and histone modifications, and the heterochromatin structure of leaf interphase nuclei of wild-type plants with those of *ddm1* and *met1* mutant plants.

Results

Hypomethylated mutants contain reduced amounts of heterochromatin

After DAPI staining of wild-type nuclei from the Landsberg *erecta* (L*er*) accession, conspicuous heterochromatic chromocenters can be distinguished. In nuclei of the hypomethylated mutants *ddm1* and *met1*, the chromocenters are smaller, indicating a reduction of heterochromatin (Figure 1A). This nuclear phenotype occurs in different organs, developmental stages and genetic backgrounds. We quantified this reduction of heterochromatin content by measuring the area and staining intensity of the chromocenters in relation to that of the entire nucleus (chromocenter fraction). The chromocenter fractions of *ddm1* and *met1* are reduced by ~25–30% in comparison to the wild type (Figure 1B). The double mutant *ddm1 met1* shows a further reduction of 20–25% compared with the single mutants, indicating an additive effect of both mutations for this feature. F_1 hybrids between wild type and either *ddm1* or *met1* mutants contain nuclei with intermediate chromocenter fractions (Figure 1B), consistent with their intermediate methylation levels (Kakutani *et al*., 1999). Since the plant phenotypes in *ddm1* appear in later generations (Kakutani *et al*., 1995), we determined (in Columbia background) whether these generations also show a further reduction in the chromocenter fraction. Although nuclei from plants of the eighth generation with a strong phenotype had smaller chromocenters than nuclei from plants of the second generation without phenotype, the difference was not significant ($P = 0.122$).

Fig. 2. Location of repetitive and single-copy sequences in leaf interphase nuclei. (**A**) Sequences corresponding to the 180 bp centromeric pAL repeat (red) are always located at chromocenters. Sequences corresponding to the pericentromeric BAC F28D6 (green) are located at chromocenters in wild type, but yield additional dispersed signals in the single and double mutants. Similar results were obtained with other pericentromeric BACs (F17A20, F10A2 and F21I2, DDBJ/EMBL/GenBank accession Nos AF147262, AF147259 and AF147261). (**B**) Schematic representation of BAC F28D6 (top). The different sequence elements are shown in accordance with the GenBank annotation; green boxes above (A–H) indicate the position and size of different PCR fragments used as probes in FISH experiments. Red signals on the nuclei, corresponding to the location of *Athila* elements, are always located at chromocenters. Green signals, corresponding to the location of PCR fragment C, are located at chromocenters in the wild type but yield dispersed signals in *ddm1*. (**C**) All tested repetitive elements (Tat*1* from the Ty3-*gypsy* group of LTR retrotransposons, Ta*1* from the Ty1-*copia* group of LTR retrotransposons, the MITE *Emi12*, the repetitive DNA element *AthE1.4* and the chromomeric repeat ATR63) are located at chromocenters in wild-type and mutant nuclei. (**D**) The *CAC1* sequence was most frequently detected at chromocenters in the wild-type and outside chromocenters in the *ddm1* mutant nuclei (arrow). The position of *CAC1* on BAC T10J7 is indicated by a yellow box in the scheme. FISH with this BAC yielded multiple signals (red), due to the presence of repetitive elements. Green signal is from four PCR fragments (green in the scheme), amplified from a sequence adjacent to *CAC1*, and indicates its original position. This signal is masked by the strong DAPI staining of chromocenters in the left image of the same wild-type nucleus. (**E**) The *FWA* sequence was usually located outside chromocenters, as detected by FISH with two BACs (T30C3 and F14M19), adjacent to the gene, in red and a probe of 10.5 kb, covering the gene, in green. (**F**) The *SUP* sequence was usually located outside chromocenters, as detected with a BAC (K14B15) that contains *SUP*, in red and a probe of 6.7 kb, covering the gene, in green. (A–C, E and F) Nuclei from plants with L*er* background; (D) nuclei from plants with Col background. Images in black and white show DAPI-stained nuclei; color images show FISH signals on the same nuclei. Bar = 5 μm.

DNA hypomethylation causes dispersion of pericentromeric sequences away from chromocenters

The reduced size of DAPI-bright chromocenters in *ddm1* and *met1* indicates that they contain less DNA than wild-type chromocenters, and the question arises which sequences are no longer within the chromocenters in *ddm1* and *met1*. We examined this by fluorescent *in situ* hybridization (FISH) using tandem and dispersed repeats, which all localize in the wild-type chromocenters. The

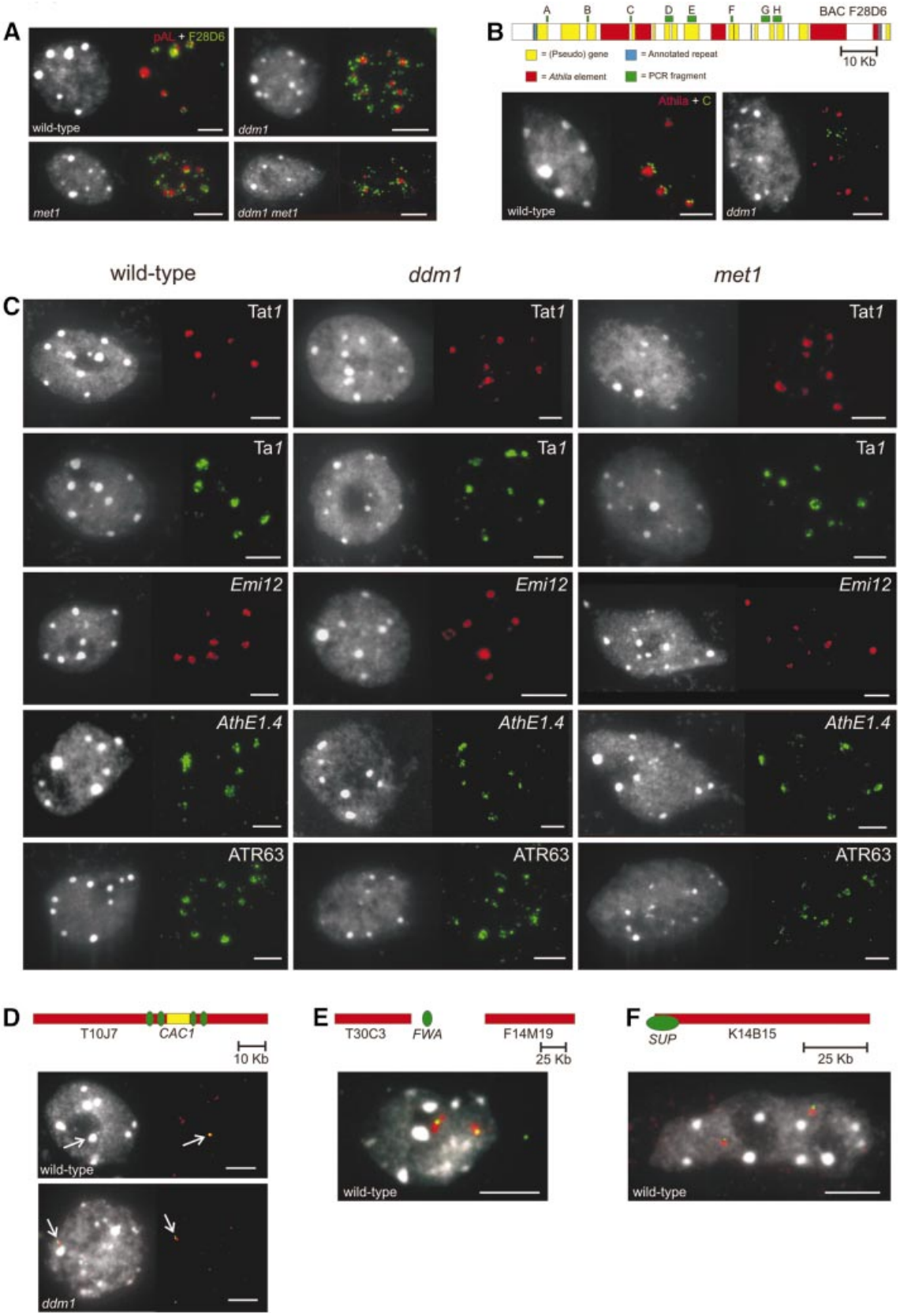


Table I. Number of FISH signals, present within and outside chromocenters, for different single-copy sequences and genotypes

Gene	Accession	Genotype	No. of scored nuclei	No. of signals in chromocenters	No. of signals out of chromocenters
CAC1	Col	Wild type	55	65	13
CAC1	Col	*ddm1*	58	30	65
FWA	L*er*	Wild type	104	24	154
FWA	L*er*	*fwa-1*	100	14	158
FWA	Col	Wild type	52	2	89
FWA	Col	*ddm1* selfed 2×	51	6	86
FWA	Col	*ddm1* selfed 8×	50	5	87
SUP	L*er*	Wild type	56	6	88
SUP	L*er*	*clk-3*	51	5	89
SUP	L*er*	*ddm1*	52	3	93

major tandem repeats (pAL1; see Figure 2A, 45S rDNA and 5S rDNA) co-localized with chromocenters in wild-type and mutants, and thus remained within the heterochromatin of *ddm1* and *met1* nuclei.

However, BAC DNA clones that represent sequences from the pericentromeric regions hybridized exclusively with chromocenters in the wild type, but showed a dispersed pattern at and around the chromocenters in the hypomethylated mutants (Figure 2A). This suggests that some pericentromeric sequences are located away from the chromocenter in the mutants. Most pericentromeric BAC clones contain many different transposable elements as well as non-transposable sequences (The Arabidopsis Genome Initiative, 2000). To determine which of these sequences are released from heterochromatin in the mutants, we probed wild-type and mutant nuclei with four highly repetitive elements mapped in pericentromeric regions and two (*Emi12* and *AthE1.4*) mapped in various regions along the chromosome arms. Each of these elements belongs to different families: *Athila* (Pélissier *et al*., 1995) and Tat*1* (Wright and Voytas, 1998) from the Ty3-*gypsy* group of LTR retrotransposons, Ta*1* (Voytas *et al*., 1990; Konieczny *et al*., 1991) from the Ty1-*copia* group of LTR retrotransposons, the miniature inverted-repeat transposable element (MITE) *Emi12* (Casacuberta *et al*., 1998), the repetitive element *AthE1.4* (Surzycki and Belknap, 1999) and the chromomeric repeat ATR63, which is derived from the heterochromatic knob hk4S (Fransz *et al*., 2000). All transposable elements hybridized to chromocenters in wild-type and mutant nuclei (Figure 2B and C). This implies that pericentromeric sequences other than transposable elements are relocated away from heterochromatin in the mutant nuclei. We tested this by FISH with different PCR fragments (A, B, C, D, E, F, G and H in Figure 2B) of BAC F28D6 (DDBJ/EMBL/GenBank accession No. AF147262) that represent putative genes and unannotated sequences. The fragments A, B, D, E, F, G and H contained low-copy sequences and yielded poor FISH signals outside the mutant chromocenters. However, fragment C, which has ~75 highly homologous sites in pericentromeric regions, is present at chromocenters in wild type but occupies more dispersed positions in *ddm1* and *met1* nuclei (Figure 2B). Thus, the dispersed signals from pericentromeric BACs in the hypomethylated mutants seem to be due to sequences separating the transposable elements.

The transposable elements tested above are inactive in wild type and largely inactive in the hypomethylated mutants. To find out whether the spatial position relative to heterochromatin might be related to transposon activity, we examined the location of the single-copy *CAC1* transposon located in the pericentromeric region of chromosome arm 2L, which is silent and methylated in Columbia (Col) wild-type plants but active and hypomethylated in the *ddm1* mutant (Miura *et al*., 2001). FISH with a combination of the BAC that contains *CAC1* (T10J7; DDBJ/EMBL/GenBank accession No. AC005897) and four PCR fragments, located on either side of the transposon (Figure 2D), revealed that activation and hypomethylation of the *CAC1* transposon in *ddm1* are correlated with its relocation away from the heterochromatin (Table I).

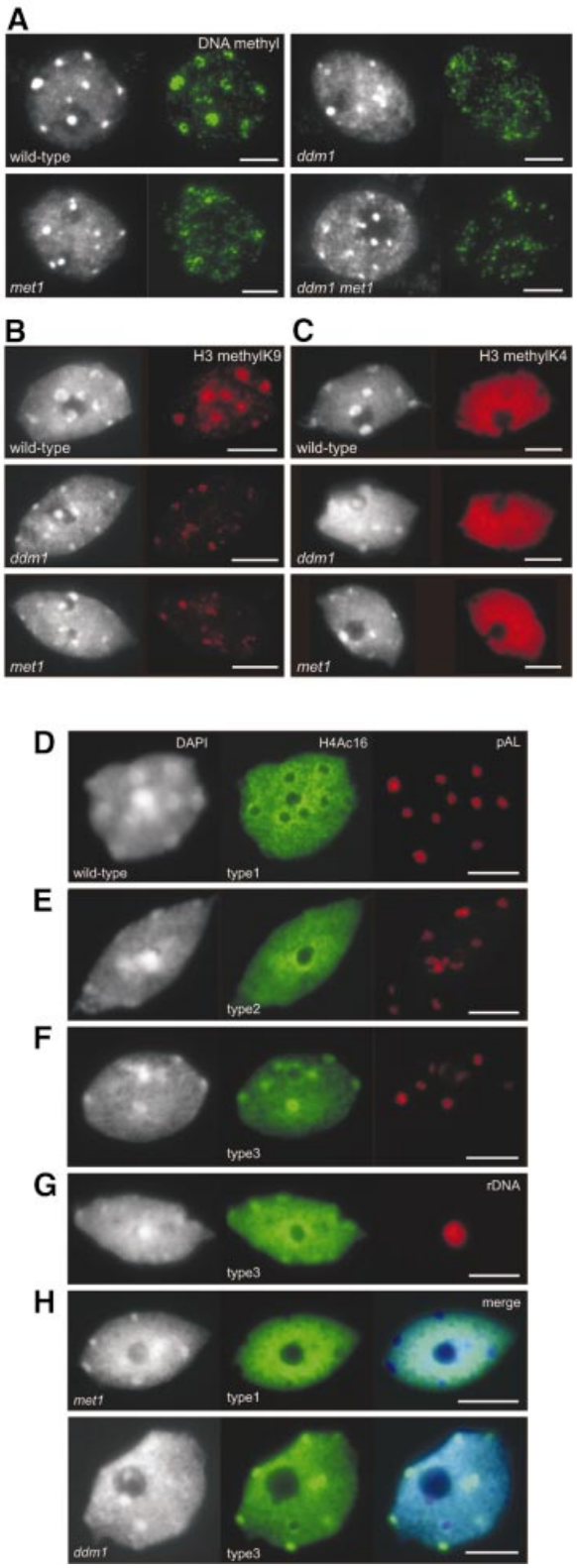
Not all silenced genes reside in chromocenters

The correlation between silencing and the nuclear position of the *CAC1* transposon prompted us to investigate whether such a correlation also exists for other genes like *FWA* (mapped at the long arm of chromosome 4) and *SUPERMAN* (*SUP*; mapped at the short arm of chromosome 3), which differ in their DNA methylation and expression levels between wild type and hypomethylation mutants (Jacobsen and Meyerowitz, 1997; Soppe *et al*., 2000).

The *FWA* sequence was localized with a combination of three probes. Two BACs, positioned on either side of the gene (T30C3, DDBJ/EMBL/GenBank accession No. AL079350; and F14M19, accession No. AL049480) were detected in red and a small probe of 10.5 kb, containing *FWA*, in green (Figure 2E). In adult wild-type plants, the *FWA* gene is not expressed and the 5′-region of the gene is strongly methylated, in contrast to its hypomethylation and constitutive expression in the *fwa-1* mutant (Soppe *et al*., 2000). For both wild type and the *fwa-1* mutant, the *FWA* sequence was detected outside chromocenters in the majority of nuclei (Table I). We also compared the location of *FWA* between Col wild type (methylated and not expressed), a *ddm1* line of the second generation (methylated and not expressed) and a *ddm1* line of the eighth generation (hypomethylated and expressed). In all these genotypes, *FWA* was located mainly outside chromocenters (Table I). Therefore, silencing and

methylation of the *FWA* gene do not mediate a shift of its nuclear position toward the chromocenters.

Similar results were obtained for the *SUP* gene. In wild-type plants, *SUP* is hypomethylated and expressed in developing flowers (Sakai *et al*., 1995). Several hypermethylated alleles of *SUP* have been found [*clark kent* (*clk*) alleles], which show decreased expression (Jacobsen and Meyerowitz, 1997). The *SUP* gene is also methylated and silenced in *ddm1* and *met1* mutant plants (Jacobsen and Meyerowitz, 1997; Jacobsen *et al*., 2000). Although *SUP* is not expressed in leaves, the pattern and extent of methylation are the same in leaves and flowers (Kishimoto *et al*., 2001). The *SUP* gene was localized in leaf nuclei by FISH with two probes: the entire BAC K14B15 (DDBJ/EMBL/GenBank accession No. AB025608) containing the *SUP* gene was detected in red and a small probe of 6.7 kb, comprising the gene, in green color (Figure 2F). In all genetic backgrounds, *SUP* was preferentially located outside chromocenters (Table I).



Decreased DNA and H3K9 methylation accompany the size reduction of chromocenters in ddm1 and met1

We compared the distribution patterns of methylated DNA in wild type and hypomethylated mutants using antibodies against 5-methylcytosine. Wild-type nuclei showed strong signals, especially at chromocenters (Figure 3A). In contrast, in nuclei of the *ddm1* and *met1* mutants, the immunosignals were dispersed and no longer clustered at chromocenters. This phenotype appeared to be stronger in

Table II. Compilation of chromatin modification data in leaf nuclei of *Arabidopsis* wild type and DNA methylation mutants

	Wild type	*ddm1*	*met1*
H4Ac5	eu^+ nu^- cc^-	eu^+ nu^- cc^-	eu^+ nu^- cc^-
H4Ac8	eu^+ nu^- cc^-	eu^+ nu^- cc^-	eu^+ nu^- cc^-
H4Ac12	eu^+ nu^- cc^-	eu^+ nu^- cc^-	eu^+ nu^- cc^-
H4Ac16 (see Table III)			
tri-/tetra-AcH4	eu^+ nu^- cc^-	eu^+ nu^- cc^-	eu^+ nu^- cc^-
H3Ac9	eu^+ nu^- cc^-	eu^+ nu^- cc^-	eu^+ nu^- cc^-
H3 methyl K4	eu^+ nu^- cc^-	eu^+ nu^- cc^-	eu^+ nu^- cc^-
H3 methyl K9	eu^- nu^- cc^+	eu^- nu^- $cc^{+/-}$	eu^- nu^- $cc^{+/-}$
DNA methylation	eu^- nu^- cc^+	eu^- nu^- $cc^{+/-}$	eu^- nu^- $cc^{+/-}$

eu, euchromatin; nu, nucleoli; cc, chromocenter. The intensity of labeling is indicated by + or –.

Fig. 3. Chromatin modifications in wild-type and hypomethylated mutant nuclei. (**A**) Immunosignals for DNA methylation (green) are strongly clustered at chromocenters in wild-type nuclei; *ddm1* and *met1* nuclei have more weakly labeled chromocenters. This effect is even stronger in the double mutant *ddm1 met1*. (**B**) Histone H3 K9 methylation. In wild-type nuclei, immunosignals for H3dimethylK9 (red) localize preferentially to chromocenters, whereas *ddm1* and *met1* nuclei showed a significantly lower intensity of labeling. (**C**) Histone H3 K4 methylation (red) occurs at euchromatin, while chromocenters and nucleoli remained unlabeled in wild-type as well as in *ddm1* and *met1* nuclei. (D–G) H4Ac16 labeling patterns (green) in wild-type nuclei. Three distinct patterns can be distinguished. (**D**) Type 1: euchromatin intensely labeled, nucleoli and chromocenters unlabeled. (**E**) Type 2: chromatin more or less uniformly labeled, nucleoli unlabeled. (**F** and **G**) Type 3: chromocenters with signal clusters, nucleoli unlabeled (inactive rDNA components of chromocenters remained unlabeled in type 2 and 3 nuclei). FISH with centromeric (pAL) or 45S rDNA repeats (red, on the right). (**H**) DNA hypomethylated mutants show similar labeling patterns as wild-type nuclei. For both mutants, labeling patterns of chromocenters correlate with the reduced size of chromocenters. DAPI staining (left), immunosignals of H4Ac16 (green, middle) and the merge of both (right). All genotypes have a L*er* background. Images in black and white show DAPI-stained 3:1 in ethanol:acetic acid (A) or formaldehyde-fixed (B–H) nuclei. Adjacent color images show immunosignals on the same nuclei. Bar = 5 μm.

the double mutant *ddm1 met1*, consistent with its further reduced chromocenter fraction (Figure 3A).

Since DNA methylation has recently been reported to be tightly correlated with histone H3 methylation (for reviews, see Rice and Allis, 2001; Lachner and Jenuwein, 2002; Richards and Elgin, 2002), we analyzed the nuclear distribution of methylated histone H3 isoforms. In wild-type nuclei, immunosignals for H3methylK9 were clustered at the chromocenters. The area and intensity of H3methylK9 signals were reduced in *ddm1* and *met1* chromocenters (Figure 3B). This indicates that methylation of H3K9 is controlled by DNA methylation. Immunolabeling of H3methylK4 gave an opposite pattern that was similar for wild-type and mutant nuclei. Euchromatin was strongly labeled, while nucleoli and chromocenters (size reduced in the mutants) were unlabeled (Figure 3C). The data suggest that the decrease in DNA methylation leads to a reduction in methylated H3K9 at chromocenters.

H4K16 acetylation in ddm1 deviates from that of wild type and met1

Apart from the effects of DNA methylation and histone methylation, chromatin structure is also modified by histone acetylation. Antibodies recognizing isoforms of histone H4 acetylated at lysine 5, 8 and 12, and histone H3 acetylated at lysine 9, all yielded similar patterns of immunosignals in wild-type nuclei. Euchromatin was intensely labeled, while nucleoli and heterochromatic chromocenters were unlabeled. A comparable pattern was observed in *ddm1* and *met1* nuclei (Table II), although the unlabeled domains were smaller than in wild type, correlating with the smaller chromocenters.

In contrast, antibodies against H4Ac16 yielded three classes of labeling patterns (Figure 3D–H; Table III). Type 1, showing strongly labeled euchromatin and unlabeled chromocenters and nucleoli, comprises 66.7% of the nuclei (Figure 3D). Type 2 displayed a uniformly labeled chromatin with unlabeled nucleoli and represents 24.2% of nuclei (Figure 3E). Type 3 is characterized by chromocenters that are more intensely labeled than euchromatin, while nucleoli and chromocenters containing inactive rDNA genes remain unlabeled. This class comprises 9.1% of nuclei (Figure 3F). The H4Ac16 patterns resemble the cell cycle-dependent modulation of acetylation at this lysine position observed in root meristems of faba bean (Jasencakova *et al*., 2000). However, leaf nuclei are mitotically inactive but frequently endopolyploid (Galbraith *et al*., 1991). Therefore, the high intensity of acetylation of H4K16 might reflect a link with DNA (endo-)replication or post-replicative processes, particularly at chromocenters.

We observed comparable labeling patterns for leaf nuclei of *ddm1* and *met1* mutants (Figure 3H). However, the proportion of nuclei with labeled chromocenters (type 2 and 3) was increased somewhat in *met1* (46.8%) and drastically in *ddm1* (80.2%) compared with the wild type (33.3%; Table III). This indicates that deacetylation of H4K16 depends on DDM1 activity and implies a functional difference between DDM1 and MET1 with respect to histone acetylation.

DNA methylation and histone H3K9 methylation, but not histone H4K16 acetylation patterns, are inherited epigenetically

Inheritance of *ddm1*-induced DNA hypomethylation is stable, even in wild-type/*DDM1* hybrid background (Kakutani *et al*., 1999). We therefore examined chromatin structure in F_1 plants, heterozygous for either *ddm1* or *met1*. The nuclei of heterozygotes showed two groups of chromocenters that differed strikingly in methylation level. One group displayed the wild-type morphology, whereas chromocenters of the other were similar to those of the mutants (Figure 4A). The difference in parental origin of chromocenters was supported by FISH with 45S rDNA. This probe hybridized to four chromocenters, of which two were heavily methylated, whereas the other two were not (data not shown). This means that the DDM1 and MET1 activities in the F_1 nuclei do not restore the wild-type level of DNA methylation in mutant-derived chromocenters. Considering the recessive nature of the *ddm1* and *met1* mutations (Vongs *et al*., 1993; E.Richards, personal communication), this indicates that DNA methylation is inherited epigenetically. When the pericentromeric BAC F28D6 was probed to *DDM1ddm1* or *MET1met1* F_1 nuclei, one half of the chromocenters showed the wild-type pattern and the other half showed the mutant pattern with more dispersed signals (Figure 4B). This indicates that the formation of pericentromeric heterochromatin requires DNA methylation as an epigenetic imprint.

Combined immunolabeling experiments for DNA and H3K9 methylation on *DDM1ddm1* F_1 nuclei showed that strong methylation of H3K9 is restricted to chromocenters containing a high level of methylated DNA (Figure 4C). This confirms that the epigenetically inherited methylation status of DNA is responsible for the reduction in methylation of H3K9 in *ddm1*-derived chromocenters. Contrary to this, strong acetylation of H4K16 at all chromocenters occurred with similar frequencies in the *DDM1ddm1* F_1 as in wild type (Table III), which demonstrates that DDM1 in heterozygous plants mediates deacetylation of H4K16 but is not able to mediate remethylation of DNA once the methylation is lost.

Discussion

Genome-wide DNA hypomethylation alters chromatin organization within the nucleus

We have demonstrated that two functionally different hypomethylated mutants display the same nuclear phenotype characterized by size-reduced chromocenters with decreased levels of DNA and H3K9 methylation, and relocation of low-copy pericentromeric sequences away from the chromocenters. The euchromatic location of single-copy genes (*SUP* and *FWA*) is not affected by changes in methylation level. Although the DNA methylation level of tandem repeats and high-copy transposons is strongly reduced, the remaining DNA methylation (Lindroth *et al*., 2001) seems to be sufficient for residual heterochromatin formation. In accordance with this, the *ddm1 met1* double mutant shows less DNA methylation than the single mutants and has smaller chromocenters (Figures 1 and 3A). The remaining methylation in the double mutant is probably due to the activity of other

Table III. H4Ac16 labeling patterns of leaf nuclei[a] of *Arabidopsis* wild type and DNA methylation mutants

Labeling patterns[b]	Wild type		*ddm1*		*met1*		F_1 (L*er* × *ddm1*)	
	n	%	*n*	%	*n*	%	*n*	%
1 nu$^-$ cc$^-$ eu$^+$	132	66.7	35	19.8	50	53.2	79	71.2
2 nu$^-$ cc$^+$ eu$^{+(+)}$	48	24.2	95	53.7	43	45.7	23	20.7
3 nu$^-$ cc^{++} eu$^+$	18	9.1	47	26.5	1	1.1	9	8.1
2+3	66	33.3	142	80.2[c]	44	46.8[d]	32	28.8
Σ	198	100.0	177	100.0	94	100.0	111	100.0

[a]4C nuclei as the major fraction of leaf nuclei, 2C and 8C nuclei showed similar results.
[b]nu, nucleoli and rDNA component of cc.
[c]$P < 0.001$.
[d]$P = 0.037$.

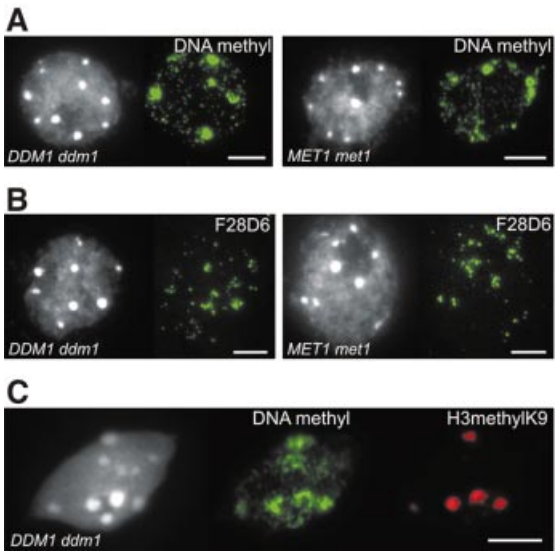


Fig. 4. The epigenetic inheritance of DNA methylation and H3K9 methylation is visible in nuclei of F_1 plants (wild type × mutant). (**A**) Half of the chromocenters show strong immunosignals for DNA methylation and the other half weak signals. (**B**) FISH signals for BAC F28D6 (green) are strongly clustered at half of the chromocenters but more dispersed around the other half. (**C**) The chromocenters with strong immunosignals for DNA methylation also show strong signals of H3K9 methylation. All genotypes have a L*er* background. Images in black and white show DAPI-stained 3:1 in ethanol:acetic acid (A and B) or formaldehyde-fixed (C) nuclei. Immunosignals are in green for DNA methylation (A and C) and in red for H3K9 methylation (C). Bar = 5 μm.

methyltransferases that partially take over the function of *MET1* (Genger *et al*., 1999). Alternatively, methylated DNA might not be required for heterochromatin formation and a critical amount of high-copy repeats might be sufficient, as in *Drosophila*, which lacks extensive DNA methylation (Henikoff, 2000). However, the relocation of low-copy pericentromeric sequences into euchromatin within nuclei of hypomethylated mutants suggests that DNA methylation is at least required for spreading of heterochromatic features into pericentromeric regions.

Unpublished data (A.Probst, O.Mittelsten Scheid, P.Fransz and J.Paszkowski, in preparation) obtained for another allele of *ddm1* indicate that even large amounts of centromeric tandem repeats are dispersed, nearly dissolving the chromocenters.

DNA maintenance methylation precedes H3K9 methylation

The decrease in DNA methylation in both hypomethylated mutants is paralleled by reduced methylation of H3K9 at chromocenters. Because only maintenance DNA methylation (mainly at CpG sites) is disturbed in the *met1* mutant, the primary cause of reduction of H3K9 methylation should be the reduction in DNA methylation. In agreement with this assumption, in F_1 plants heterozygous for *ddm1*, only chromocenters that have reduced DNA methylation also have reduced methylation of H3K9. Therefore, we propose that maintenance CpG methylation directs histone H3K9 methylation. This seems to contradict previous results in *Neurospora crassa* (Tamaru and Selker, 2001) and *Arabidopsis* (Jackson *et al*., 2002), which show that DNA methylation is dependent on H3K9 methylation, and disagrees with the suggestion of Gendrel *et al*. (2002) that the loss of DNA methylation in *ddm1* might be a consequence of reduced H3K9 methylation in heterochromatin. This discrepancy might be explained by a difference in function between the methylases involved because the histone methylation-dependent chromomethylase (CMT3) of *Arabidopsis* specifically methylates non-CpG sites (Lindroth *et al*., 2001). If CpG DNA methylation induces H3K9 methylation and this, in turn, induces CpNpG methylation, the positive feedback might induce spreading of heterochromatin from high-copy repeats to low-copy pericentromeric sequences.

Our conclusion that maintenance DNA methylation precedes H3K9 methylation concerns the assembly of wild-type heterochromatic chromocenters. In a chromatin immunoprecipitation study of H3K9 methylation in the *ddm1* and *met1* mutants, Johnson *et al*. (2002) found a loss of H3K9 methylation in *ddm1*, but found that the majority of H3K9 methylation was retained in the *met1* mutant. In contrast, using immunolabeling experiments, we observed similar losses of H3K9 methylation signals at chromocenters in both the *ddm1* and the *met1* mutants. One possible explanation for this difference is that the particular sequences assayed by chromatin immunoprecipitation may not have shown the same global loss that we observed by immunolabeling studies. A second possibility is that the loss of H3K9 methylation immunosignals is in part due to dislocation of pericentromeric regions away from the chromocenters, as well as an actual loss of H3K9 methylation from the chromatin. However, the reduction of H3K9 methylation at remnant chromocenters (largely

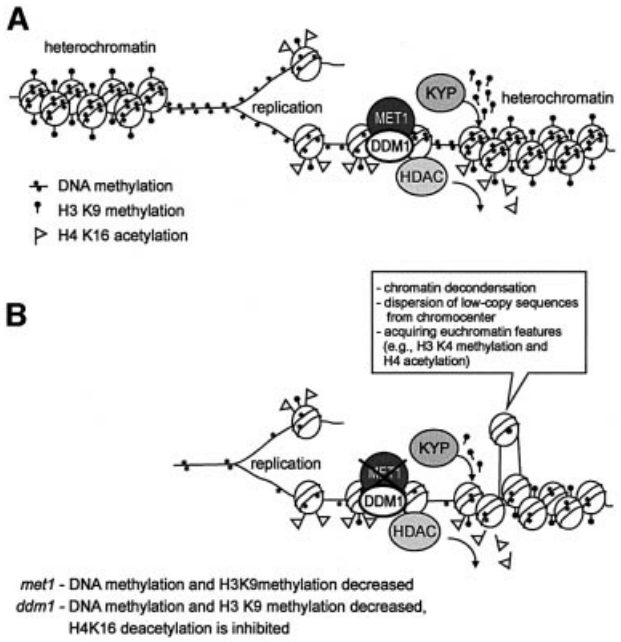


Fig. 5. A model for heterochromatin assembly. (**A**) In wild-type *Arabidopsis* nuclei, strong methylation of DNA and H3K9, followed by histone deacetylation, leads after replication to re-establishment of heterochromatin by DDM1, MET1, a histone H3K9-specific methylase (KYP) and a histone deacetylase. DNA maintenance methylation mediated by MET1 and supported by DDM1 directs H3K9 methylation. H4K16 deacetylation at newly replicated nucleosomes is mediated by DDM1. (**B**) In the *met1* mutant, maintenance DNA methylation is severely reduced, leading to decreased H3K9 methylation. Below a certain threshold, low-copy sequences disperse from heterochromatin and acquire euchromatin features. In the absence of DDM1, H4K16 deacetylation is additionally impaired.

composed of transcriptionally inactive sequences) in *met1* mutants seems most likely to be a direct loss of H3K9 methylation and not due to dislocation.

DDM1 is required for maintenance methylation activity by MET1 and additionally for deacetylation of H4K16

Genomic DNA methylation patterns are maintained immediately after replication by the activities of DNA methyltransferase(s) with a high preference for hemimethylated DNA (Bestor, 1992). The high level of H4 acetylation at this stage (Taddei *et al*., 1999; Jasencakova *et al*., 2000, 2001) might facilitate this process. The additive effect of *ddm1* and *met1* on chromocenter size reduction in the double mutant *ddm1 met1* (Figure 1B) implies that each gene controls DNA maintenance methylation by different mechanisms. Methylation of DNA by *MET1* is probably supported by chromatin remodeling factors such as *DDM1*, as proposed by Jeddeloh *et al*. (1999). This support might be more important for highly repetitive sequences in strongly condensed heterochromatic regions than for single-copy genes in euchromatic regions, since, in *ddm1* mutants, high-copy sequences have already become hypomethylated in the first generation, whereas the single-copy sequences yielding phenotypes may become hypomethylated only in later generations.

Immunolabeling of nuclei from F_1 hybrids heterozygous for *ddm1* or *met1* revealed the inability of DDM1 and MET1 to re-establish DNA methylation of chromocenters once it has been lost (Figure 4A). This confirms the stable inheritance of DNA hypomethylation by *ddm1* (Kakutani *et al*., 1999), and is consistent with a role of *DDM1*and *MET1* in maintenance methylation. The inability to remethylate repetitive sequences does not prevent *de novo* methylation of multicopy transgenes (Jakowitsch *et al*., 1999; Pélissier *et al*., 1999) and endogenous genes (Melquist *et al*., 1999) in *ddm1* and *met1*. It is possible that such repetitive genes require transcription to be methylated *de novo* in the process of TGS or PTGS. Transcription is lacking for most hypomethylated centromeric and pericentromeric repetitive sequences, thus preventing their *de novo* methylation.

The increased number of nuclei with H4K16 acetylation at chromocenters of *ddm1* plants indicates that DDM1, in addition to its role in DNA methylation, is also involved in histone deacetylation, presumably after completion of maintenance DNA methylation. Similarly, a DDM1-like factor (ISWI) of *Drosophila* was found to counteract H4K16 acetylation (Corona *et al*., 2002).

A model for the assembly of constitutive heterochromatin in Arabidopsis

The characteristics of constitutive heterochromatin need to be preserved through cell divisions. We propose a central role for DDM1, MET1, a H3K9-specific histone methylase (KYP) and a histone deacetylase (H4K16-specific in *Arabidopsis*) in the reassembly of heterochromatin, directly after DNA replication. (A complex of DNA methyltransferase and histone deacetylase has been proposed for mammals, see Rountree *et al*., 2000.) DNA maintenance methylation at CpG sites is performed when newly replicated nucleosomes are still accessible due to acetylated H4K16. During or after maintenance methylation of DNA, H3K9 methylation, directed by methylated DNA, might complete heterochromatin assembly, including binding of HP1-like proteins (Bannister *et al*., 2001; Gaudin *et al*., 2001; Lachner *et al*., 2001) to H3K9. Then DDM1 could mediate deacetylation of H4K16 (Figure 5A). When either DDM1 or MET1 is lacking, DNA methylation is reduced, causing reduced H3K9 methylation. At pericentromeric regions with low-copy sequences, DNA methylation and H3K9 methylation can fall below a critical threshold. As a consequence, these regions acquire euchromatin features (e.g. H3methylK4) and disperse from chromocenters (Figure 5B). If DDM1 is lacking, deacetylation of H4K16 is prevented additionally.

For other eukaryotic organisms, this model has to be modified according to their post-replicative requirement for H4 acetylation. Furthermore, it has to be considered that H3K9 methylation is not sufficient to determine constitutive heterochromatin in large plant genomes (A.Houben, D.Demidov, D.Gernand, A.Meister, C.R.Leach and I.Schubert, in preparation). Most likely, the ratio between H3methylK4 and H3methylK9 is essential. Within constitutive heterochromatin, H3K4 remains (largely) unmethylated, while euchromatin contains strongly methylated H3K4, independent of the level of H3K9 methylation. Thus, constitutive heterochromatin can be molecularly identified by the presence of a

threshold amount of (usually highly methylated) tandem repeats, and an excess of methylated H3K9 over methylated H3K4 (Noma *et al*., 2001). The model can be further tested/refined using (transgenic) mutants of *Arabidopsis*, affected in histone acetylation and methylation (Tian and Chen, 2001; Jackson *et al*., 2002).

Materials and methods

Plant material

The *ddm1-2* and *met1* mutants were originally obtained in the Col accession and later transferred to the L*er* background by repeated backcrossing (Jacobsen *et al*., 2000). The double mutant *ddm1 met1* was created by crossing plants with the single mutations. True double mutants were identified in F_2 by PCR, restriction digest and DNA sequence analysis of the mutations. Plants heterozygous for *ddm1* or *met1* were obtained from the crosses *cer2* × *ddm1* and *cer2* × *met1*, respectively. The *cer2* marker (bright green stems and siliques) was used to distinguish F_1 plants from self-pollinated progeny. The mutants *clk-3* (Jacobsen and Meyerowitz, 1997) and *fwa-1* (Soppe *et al*., 2000) were both in the L*er* accession.

Plants were grown either in the greenhouse with a long-day regime or in a growth chamber under continuous light. Young rosette leaves, flower buds and root tips were harvested, fixed in ethanol/acetic acid (3:1) and stored at –20°C. For immunodetection of histones, the tissue was fixed in 2% formaldehyde in Tris buffer. Nuclear suspensions were produced and processed for flow sorting as described previously (Jasencakova *et al*., 2000, 2001). Nuclei were stained with DAPI.

FISH

Probes used: BACs were obtained from the Arabidopsis Biological Resource Center. For the amplification of pericentromeric PCR fragments A–H, BAC F28D6 (DDBJ/EMBL/GenBank accession No. AF147262) was used as template (Figure 2B). The four PCR fragments used to locate *CAC1* were based on nucleotides 41 909–44 519, 46 209–48 649, 58 108–60 468 and 62 069–64 377 from BAC T10J7 (accession No. AC005897). The different PCR fragments used for FISH detection of different repetitive elements were based on nucleotides 293–2704 from the BAC T1J24 sequence (accession No. AF147263) for *Athila*, nucleotides 17–2044 from the Tat*1* sequence (accession No. AF056631), nucleotides 1269–3124 from the Ta*1-3* sequence (accession No. X13291), nucleotides 26 751–27 824 from the FCA contig fragment No. 2 (accession No. Z97337) for *Emi12*, nucleotides 30 737–32 974 from P1 clone MHK7 (accession No. AB011477) for *AthE1.4* and nucleotides 22 121–23 847 from the BAC T5H22 sequence (accession No. AF096372) for the chromomeric repeat ATR63. PCR conditions and primer sequences can be obtained from the authors on request. Probes for detection of the *SUP* and *FWA* sequences were a 6.7 kb *SUP* genomic DNA fragment cloned into pCGN1547 and a 10.5 kb genomic DNA fragment cloned into pCAMBIA 2300, respectively. The 180 bp centromeric repeat sequence was detected with pAL1 (Martínez-Zapater *et al*., 1986).

For BLAST analyses of PCR fragment sequences, NCBI BLAST2.0 was used.

FISH experiments were performed according to Schubert *et al*. (2001) with the antibodies goat anti-avidin conjugated with biotin (1:200; Vector Laboratories) and avidin conjugated with Texas Red (1:1000; Vector Laboratories) for the detection of biotin-labeled probes, and mouse anti-DIG (1:250; Roche) and goat anti-mouse conjugated with Alexa^{488} (1:200; Molecular Probes) for the detection of DIG-labeled probes.

5-methylcytosine immunodetection

Slide preparations were baked at 60°C for 30 min, denaturated in 70% formamide, 2× SSC, 50 mM sodium phosphate pH 7.0 at 80°C for 3 min, washed in ice-cold PBS (10 mM sodium phosphate pH 7.0, 143 mM NaCl) for 5 min, incubated in 1% bovine serum albumin in PBS for 30 min at 37°C and subsequently incubated with mouse antiserum raised against 5-methylcytosine (Podesta *et al*., 1993; 1:250) in TNB (100 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% blocking reagent; Roche). Mouse antibodies were detected using rabbit anti-mouse–FITC (1:1000; Sigma), followed by goat anti-rabbit–Alexa^{488} (1:200; Molecular Probes).

Histone immunodetection

Antibodies used were rabbit polyclonal antisera recognizing specifically modified lysine residues of histones H3 and H4 [R41 (H4Ac5; 1:100), R232 (H4Ac8; 1:100), R101 (H4Ac12; 1:100), R252 (H4Ac16; 1:1000), R243 (preferentially recognizing tri- and tetra-acetylated H4; 1:200) (Turner and Fellows, 1989; Turner *et al*., 1989; Belyaev *et al*., 1996; Stein *et al*., 1997; White *et al*., 1999)], anti-acetyl histone H3 (Lys9; 1:200), anti-dimethyl-histone H3 (Lys4; 1:200–1:500) and anti-dimethyl-histone H3 (Lys9; 1:100) (from Upstate).

The immunolabeling procedure for histones was as described previously (Jasencakova *et al*., 2000, 2001). After post-fixation in 4% paraformaldehyde/PBS, subsequent washes in PBS and blocking at 37°C, slides were exposed to primary antisera for 1 h at 37°C or overnight at 4°C. After washes in PBS, the incubation with secondary antibodies, goat anti-rabbit–FITC (1:80; Sigma) or goat anti-rabbit–rhodamine (1:100; Jackson ImmunoResearch Labs) was performed at 37°C. Nuclei were counterstained with DAPI (2 μg/ml, in Vectashield; Vector).

For a combined detection of H3K9 methylation and DNA methylation, nuclei were post-fixed after histone detection, denatured and incubated with mouse anti-5mC, followed by goat anti-mouse conjugated with biotin (1:600; Jackson ImmunoResearch Labs) and streptavidin conjugated with FITC (1:1000).

Microscopy and image processing

Preparations were analyzed with a Zeiss Axiophot 2 epifluorescence microscope equipped with a cooled CCD camera (Photometrics). Fluorescence images for each fluorochrome were captured separately using the appropriate excitation filters. The images were pseudocolored, merged and processed with Adobe Photoshop software (Adobe Systems).

Measuring of chromocenter fractions

Digital images in gray scale were analyzed with the freeware program NIH-image 1.62. Special macros were written to measure the size and average staining intensity of nuclei and chromocenters. The chromocenter value was divided by the whole nucleus value and yielded the chromocenter fraction.

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References

Bannister,A.J., Zegerman,P., Partridge,J.F., Miska,E.A., Thomas,J.O., Allshire,R.C. and Kouzarides,T. (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature*, **410**, 120–124.

Belyaev,N.D., Keohane,A.M. and Turner,B.M. (1996) Differential underacetylation of histones H2A, H3 and H4 on the inactive X chromosome in human female cells. *Hum. Genet.*, **97**, 573–578.

Bestor,T.H. (1992) Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain. *EMBO J.*, **11**, 2611–2617.

Briggs,S.D., Bryk,M., Strahl,B.D., Cheung,W.L., Davie,J.K., Dent,S.Y.R., Winston,F. and Allis,C.D. (2001) Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in *Saccharomyces cerevisiae*. *Genes Dev.*, **15**, 3286–3295.

Casacuberta,E., Casacuberta,J.M., Puigdomènech,P. and Monfort,A. (1998) Presence of miniature inverted-repeat transposable elements (MITEs) in the genome of *Arabidopsis thaliana*: characterisation of the *Emigrant* family of elements. *Plant J.*, **16**, 79–85.

Corona,D.F.V., Clapier,C.R., Becker,P.B. and Tamkun,J.W. (2002) Modulation of ISWI function by site-specific histone acetylation. *EMBO rep.*, **3**, 242–247.

Dennis,K., Fan,T., Geiman.,T., Yan,Q. and Muegge,K. (2001) Lsh, a member of the SNF2 family, is required for genome-wide methylation. *Genes Dev.*, **15**, 2940–2944.

Finnegan,E.J. and Kovac,K.A. (2000) Plant DNA methyltransferases. *Plant Mol. Biol.*, **43**, 189–201.

Finnegan,E.J., Peacock,W.J. and Dennis,E.S. (1996) Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. *Proc. Natl Acad. Sci. USA*, **93**, 8449–8454.

Finnegan,E.J., Genger,R.K., Peacock,W.J. and Dennis,E.S. (1998) DNA methylation in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **49**, 223–247.

Fransz,P.F., Armstrong,S., de Jong,J.H., Parnell,L.D., van Drunen,C., Dean,C., Zabel,P., Bisseling,T. and Jones,G.H. (2000) Integrated cytogenetic map of chromosome arm 4S of *A.thaliana*: structural organization of heterochromatic knob and centromere region. *Cell*, **100**, 367–376.

Fransz,P., de Jong,J.H., Lysak,M., Ruffini Castiglione,M. and Schubert,I. (2002) Interphase chromosomes in *Arabidopsis* are organised as well-defined chromocenters from which euchromatin loops emanate. *Proc. Natl Acad. Sci. USA*, **99**, 14584–14589.

Galbraith,D.W., Harkins,K.R. and Knapp,S. (1991) Systemic endopolyploidy in *Arabidopsis thaliana*. *Plant Physiol.*, **96**, 985–989.

Gaudin,V., Libault,M., Pouteau,S., Juul,T., Zhao,G., Lefebvre,D. and Grandjean,O. (2001) Mutations in *LIKE HETEROCHROMATIN PROTEIN 1* affect flowering time and plant architecture in *Arabidopsis*. *Development*, **128**, 4847–4858.

Gendrel,A.V., Lippman,Z., Yordan,C., Colot,V. and Martienssen,R. (2002) Dependence of heterochromatic histone H3 methylation patterns on the *Arabidopsis* gene *DDM1*. *Science*, **297**, 1871–1873.

Genger,R.K., Kovac,K.A., Dennis,E.S., Peacock,W.J. and Finnegan,E.J. (1999) Multiple DNA methyltransferase genes in *Arabidopis thaliana*. *Plant Mol. Biol.*, **41**, 269–278.

Henikoff,S. (2000) Heterochromatin function in complex genomes. *Biochim. Biophys. Acta*, **1470**, O1–8.

Heslop-Harrison,J.S., Murata,M., Ogura,Y., Schwarzacher,T. and Motoyoshi,F. (1999) Polymorphisms and genomic organization of repetitive DNA from centromeric regions of *Arabidopsis* chromosomes. *Plant Cell*, **11**, 31–42.

Hirochika,H., Okamoto,H. and Kakutani,T. (2000) Silencing of retrotransposons in *Arabidopsis* and reactivation by the *ddm1* mutation. *Plant Cell*, **12**, 357–368.

Jackson,J., Lindroth,A.M., Cao,X. and Jacobsen,S.E. (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. *Nature*, **416**, 556–560.

Jacobsen,S.E. and Meyerowitz,E.M. (1997) Hypermethylated *SUPERMAN* epigenetic alleles in *Arabidopsis*. *Science*, **277**, 1100–1103.

Jacobsen,S.E., Sakai,H., Finnegan,E.J., Cao,X. and Meyerowitz,E.M. (2000) Ectopic hypermethylation of flower-specific genes in *Arabidopsis*. *Curr. Biol.*, **10**, 179–186.

Jakowitsch,J., Papp,I., Moscone,E.A., van der Winden,J., Matzke,M. and Matzke,A.J.M. (1999) Molecular and cytogenetic characterization of a transgene locus that induces silencing and methylation of homologous promoters *in trans*. *Plant J.*, **17**, 131–140.

Jasencakova,Z., Meister,A., Walter,J., Turner,B. M. and Schubert,I.(2000) Histone H4 acetylation of euchromatin and heterochromatin is cell cycle dependent and correlated with replication rather than with transcription. *Plant Cell*, **12**, 2087–2100.

Jasencakova,Z., Meister,A. and Schubert,I. (2001) Chromatin organization and its relation to replication and histone acetylation during the cell cycle in barley. *Chromosoma*, **110**, 83–92.

Jeddeloh,J.A., Stokes,T.L. and Richards,E.J. (1999) Maintenance of genomic methylation requires a SW12/SNF2 protein. *Nat. Genet.*, **22**, 94–97.

Johnson,L.M., Cao,X. and Jacobsen,S.E. (2002) Interplay between two epigenetic marks: DNA methylation and histone H3 lysine 9 methylation. *Curr. Biol.*, **12**, 1360–1367.

Jost,J.P. and Saluz,H.P. (eds) (1993) *DNA Methylation: Molecular Biology and Biological Significance*. Birkhäuser Verlag, Basel, Switzerland.

Kakutani,T., Jeddeloh,J.A. and Richards,E.J. (1995) Characterization of an *Arabidopsis thaliana* DNA hypomethylation mutant. *Nucleic Acids Res.*, **23**, 130–137.

Kakutani,T., Jeddeloh,J.A., Flowers,S.K., Munakata,K. and Richards,E.J. (1996) Developmental abnormalities and epimutations associated with DNA hypomethylation mutations. *Proc. Natl Acad. Sci. USA*, **93**, 12406–12411.

Kakutani,T., Munakata,K., Richards,E.J. and Hirochika,H. (1999) Meiotically and mitotically stable inheritance of DNA hypomethylation induced by *ddm1* mutation of *Arabidopsis thaliana*. *Genetics*, **151**, 831–838.

Kishimoto,N., Sakai,H., Jackson,J., Jacobsen,S.E., Meyerowitz,E.M., Dennis,E.S. and Finnegan,E.J. (2001) Site specificity of the *Arabidopsis* MET1 DNA methyltransferase demonstrated through hypermethylation of the *superman* locus. *Plant Mol. Biol.* **46**, 171–183.

Konieczny,A., Voytas,D.F., Cummings,M.P. and Ausubel,F.M. (1991) A superfamily of *Arabidopsis thaliana* retrotransposons. *Genetics*, **127**, 801–809.

Lachner,M. and Jenuwein,T. (2002) The many faces of histone lysine methylation. *Curr. Opin. Cell Biol.*, **14**, 286–296.

Lachner,M., O'Carroll,D., Rea,S., Mechtler,K. and Jenuwein,T. (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature*, **410**, 116–120.

Lindroth,A.M., Cao,X., Jackson,J.P., Zilberman,D., McCallum,C.M., Henikoff,S. and Jacobsen,S.E. (2001) Requirement of *CHROMOMETHYLASE3* for maintenance of CpXpG methylation. *Science*, **292**, 2077–2080.

Maluszynska,J. and Heslop-Harrison,J.S. (1991) Localization of tandemly repeated DNA sequences in *Arabidopsis thaliana*. *Plant J.*, **1**, 159–166.

Martínez-Zapater,J.M., Estelle,M.A. and Somerville,C.R. (1986) A highly repeated DNA sequence in *Arabidopsis thaliana*. *Mol. Gen. Genet.*, **204**, 417–423.

Melquist,S., Luff,B. and Bender,J. (1999) *Arabidopsis PAI* gene arrangements, cytosine methylation and expression. *Genetics*, **153**, 401–413.

MittelstenScheid,O., Afsar,K. and Paszkowski,J. (1998) Release of epigenetic gene silencing by *trans*-acting mutations in *Arabidopsis*. *Proc. Natl Acad. Sci. USA*, **95**, 632–637.

Miura,A., Yonebayashi,S., Watanabe,K., Toyama,T., Shimada,H. and Kakutani,T. (2001) Mobilization of transposons by a mutation abolishing full DNA methylation in *Arabidopsis*. *Nature*, **411**, 212–214.

Morel,J.B., Mourrain,P., Béclin,C. and Vaucheret,H. (2000) DNA methylation and chromatin structure affect transcriptional and post-transcriptional transgene silencing in *Arabidopsis*. *Curr. Biol.*, **10**, 1591–1594.

Noma,K., Allis,C.D. and Grewal,S.I.S. (2001) Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. *Science*, **293**, 1150–1155.

Pélissier,T., Tutois,S., Deragon,J.M., Tourmente,S., Genestier,S. and Picard,G. (1995) *Athila*, a new retroelement from *Arabidopsis thaliana*. *Plant Mol. Biol.*, **29**, 441–452.

Pélissier,T., Thalmeir,S., Kempe,D., Sänger,H.L. and Wassenegger,M. (1999) Heavy *de novo* methylation at symmetrical and non-symmetrical sites is a hallmark of RNA-directed DNA methylation. *Nucleic Acids Res.*, **27**, 1625–1634.

Podesta,A., Ruffini Castiglione,M., Avanzi,S. and Montagnoli,G. (1993) Molecular geometry of antigen binding by a monoclonal antibody against 5-methylcytidine. *Int. J. Biochem.*, **25**, 929–933.

Rice,J.C. and Allis,C.D. (2001) Histone methylation versus histone acetylation: new insights into epigenetic regulation. *Curr. Opin. Cell Biol.*, **13**, 263–273.

Richards,E.J. and Elgin,S.C.R. (2002) Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. *Cell*, **108**, 489–500.

Ronemus,M.J., Galbiati,M., Ticknor,C., Chen,J. and Dellaporta,S.L. (1996) Demethylation-induced developmental pleiotropy in *Arabidopsis*. *Science*, **273**, 654–657.

Rountree,M.R., Bachman,K.E. and Baylin,S.B. (2000) DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. *Nat. Genet.*, **25**, 269–277.

Sakai,H., Medrano,L.J. and Meyerowitz,E.M. (1995) Role of *SUPERMAN* in maintaining *Arabidopsis* floral whorl boundaries. *Nature*, **378**, 199–203.

Schubert,I., Fransz,P.F., Fuchs,J. and de Jong,J.H. (2001) Chromosome painting in plants. *Methods Cell Sci.*, **23**, 57–69.

Singer,T., Yordan,C. and Martienssen,R.A. (2001) Robertson's *Mutator* transposons in *A.thaliana* are regulated by the chromatin-remodeling gene *Decrease in DNA Methylation* (*DDM1*). *Genes Dev.*, **15**, 591–602.

Soppe,W.J.J., Jacobsen,S.E., Alonso-Blanco,C., Jackson,J.P., Kakutani,T., Koornneef,M. and Peeters,A.J.M. (2000) The late flowering phenotype of *fwa* mutants is caused by gain-of-function epigenetic alleles of a homeodomain gene. *Mol. Cell*, **6**, 791–802.

Stein,P., Worrad,D.M., Belyaev,N.D., Turner,B.M. and Schultz,R.M. (1997) Stage-dependent redistribution of acetylated histones in nuclei of the early preimplantation mouse embryo. *Mol. Reprod. Dev.*, **47**, 421–429.

Surzycki,S.A. and Belknap,W.R. (1999) Characterization of repetitive DNA elements in *Arabidopsis*. *J. Mol. Evol.*, **48**, 684–691.

Taddei,A., Roche,D., Sibarita,J.-B., Turner,B.M. and Almouzni,G. (1999) Duplication and maintenance of heterochromatin domains. *J. Cell Biol.*, **147**, 1153–1166.

Tamaru,H. and Selker,E.U. (2001) A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. *Nature*, **414**, 277–283.

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*, **408**, 796–815.

Tian,L. and Chen,Z.J. (2001) Blocking histone deacetylation in *Arabidopsis* induces pleiotropic effects on plant gene regulation and development. *Proc. Natl Acad. Sci. USA*, **98**, 200–205.

Turner,B.M. and Fellows,G. (1989) Specific antibodies reveal ordered and cell-cycle-related use of histone-H4 acetylation sites in mammalian cells. *Eur. J. Biochem.*, **179**, 131–139.

Turner,B.M., O'Neill,L.P. and Allan,I.M. (1989) Histone H4 acetylation in human cells. Frequency of acetylation at different sites defined by immunolabeling with site-specific antibodies. *FEBS Lett.*, **253**, 141–145.

Vongs,A., Kakutani,T., Martienssen,R.A. and Richards,E.J. (1993) *Arabidopsis thaliana* DNA methylation mutants. *Science*, **260**, 1926–1928.

Voytas,D.F., Konieczny,A., Cummings,M.P. and Ausubel,F.M. (1990) The structure, distribution and evolution of the Ta*1* retrotransposable element family of *Arabidopsis thaliana*. *Genetics*, **126**, 713–721.

White,D.A., Belyaev,N.D. and Turner,B.M. (1999) Preparation of site-specific antibodies to acetylated histones. *Methods*, **19**, 417–421.

Wright,D.A. and Voytas,D.F. (1998) Potential retroviruses in plants: Tat1 is related to a group of *Arabidopsis thaliana* Ty3/*gypsy* retrotransposons that encode envelope-like proteins. *Genetics*, **149**, 703–715.

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6.4. JASENCAKOVA Z, SOPPE WJJ, MEISTER A, GERNAND D, TURNER BM, SCHUBERT I

Histone modifications in *Arabidopsis* – high methylation of H3 lysine 9 is dispensable for constitutive heterochromatin.

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Histone modifications in *Arabidopsis* – high methylation of H3 lysine 9 is dispensable for constitutive heterochromatin

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Summary

N-terminal modifications of nucleosomal core histones are involved in gene regulation, DNA repair and recombination as well as in chromatin modeling. The degree of individual histone modifications may vary between specific chromatin domains and throughout the cell cycle. We have studied the nuclear patterns of histone H3 and H4 acetylation and of H3 methylation in *Arabidopsis*. A replication-linked increase of acetylation only occurred at H4 lysine 16 (not for lysines 5 and 12) and at H3 lysine 18. The last was not observed in other plants. Strong methylation at H3 lysine 4 was restricted to euchromatin, while strong methylation at H3 lysine 9 occurred preferentially in heterochromatic chromocenters of *Arabidopsis* nuclei. Chromocenter appearance, DNA methylation and histone modification patterns were similar in nuclei of wild-type and *kryptonite* mutant (which lacks H3 lysine 9-specific histone methyltransferase), except that methylation at H3 lysine 9 in heterochromatic chromocenters was reduced to the same low level as in euchromatin. Thus, a high level of H3methylK9 is apparently not necessary to maintain chromocenter structure and does not prevent methylation of H3 lysine 4 within *Arabidopsis* chromocenters.

Keywords: *Arabidopsis*, heterochromatin, histone acetylation, histone methylation, DNA methylation, histone methyltransferase mutant (*kyp*).

Introduction

Dynamic modifications of chromatin structure are essential for correct regulation of vital nuclear processes such as transcription, replication, repair, or recombination of DNA. Specific post-translational histone modifications like acetylation, methylation, phosphorylation, ubiquitination and combinations thereof provide, in concert with DNA methylation, an epigenetic code that acts upstream of chromatin functions (Lachner and Jenuwein, 2002; Richards and Elgin, 2002; Strahl and Allis, 2000; Turner, 2000). Therefore, the distribution of histone isoforms is not uniform within eukaryotic genomes. Usually, acetylated histones are enriched within euchromatin and diminished in heterochromatin domains with some exceptions (e.g. H4Ac12 in *Drosophila*, Turner *et al.*, 1992). In contrast to the well-established linkage between increased histone acetylation and ongoing transcription at the level of single genes (for review, see Brown *et al.*, 2000; Cheung *et al.*, 2000), studies at the level of large chromatin domains revealed cell cycle-dependent modulations, correlated with replication of euchromatin and heterochromatin in mammals (Taddei *et al.*, 1999) and plants (Jasencakova *et al.*, 2000, 2001). This is apparently due to evolutionarily conserved 'deposition-related' acetylation of H4 at lysines 5 and 12 (Sobel *et al.*, 1995) at newly replicated chromatin, which might be extended to lysine 16 in the dicot *Vicia faba* (Jasencakova *et al.*, 2000) or to lysine 8 in the monocot barley (Jasencakova *et al.*, 2001). Contrary to H4 acetylation, the nuclear patterns of H3 acetylation remained fairly constant through the cell cycle (Jasencakova *et al.*, 2000, 2001). While molecular analysis of chromatin modifications characterize either the total chromatin or regions of individual DNA sequences, a cytological approach provides a global view on entire nuclei and specific domains therein.

Constitutive heterochromatin, cytologically defined as highly condensed chromatin (Heitz, 1928), is usually transcriptionally inactive and shows, in addition to underacetylated histones H3 and H4 and low methylated lysine 4 of H3, highly methylated lysine 9 of histone H3 in fission yeast

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(Noma *et al.*, 2001), *Drosophila* (Schotta *et al.*, 2002), mammals (Peters *et al.*, 2001) and *Arabidopsis* (Soppe *et al.*, 2002). H3methylK9 recruits the highly conserved heterochromatin-associated protein HP1 (Bannister *et al.*, 2001; Jackson *et al.*, 2002; Lachner *et al.*, 2001) and is thereby involved in heterochromatin assembly (Nakayama *et al.*, 2001; Zhang and Reinberg, 2001).

A better understanding of the significance of specific histone modifications can be obtained by the study of mutants. Cytological investigations of *Arabidopsis* mutants with reduced DNA methylation (*ddm1* and *met1*) have shown that reduced overall maintenance methylation at CpG sites is accompanied in both mutants by size reduction of heterochromatic chromocenters (due to dislocation of sequences of pericentromeric regions away from the chromocenters) and by disappearance of strong DNA and H3K9 methylation from the mutant chromocenters. These features were shown to be epigenetically inherited in mutant-derived chromocenters of F_1 plants heterozygous for *ddm1* or *met1* (Soppe *et al.*, 2002). Because MET1 is a major maintenance methylase with strong preference for CpG sites (Jackson *et al.*, 2002), the most likely interpretation of these findings is that reduced CpG methylation represents an imprint controlling heterochromatin assembly and H3K9 methylation at chromocenters (Soppe *et al.*, 2002). Recently, a mutant (*kyp*) of KRYPTONITE, an H3K9-specific histone methyltransferase, was described which showed reduced H3methylK9 and CpNpG methylation but no other phenotypes except suppression of gene silencing at the *superman (sup)* locus (Jackson *et al.*, 2002).

Here, we report on the distribution of post-translationally acetylated and methylated histones at distinct chromatin domains (heterochromatic chromocenters, euchromatin, nucleoli) within nuclei of different cell cycle (including pachytene chromosomes) and ploidy stages of *Arabidopsis thaliana*. The data provide an interesting comparison with the results recently obtained for larger plant genomes (Jasencakova *et al.*, 2000, 2001). In addition, we investigated the *kyp* mutant as to the degree of DNA methylation, H3 and H4 acetylation and H3 methylation within its pericentromeric heterochromatin, to find out whether the absence of KRYPTONITE has an influence on assembly and H3methylK9 content of chromocenters and/or on other chromatin modifications.

Results

H4 acetylation (except at K16) is not detectable at nucleoli and chromocenters in A. thaliana

Performing immunolabeling experiments on formaldehyde-fixed flow-sorted interphase nuclei (Figure 1), we have tested whether or not the nuclear distribution of modified histone isoforms in *Arabidopsis* is comparable to that observed for larger and more complex plant genomes (Jasencakova *et al.*, 2000, 2001). Antibodies recognizing isoforms of histone H4 acetylated at lysine positions 5 (H4Ac5, Figure 2a), 8 (H4Ac8, Figure 2b), 12 (H4Ac12, Figure 2c) as well as tri-and tetra-acetylated H4, yielded the same pattern of immunosignals on root tip and leaf nuclei of *A. thaliana*. Euchromatin was intensely labeled, while nucleoli and heterochromatic chromocenters remained unlabeled during all cell cycle stages (G1, S, late S/earlyG2, G2) in root tips and at 2, 4 and 8C ploidy-levels in leaves. The lack of any detectable cell cycle-dependent modulation and the general absence of signals for these acetylated isoforms of H4 from nucleoli of G1 and G2 nuclei of *A. thaliana* are in contrast to the situation observed in field bean and barley (Jasencakova *et al.*, 2000, 2001).

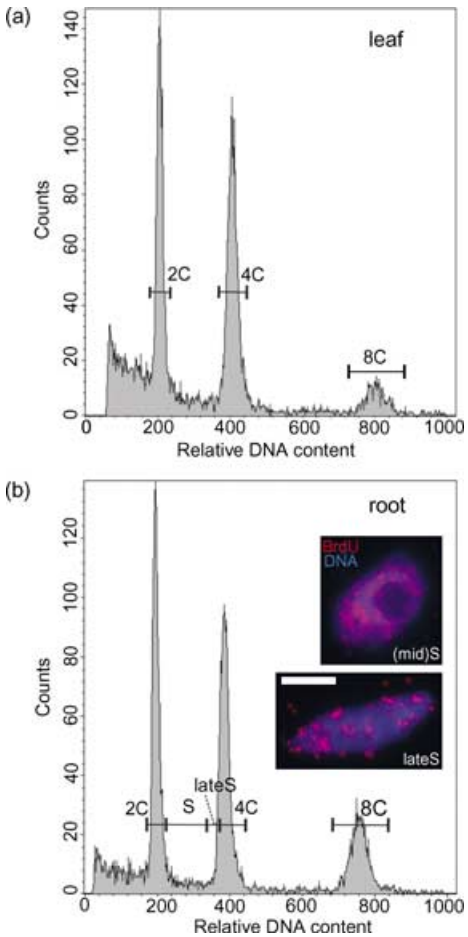

Figure 1. Histograms of the relative DNA content of unsynchronized *Arabidopsis* nuclei after DAPI staining and flow-cytometric analysis.
The gates used for sorting the nuclei are indicated.(a) leaf; (b) root nuclei, inserts show replication patterns of (mid)S and late S nuclei after BrdU incorporation. Bar = 5 μm.

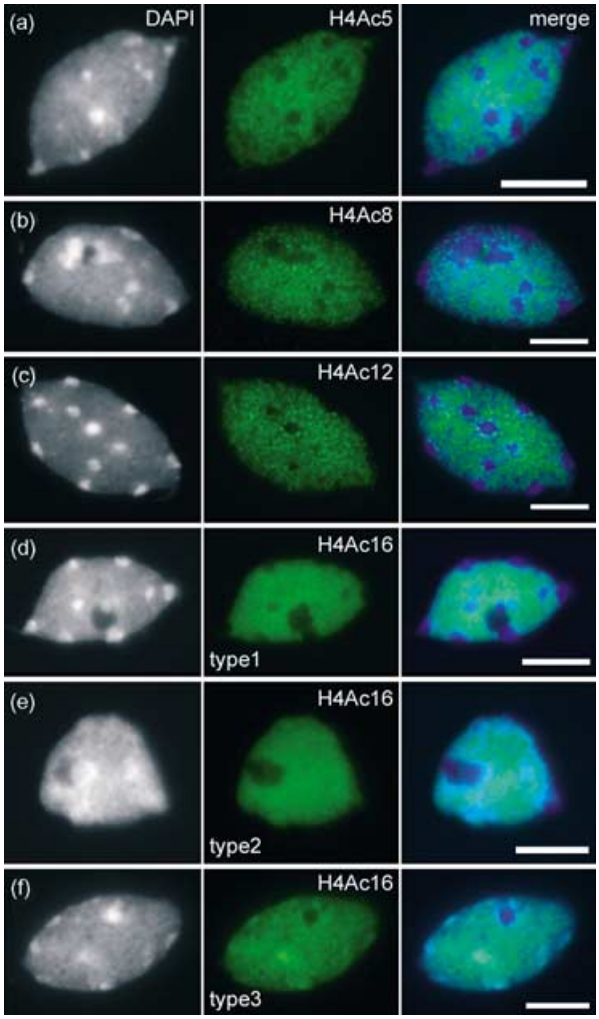

Figure 2. Distribution of acetylated histone H4 in *A. thaliana* nuclei.
DAPI staining in black and white, left; immunosignals in green, middle; and merge of both, right.
(a) H4Ac5 (b) H4Ac8 (c) H4Ac12. Note the absence of signals from nucleoli and DAPI-positive chromocenters.
(d) H4Ac16 type 1: euchromatin intensely labeled, nucleolus and chromocenters unlabeled.
(e) H4Ac16 type 2: chromatin uniformly labeled, nucleolus unlabeled.
(f) H4Ac16 type 3: chromocenters with signal clusters, nucleolus unlabeled (inactive rDNA components of chromocenters remained unlabeled in type 2 and 3 nuclei). Bars = 5 μm.

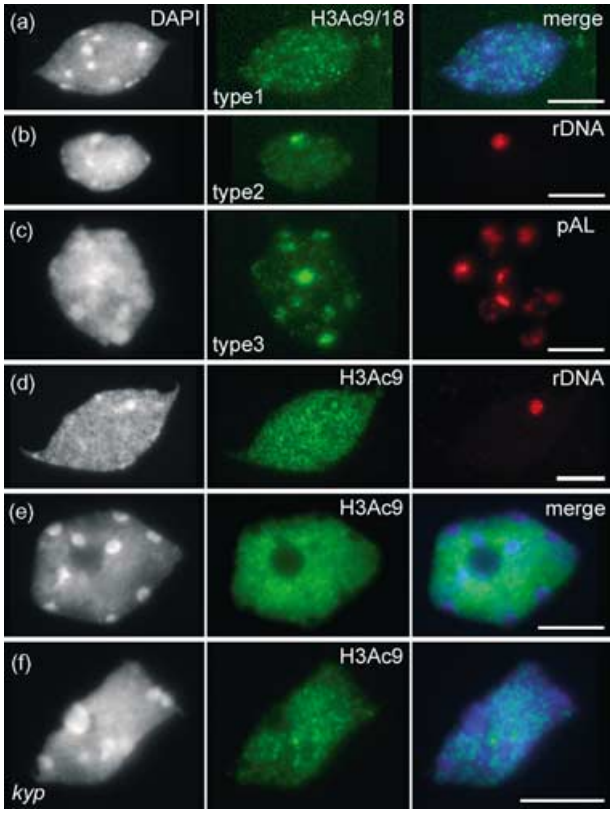

Figure 3. Distribution of histone H3 acetylated at lysines 9/18 (a–c) and 9 (d–f) in *A. thaliana* nuclei.
DAPI staining left; immunosignals, middle; merge of both (a, e, f), or FISH signals for rDNA (b, d) and centromeric sequences (c), right.
(a) H3Ac9/18 type 1: uniformly labeled chromatin.
(b) H3Ac9/18 type 2: strongest labeling at rDNA-containing chromocenter.
(c) H3Ac9/18 type 3: signal clusters at chromocenters.
(d) H3Ac9 labeling of an endopolyploid root nucleus, part of rDNA-containing chromocenter shows strong immunosignals.
(e) H3Ac9 labeling of a leaf nucleus with unlabeled nucleolus and chromocenters.
(f) H3Ac9 labeling of a *kyp* mutant leaf nucleus is similar as that of wild type, with unlabeled nucleolus and chromocenters. Bars = 5 μm.

Acetylation of K16 of H4 at heterochromatin is cell cycle dependent

Contrary to the above, antibodies against H4Ac16 yielded three distinct labeling patterns in leaf as well as in root nuclei of *Arabidopsis* (Figure 2d–f). This resembles the cell cycle-dependent modulation of acetylation at this lysine position observed in faba bean (Jasencakova *et al.*, 2000). Three types of labeling patterns could be distinguished, mainly differing in the labeling intensity of chromocenters, which were either unlabeled (type 1), labeled equally strong as euchromatin (type 2), or stronger than euchromatin (type 3).

In roots, unlabeled chromocenters occurred most frequently in G1/2C nuclei and were less abundant in late S, 4C and 8C nuclei. Brightly labeled chromocenters (except at regions containing rDNA components) appeared rarely in 2C nuclei but more frequently in late S, 4C and 8C nuclei (Table 1a). Among mitotically inactive leaf nuclei of 2, 4, and 8C DNA content, the frequency of the three patterns was rather constant. The majority showed unlabeled chromocenters (>64%) or uniformly labeled chromatin (24.2–28.9%). Only a minority (3.5–11.2%) revealed strongly labeled (peri-)centromeric heterochromatin (Table 1b).

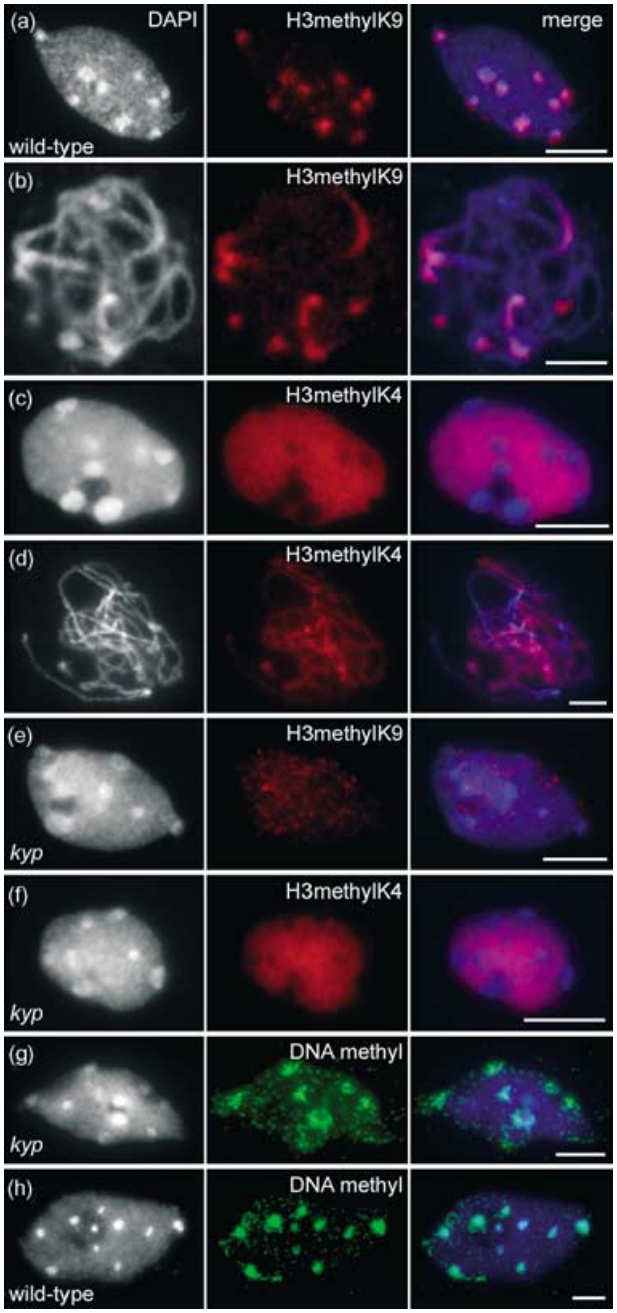

Figure 4. Distribution of methylated DNA and histone H3 methylated at lysine 9 and 4 in *A. thaliana* wild-type and *kyp* nuclei.
DAPI staining, left; immunosignals, middle; merge of both, right. Wild-type (a–d, h); *kyp* nuclei in (e–g).
(a) H3methylK9 labeling of a root nucleus: chromocenters are preferentially labeled.
(b) H3methylK9 labeling of pachytene chromosomes: DAPI-positive pericentromeric heterochromatin and terminal NORs are preferentially labeled.
(c) H3methylK4 labeling of a leaf nucleus: intense immunosignals at euchromatin, while nucleolus and chromocenters are unlabeled.
(d) H3methylK4 labeling of pachytene chromosomes: euchromatin remains intensely labeled.
(e) H3methylK9 labeling of a *kyp* leaf nucleus: the weak immunosignals are uniformly distributed and chromocenters show no preferential labeling.
(f) H3methylK4 labeling of a *kyp* leaf nucleus: similar as in wild type, nucleolus and chromocenters remain unlabeled.

Histone H3 acetylation alters along the cell cycle in A. thaliana

No pronounced cell cycle-dependent variation of H3 acetylation has been observed in faba bean and barley (Jasencakova *et al.*, 2000, 2001). However, in *Arabidopsis* nuclei, antibodies against H3Ac9/18 yielded three distinct labeling patterns (Figure 3a–c). Nuclei were either uniformly labeled (type 1) or the largest, rDNA-containing chromocenter(s), associated with nucleoli, were more intensely labeled than the remaining chromatin (type 2), or all chromocenters were more strongly labeled than euchromatin (type 3). The frequency of these patterns varied depending on the cell cycle stage and the ploidy level of the root tip nuclei (Table 2). Uniformly labeled nuclei occurred most frequently during S-phase (59%) but less frequent among 2C (31.6%), 4C (21.1%), and 8C (9.9%) nuclei. The percentages of strongly labeled rDNA-containing heterochromatin and chromocenters increased with increasing ploidy levels (Table 2).

With an antibody recognizing specifically H3 acetylated at K9, the majority of root tip nuclei showed no labeled chromocenters. Only among 8C nuclei, approximately 60% showed strong labeling at those chromocenters that contain condensed rDNA, while nucleoli consistently remained free of signals (Table 3; Figure 3d). The strong labeling at these chromocenters was restricted to parts of the area covered by rDNA signals after fluorescent *in situ* hybridization (FISH). Interestingly, all chromocenters and nucleoli of leaf nuclei consistently revealed less H3Ac9 than the remaining chromatin (Figure 3e) and no strong labeling at rDNA-containing chromocenters was detected. This suggests tissue-specific differences as to H3K9 acetylation of chromatin containing inactive rDNA in *Arabidopsis*. The deviating patterns obtained with anti-H3Ac9/18 and anti-H3Ac9 antibodies likely reflect acetylation of K18.

Wild-type chromocenters have a high level of H3methylK9

Strong methylation of lysine 9 of histone H3 was found to be a typical feature of heterochromatin in non-plant eukaryotes, while methylation of lysine 4 of H3 occurred preferentially within transcriptionally competent chromatin (Litt *et al.*, 2001; Noma *et al.*, 2001).

Immunostaining with antibodies directed against dimethylated K9 of H3 revealed signals preferentially at heterochromatic chromocenters (Figure 4a) of root tip, leaf and

Figure 4. continued
(g) DNA methylation of a *kyp* leaf nucleus: immunosignals for 5mC are located preferentially at chromocenters (except those associated with the nucleolus which contain rDNA).
(h) The same DNA-methylation pattern occurred in wild-type nuclei. Bars = 5 μm.

Table 1 H4Ac16 labeling patterns
(a) wild-type (Col) root tip nuclei

	DNA content/cell cycle stage									
	2C		2C-4C (S)		<4C (late S)		4C		8C	
Labeling patterns[1]	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
$nu^{-}cc^{-}eu^{+}$	172	44.0	171	43.2	106	26.4	111	28.9	89	16.8
$nu^{-}cc^{+}eu^{+(+)}$	199	50.9	182	46.0	183	45.5	139	36.2	165	31.1
$nu^{-}cc^{++}eu^{+}$	20	5.1	43	10.9	113	28.1	134	34.9	276	52.1
Σ	391	100.0	396	100.0	402	100.0	384	100.0	530	100.0

(b) wild-type (Ler) leaf nuclei

	DNA content					
	2C		4C		8C	
Labeling patterns[1]	*n*	%	*n*	%	*n*	%
$nu^{-}cc^{-}eu^{+}$	192	67.6	132	66.7	199	64.0
$nu^{-}cc^{+}eu^{+(+)}$	82	28.9	48	24.2	77	24.8
$nu^{-}cc^{++}eu^{+}$	10	3.5	18	9.1	35	11.2
Σ	284	100.0	198	100.0	311	100.0

(c) *kyp* leaf nuclei

	DNA content					
	2C		4C		8C	
Labeling patterns[1]	*n*	%	*n*	%	*n*	%
$nu^{-}cc^{-}eu^{+}$	107	85.6	94	80.3	85	75.9
$nu^{-}cc^{+}eu^{+(+)}$	16	12.8	16	13.7	19	17.0
$nu^{-}cc^{++}eu^{+}$	2	1.6	7	6.0	8	7.1
Σ	125	100.0	117	100.0	112	100.0

[1]nu: nucleoli and rDNA component of cc; cc: chromocenter; eu: euchromatin; intensity of labeling is indicated by (+) or (−).

Table 2 H3Ac9/18 labeling patterns of *Arabidopsis thaliana* wild-type (Col) root tip nuclei

	DNA content/cell cycle stage									
	2C		2C-4C (S)		<4C (late S)		4C		8C	
Labeling patterns[1]	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
uni	106	31.6	184	59.0	123	37.8	69	21.1	35	9.9
$rDNA^{++}cc^{(+)}eu^{(+)}$	154	46.0	92	29.5	101	31.1	124	37.9	86	24.4
$rDNA^{++}cc^{++}eu^{(+)}$	75	22.4	36	11.5	101	31.1	134	41.0	231	65.6
Σ	335	100.0	312	100.0	325	100.0	327	100.0	352	100.0

[1]uni: uniform labeling; cc: chromocenter; eu: euchromatin; rDNA: rDNA containing parts of chromocenters; intensity of labeling is indicated by (+) or (−).

Table 3 H3Ac9 labeling patterns of *Arabidopsis thaliana* wild-type (Col) root tip nuclei

	DNA content/cell cycle stage									
	2C		2C-4C (S)		<4C (late S)		4C		8C	
Labeling patterns[1,2]	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
rDNA$^+$cc$^-$eu$^+$	5	5.3	7	8.0	18	16.7	15	15.3	73	60.3
rDNA$^-$cc$^-$eu$^+$	87	91.6	78	88.6	86	79.6	82	83.7	47	38.8
uni	3	3.1	3	3.4	4	3.7	1	1.0	1	0.8
Σ	95	100.0	88	100.0	108	100.0	98	100.0	121	100.0

[1]rDNA: rDNA containing parts of chromocenters; cc: chromocenter; eu: euchromatin; uni: uniform labeling; intensity of labeling is indicated by (+) or (–).
[2]Nucleoli remained unlabeled in all patterns.

flower bud nuclei of different cell cycle and endopolyploidy stages in *Arabidopsis*. The strong labeling of heterochromatin also persisted during mitotic and meiotic divisions (Figure 4b). In contrast, antibodies against dimethylated K4 of H3 revealed the most intense signals at euchromatin, while nucleoli and chromocenters remained unlabeled (Figure 4c). A corresponding pattern was observed for mitotic and meiotic chromosomes (Figure 4d). This confirmed previous data obtained for wild-type leaf nuclei (Soppe *et al.*, 2002) and is now extended for nuclei of other organs and cell cycle stages.

H3methylK9 is strongly reduced in chromocenters of kyp *nuclei*

Wild-type labeling patterns of the different histone modifications were compared with that of the *kyp* mutant (Jackson *et al.*, 2002) to analyze the influence of a lack of the H3K9-specific histone methyltransferase KRYPTONITE. The H4Ac16 labeling patterns and their frequencies in leaf nuclei of the *kyp* mutant were similar as in wild type (Table 1c). This suggests that *kyp* does not influence H4K16 acetylation, contrary to the situation observed for the hypomethylation mutant *ddm1* (decrease in DNA methylation), which revealed a strongly decreased postreplicative deacetylation at this lysine position within chromocenters (Soppe *et al.*, 2002). Immunodetection of H3Ac9 revealed that chromocenters remained unlabeled as in wild type (Figure 3f).

Immunolabeling of H3methylK9 in leaf nuclei of the *kyp* mutant yielded only small and uniformly distributed signal foci (Figure 4e) and no longer signal clusters at chromocenters as in wild type (Figure 4a). The distribution of H3methylK4 was as in wild type, with euchromatin most intensely labeled (Figure 4f). Size and shape of chromocenters in *kyp* nuclei were similar as in wild-type nuclei after DAPI-staining, although the proportion of chromocenters per nucleus was slightly higher in mutant nuclei (Table 4).

Table 4 Chromocenter percentages in wild-type Ler and *kyp* leaf nuclei

	% cc[1]	*n*[2]
Ler	13.46	60
kyp	14.60*	60

[1]% cc: percentage of area and staining intensity of chromocenters in relation to the entire nucleus.
[2]Number of measured nuclei.
*$P < 0.05$.

Also, the DNA methylation pattern after immunodetection of 5-methyl-cytosine was similar to that described for wild-type nuclei, i.e. intense immunosignals were observed preferentially at chromocenters except for those regions containing rDNA (Figure 4g,h). The latter two observations are in contrast with the significantly reduced size and the absence of pronounced DNA methylation from chromocenters within nuclei of the hypomethylation mutants *ddm1* and *met1* (Soppe *et al.*, 2002). The appearance of wild-type-like chromocenters in *kyp* nuclei suggests that high amounts of H3methylK9 are not necessarily required for the formation of constitutive heterochromatin in *Arabidopsis*.

Discussion

Nuclear histone acetylation patterns in Arabidopsis *differ from that of other plants*

In field bean (Jasencakova *et al.*, 2000), barley (Jasencakova *et al.*, 2001) and mammals (Taddei *et al.*, 1999) there is a cell cycle-dependent modulation of histone acetylation intensity, particularly at lysines 5 and 12 of H4 with pronounced, probably 'deposition-related' acetylation (Sobel *et al.*, 1995) at eu- and heterochromatin around the time of DNA replication, followed by a strong deacetylation at heterochromatin toward mitosis. In contrast, only K16 of H4 showed

such a pattern in *Arabidopsis* as also observed in field bean. Pronounced acetylation of this residue within chromocenters of a considerable number of endopolyploid nuclei probably indicates delayed deacetylation after endo-reduplication. The strong H4 acetylation within nucleoli of G1 and G2 nuclei observed in other plants was consistently lacking in *Arabidopsis* and also the rDNA-containing proportions of chromocenters were free of acetylation signals. However, H3K18 (but not K9) revealed a similar dynamics of acetylation as H4K16 in *Arabidopsis*, while H3 acetylation patterns of other plants did not show a clear cell cycle-dependency (Belyaev *et al.*, 1998; Jasencakova *et al.*, 2000, 2001). In case of H3K18, acetylation also involved rDNA-containing chromocenters. Apparently, acetylation of H4K16 and H3K18 are sufficient in *Arabidopsis* to enable post-replicative processes such as maintenance methylation of DNA and H3K9 at chromocenters or post-replication repair.

Chromocenter formation does not require a high level of H3methylK9

Strong methylation of H3K9 is mainly restricted to heterochromatin in *Arabidopsis*, but also occurs along the euchromatic chromosome arms in plants with large genomes. In contrast, H3methylK4 is present at euchromatin, while heterochromatin domains are unlabeled independent of genome size (Houben *et al.*, unpublished). Therefore, a high ratio between H3methylK9 and H3methylK4 seems to be more relevant for proper assembly of constitutive heterochromatin than high absolute levels of H3methylK9. This gains further support from our data obtained for the *kyp* mutant, since in mutant nuclei size and shape of chromocenters and the distribution of acetylated histones H3 and H4 and of methylated H3K4 are similar as in wild-type nuclei, while the level of H3methylK9 is severely decreased and no longer distinguishable between euchromatin and chromocenters. The latter was not surprising since KRYPTONITE is a major H3K9-specific histone methyltransferase in *Arabidopsis* (Jackson *et al.*, 2002; Johnson *et al.*, 2002). The lack of a morphological and cytological phenotype of the *kyp* mutant (even after extensive inbreeding), except for suppression of an epigenetically silenced allele of *SUPERMAN* (*clk*) which became activated because methylation of specific CpNpG sites by the chromomethylase CMT3 was suppressed by reduced H3methylK9 (Jackson *et al.*, 2002), tempts to speculate that strong methylation of H3K9 is not really essential for the structure of constitutive heterochromatin in *Arabidopsis*. However, H3methylK9 is essential for gene silencing by heterochromatinization within cytologically euchromatic regions.

CpNpG methylation in the *kyp* mutant is reduced compared to the wild type, but no effects on CpG methylation at centromeric repeat sequences were found (Jackson *et al.*, 2002; Johnson *et al.*, 2002). This agrees with our observation that immunosignals for 5-methyl-cytosine are of similar intensity and distribution in *kyp* and wild-type nuclei, and emphasizes the importance of CpG maintenance methylation as an imprint for correct chromocenter formation. These data suggest that the recently proposed dependence of DNA methylation inheritance on H3K9 methylation (Gendrel *et al.*, 2002) concerns only a minor fraction of DNA methylation within the *Arabidopsis* genome, consisting of non-CpG sites.

In null mutants for SU(VAR)3-9, an H3K9-specific histone methylase in *Drosophila*, H3methylK9 and the concentration of the heterochromatin-associated protein HP1 are strongly reduced within the heterochromatic chromocenters, which otherwise are of wild-type appearance (Schotta *et al.*, 2002). Also, in mitotic chromosomes and in nuclei of primary mouse embryonic fibroblasts (double null mutants for the histone methyltransferases *Suv39h1* and *Suv39h2*), the DAPI-positive heterochromatin was present but, in contrast to wild-type nuclei, not associated with signal clusters for H3methylK9 (Peters *et al.*, 2001). Therefore, in *Arabidopsis* as in *Drosophila* and in mouse, a high level of H3methylK9 is apparently dispensable for the heterochromatic structure of chromocenters. Moreover, the reduction of H3methylK9 (to the level remaining within euchromatin) is not accompanied by an increase in H3methylK4 (up to the level of euchromatin) within the chromocenters of *kyp* nuclei (Figure 4e,f). In contrast to previous assumptions based on immunoprecipitation data for fission yeast (Noma *et al.*, 2001) and *Arabidopsis* (Gendrel *et al.*, 2002), methylation of K9 is not the reason for the low level of H3methylK4 at heterochromatic chromocenters (because H3K4 methylation is not increased in *kyp* chromocenters), and therefore, methylation of these lysine residues within euchromatic and heterochromatic regions does not generally exhibit simple reciprocity (similar results were obtained by ChIP analyses; S. E. Jacobsen, personal communication). The lack of detectable H3Ac9 within the chromocenters of *kyp* nuclei shows that reduced methylation is not accompanied by higher acetylation at this residue. Taken together, these data again raise the question whether highly methylated H3K9 is important for assembly of constitutive heterochromatin and, if yes, how it is compensated within the H3K9-specific histone methylase mutants.

Experimental procedures

Plant material, isolation and flow-sorting of nuclei

Arabidopsis thaliana accessions Columbia (Col), Landsberg erecta (Ler), and the *kyp* mutant (*kyp-2* in clk3/gL-1 background) in Ler (Jackson *et al.*, 2002) were used. Seeds were germinated in Petri dishes on wet filter paper placed on 0.5% agar. After 2 days at 4°C, they were allowed to germinate 5–6 days at room temperature until roots were approximately 1 cm long. Root tip nuclei were isolated

from Col and leaf nuclei from Ler and the *kyp* mutant. Roots were fixed for 20 min in 2% and young leaves in 4% formaldehyde in Tris buffer (10 mM Tris, 10 mM Na_2EDTA, 100 mM TritonX-100, pH 7.5). Nuclei were isolated as described (Schubert *et al.*, 1993). Nuclear suspensions were stained with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg ml^{-1}) and processed for flow-sorting according to Jasencakova *et al.* (2000, 2001). The gates for sorting were set according to the histograms obtained for each suspension of nuclei. Representative histograms of root tip and leaf nuclei are shown in Figure 1. About 1000 nuclei of each fraction were sorted onto microscopic slides into a drop containing 100 mM Tris, 50 mM KCl, 2 mM $MgCl_2$, 0.05% Tween 20 and 5% sucrose (Kubaláková *et al.*, 1997), air-dried at room temperature for several hours and used for immunolabeling and/or FISH, or stored at −20°C until use.

Chromosome preparation

Flower buds of appropriate size were fixed for 20 min with ice cold 4% (w/v) paraformaldehyde in PBS (pH 7.3). After washing thrice for 10 min in PBS, the flower buds were digested at 37°C for 40 min with a mixture of 2.5% pectinase, 2.5% cellulase 'Onozuka R-10' and 2.5% Pectolyase Y-23 (w/v) dissolved in PBS. After washing thrice for 10 min in PBS, anthers were dissected from flower buds and squashed in a drop of Tris-buffer. After freezing in liquid nitrogen, the coverslips were removed and the slides were transferred immediately into PBS.

Measurement of chromocenter fractions

Young rosette leaves were fixed in ethanol/acetic acid (3 : 1) and stored at −20°C until use. After washing in water and citrate buffer (10 mM Na-citrate, pH 4.5), the leaves were digested at 37°C for 2–3 h in a mix containing 0.3% (w/v) pectolyase, 0.3% (w/v) cytohelicase, and 0.3% (w/v) cellulase dissolved in citrate buffer. The leaves were washed in citrate buffer and nuclear suspensions were spread in a drop of 45% acetic acid at 45°C, acetic acid was then removed by ice-cold ethanol/acetic acid (3 : 1) fixative and the slides were air-dried. Prior to measurements, nuclei were stained with DAPI (2 μg ml^{-1} in Vectashield). To quantify chromocenter fractions in wild type and *kyp*, digital images of nuclei in gray-scale were analyzed with the freeware program NIH-IMAGE 1.62. Special macros were written to measure the size and average staining intensity of nuclei and chromocenters. The chromocenter value was divided by the whole nucleus value and yielded the chromocenter fraction.

Replication labeling

For labeling of replicating chromatin, the roots of germinating plants were incubated in 5-bromo-2′-deoxyuridine (BrdU, 100 μM), fluorodeoxyuridine (0.1 μM) and uridine (5 μM) for 40 min in the dark. After rinsing, the roots were fixed in 2% formaldehyde/Tris buffer before isolation and flow-sorting of nuclei. For the detection of BrdU (Jasencakova *et al.*, 2001) mouse anti-BrdU (Becton Dickinson, 1 : 20), anti-mouse conjugated with biotin (Jackson Immuno Research Laboratories, 1 : 600), and avidin conjugated with Texas Red (Vector, 1 : 800) were used.

Histone immunolabeling

The following rabbit polyclonal antisera against modified histones were used: R41 (H4Ac5, 1 : 100), R232 (H4Ac8, 1 : 100), R101 (H4Ac12, 1 : 100), R252 (H4Ac16, 1 : 1000), R243 (preferentially recognizing tri- and tetra-acetylated H4, 1 : 200), R47 (H3Ac9/18, 1 : 200) (Belyaev *et al.*, 1996; Stein *et al.*, 1997; Turner and Fellows, 1989; Turner *et al.*, 1989; White *et al.*, 1999), anti-acetyl histone H3 (Lys9, 1 : 200), anti-dimethyl histone H3 (Lys4, 1 : 200–1 : 500), anti-dimethyl histone H3 (Lys9, 1 : 100) (Upstate). The specificity of the sera against the corresponding acetylated histone isoforms of plants has been shown by Buzek *et al.* (1998) and Vyskot *et al.* (1999) and against the methylated isoforms of plant H3 by Gendrel *et al.* (2002). Goat anti-rabbit FITC (1 : 80, Sigma), or goat anti-rabbit rhodamine (1 : 100, Jackson Immuno Research Laboratories) were applied as secondary antibodies. The immunolabeling procedure was as described (Jasencakova *et al.*, 2000, 2001). After post-fixation in 4% paraformaldehyde/PBS, subsequent washes in PBS, and blocking at 37°C, slides were exposed to primary antisera for 1 h at 37°C, or overnight at 4°C. After washes in PBS (at room temperature), the incubation with secondary antibodies was done at 37°C. Nuclei were counterstained with DAPI (1 μg ml^{-1}, in Vectashield, Vector).

Fluorescent in situ *hybridization (FISH)*

The following probes were used to identify specific chromatin domains within interphase nuclei:rDNA (*A. thaliana* BAC T15P10 (AF167571) containing 45S rDNA repeats) and pAL (180 bp centromere-specific repeat, Martinez-Zapater *et al.*, 1986). Probes were labeled with biotin-16-dUTP using a nick translation kit (Roche Biochemicals) according to manufacturer's instructions. After evaluation and capturing of images obtained from immunostaining, the slides were processed for FISH as described (Jasencakova *et al.*, 2000, 2001). Briefly, after removing the coverslips and washing in TNT (100 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20), the preparations were dehydrated through an ethanol series, baked for 30 min at 60°C, post-fixed in 4% paraformaldehyde/PBS, washed in PBS, dehydrated again and air-dried. When 45S rDNA was used as a probe, RNase treatment (100μg ml^{-1} in 2× SSC, 30 min at 37°C) was performed after baking and prior to post-fixation. The hybridization mixture containing probe, 50% formamide, 10% dextran sulfate and 2× SSC was heated at 80°C for 10 min, cooled on ice, applied on slides and denatured for 1 min at 80°C. After overnight hybridization at 37°C, the slides were washed 3 × 5 min in 50% formamide/2× SSC at 42°C, followed by washing in 2× SSC at room temperature. Detection of FISH signals was according to Fransz *et al.* (1996) using avidin-Texas Red (Vector) 1 : 1000 and goat biotin-conjugated-anti-avidin (Vector) 1 : 200, followed by avidin-Texas Red 1 : 1000. Incubation with antibodies was carried out at 37°C, for 40 min. Nuclei were counterstained with DAPI (see above).

5-methyl-cytosine immunodetection

The detection was done using mouse antiserum raised against 5-methyl-cytosine (Podesta *et al.*, 1993; kindly provided by M. Ruffini Castiglione, 1 : 250) in TNB (100 mM Tris–HCl, pH 7.5; 150 mM NaCl; 0.5% blocking reagent, Roche), followed by rabbit anti-mouse FITC (1 : 1000, Sigma), and goat anti-rabbit Alexa^{488} (1 : 200, Molecular Probes). The nuclei were counterstained with DAPI.

Microscopy, image processing, data evaluation

The slides were inspected using a Zeiss Axiophot 2 epifluorescence microscope equipped with a cooled CCD camera (Photometrics). Images were captured using IPLab Spectrum software, pseudocolored and merged in Adobe Photoshop.

To determine the frequency of immunolabeling patterns, approximately 100 nuclei of each flow-sorted fraction from root and leaf nuclear suspensions were evaluated per experiment.

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References

Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C. and Kouzarides, T. (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature*, **410**, 120–124.

Belyaev, N.D., Houben, A., Baranczewski, P. and Schubert, I. (1998) The acetylation patterns of histones H3 and H4 along *Vicia faba* chromosomes are different. *Chromosome Res.* **6**, 59–63.

Belyaev, N.D., Keohane, A.M. and Turner, B.M. (1996) Differential underacetylation of histones H2A, H3 and H4 on the inactive X chromosome in human female cells. *Hum. Genet.* **97**, 573–578.

Brown, C.E., Lechner, T., Howe, L. and Workman, J.L. (2000) The many HATs of transcriptional co-activators. *Trends Biochem. Sci.* **25**, 15–19.

Buzek, J., Riha, K., Siroky, J., Ebert, I., Greilhuber, J. and Vyskot, B. (1998) Histone H4 underacetylation in plant facultative heterochromatin. *Biol. Chem.* **397**, 1235–1241.

Cheung, W.L., Briggs, S.D. and Allis, C.D. (2000) Acetylation and chromosomal functions. *Curr. Opin. Cell Biol.* **12**, 326–333.

Fransz, P., Alonso-Blanco, C., Liharska, T.B., Peeters, A.J.M., Zabel, P. and de Jong, J.H. (1996) High-resolution physical mapping in *Arabidopsis thaliana* and tomato by fluorescence *in situ* hybridization to extended DNA fibers. *Plant J.* **9**, 421–430.

Gendrel, A.V., Lippman, Z., Yordan, C., Colot, V. and Martienssen R. (2002) Dependence of heterochromatic histone H3 methylation patterns on the *Arabidopsis* gene DDM1. *Science*, **297**, 1871–1873.

Heitz, E. (1928) Das Heterochromatin der Moose. *Jahrb. Wiss. Botanik*, **69**, 762–818.

Jackson, J.P., Lindroth, A.M., Cao, X. and Jacobsen, S.E. (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. *Nature*, **416**, 556–560.

Jasencakova, Z., Meister, A. and Schubert, I. (2001) Chromatin organization and its relation to replication and histone acetylation during the cell cycle in barley. *Chromosoma*, **110**, 83–92.

Jasencakova, Z., Meister, A., Walter, J., Turner, B.M. and Schubert, I. (2000) Histone acetylation of euchromatin and heterochromatin is cell cycle dependent and correlated with replication rather than with transcription. *Plant Cell*, **12**, 2087–2100.

Johnson, L.M., Cao, X. and Jacobsen, S.E. (2002) Interplay between two epigenetic marks: DNA methylation and histone H3 lysine 9 methylation. *Curr. Biol.* **12**, 1360–1367.

Kubaláková, M., Macas, J. and Dolezel, J. (1997) Mapping of repeated DNA sequences in plant chromosomes by PRINS and C-PRINS. *Theor. Appl. Genet.* **94**, 758–763.

Lachner, M. and Jenuwein, T. (2002) The many faces of histone lysine methylation. *Curr. Opin. Cell Biol.* **14**, 286–298.

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K. and Jenuwein, T. (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature*, **410**, 116–120.

Litt, M.D., Simpson, M., Gaszner, M., Allis, C.D. and Felsenfeld, G. (2001) Correlation between histone lysine methylation and developmental changes at the chicken β-globin locus. *Science*, **293**, *2453–2455*.

Martinez-Zapater, J.M., Estelle, M.A. and Sommerville, C.R. (1986) A highly repeated DNA sequence in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **204**, 417–423.

Nakayama, J., Rice, J.C., Strahl, B.D., Allis, C.D. and Grewal, S.I.S. (2001) Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science*, **292**, 110–113.

Noma, K., Allis, C.D. and Grewal, S.I.S. (2001) Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. *Science*, **293**, 1150–1155.

Peters, A.H.F.M., O'Carroll, D., Scherthan, H. *et al.* (2001) Loss of the *Suv39h* histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell*, **107**, 323–337.

Podesta, A., Ruffini Castiglione, M., Avanzi, S. and Montagnoli. G. (1993) Molecular geometry of antigen binding by a monoclonal antibody against 5-methylcytidine. *Int. J. Biochem.* **25**, 929–933.

Richards, E.J. and Elgin, S.C.R. (2002) Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. *Cell*, **108**, 489–500.

Schotta, G., Ebert, A., Krauss, V., Fischer, A., Hoffmann, J., Rea, S., Jenuwein, T., Dorn, R. and Reuter, G. (2002) Central role of *Drosophila* SU (VAR) 3-9 in histone H3-K9 methylation and heterochromatic gene silencing. *EMBO J.* **21**, 1121–1131.

Schubert, I., Dolezel, J., Houben, A., Scherthan, H. and Wanner, G. (1993) Refined examination of plant metaphase chromosome structure at different levels made feasible by new isolation methods. *Chromosoma*, **102**, 96–101.

Sobel, R.E., Cook, R.G., Perry, C.A., Annunziato, A.T. and Allis, C.D. (1995) Conservation of deposition-related acetylation sites in newly synthesized histones H3 and H4. *Proc. Natl. Acad. Sci. USA*, **92**, 1237–1241.

Soppe, W.J.J., Jasencakova, Z., Houben, A., Kakutani, T., Meister, A., Huang, M.S., Jacobsen, S.E., Schubert, I. and Fransz, P.F. (2002) DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in *Arabidopsis*. *EMBO J.* **21**, 6549–6559.

Stein, P., Worrad, D.M., Belyaev, N.D., Turner, B.M. and Schultz, R.M. (1997) Stage-dependent redistribution of acetylated histones in nuclei of the early pre-implantation mouse embryo. *Mol. Reprod. Dev.* **47**, 421–429.

Strahl, B.D. and Allis, C.D. (2000) The language of covalent histone modifications. *Nature*, **413**, 41–45.

Taddei, A., Roche, D., Sibarita, J.-B., Turner, B.M. and Almouzni, G. (1999) Duplication and maintenance of heterochromatin domains. *J. Cell Biol.* **147**, 1153–1166.

Turner, B.M. (2000) Histone acetylation and an epigenetic code. *Bioessays*, **22**, 836–845.

Turner, B.M., Birley, A.J. and Lavender, J. (1992) Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in *Drosophila* polytene nuclei. *Cell*, **69**, 375–384.

Turner, B.M. and Fellows, G. (1989) Specific antibodies reveal ordered and cell-cycle-related use of histone-H4 acetylation sites in mammalian cells. *Eur. J. Biochem.* **179**, 131–139.

Turner, B.M., O'Neill, L.P. and Allan, I.M. (1989) Histone H4 acetylation in human cells: frequency of acetylation at different sites

defined by imunolabelling with site-specific antibodies. *FEBS Lett.* **253**, 141–145.

Vyskot, B., Siroky, J., Hladilova, R., Belyaev, N.D. and Turner, B.M. (1999) Euchromatic domains in plant chromosomes as revealed by H4 histone acetylation and early DNA replication. *Genome*, **42**, 343–350.

White, D.A., Belyaev, N.D. and Turner, B.M. (1999) Preparation of site-specific antibodies to acetylated histones. *Methods*, **19**, 417–421.

Zhang, Y. and Reinberg, D. (2001) Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Gen. Dev.* **15**, 2343–2360.

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass diese Arbeit von mir bisher weder der Mathematisch-Naturwissenschaftlich-Technischen Fakultät der Martin-Luther-Universität Halle-Wittenberg noch einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion eingereicht wurde.
Ich erkläre ferner, dass ich diese Arbeit selbstständig und nur unter Zuhilffenahme der angegeben Hilfsmittel und Literatur angefertigt habe.

Gatersleben, den 25.02. 2003 _____

Z. Jasenčáková