The architecture of interphase chromosomes and gene positioning are altered by changes in DNA methylation and histone acetylation

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Summary

Wheat nuclei have a remarkably well defined interphase organisation, and we have made use of this to determine the relationship between interphase chromosome organisation, the positioning of specific transgenes and induced changes in DNA methylation and histone acetylation, using in situ hybridisation and confocal 3D imaging. After germinating seeds either in the presence of 5-Azacytidine (5-AC), which leads to DNA hypomethylation, or trichostatin A (TSA), which results in histone hyperacetylation, the architecture of the interphase chromosome arms changes significantly even though the overall Rabl configuration is maintained. This suggests that specific chromosome segments are remodelled by these treatments but that there is a strong link of both centromeres and telomeres to the nuclear envelope. In lines carrying multiple transgene integrations at widely separated sites, we show that the multiple transgenes, which are usually colocalised during interphase, are dispersed after 5-AC or TSA treatment and that there is an increase in transgene activity. This suggests that the colocalisation/dispersion of the transgenes may be a function of specific interphase chromosome organisation and that these lines containing multiple transgene copies may all be partially transcriptionally repressed.

Key words: Interphase chromosomes, Transgenes, DNA methylation, Histone acetylation, Chromatin remodelling

Introduction

In most eukaryotes the chromosomes are clearly visible when they are condensed during mitosis, but they are also highly organised in distinct, non-overlapping territories during interphase. In wheat, genomic in situ hybridisation, as well as labelling with specific probes to the centromeres and telomeres, has shown that the interphase chromosome territories are highly organised as parallel linear regions stretching across the nucleus, the two arms of each chromosome lying along side each other, with the centromeres and telomeres located at opposite poles of the nucleus in a Rabl configuration (Abranches et al., 1998).

One current model for higher-order chromosome organisation suggests that active genes are located on loops that may be mobile within the nucleus (Cook, 1995). It has furthermore been suggested that these loops may be pulled through fixed polymerases during transcription and replication, rather than the polymerases moving along fixed DNA strands (Dickinson et al., 1990; Jackson et al., 1993; Iborra et al., 1996; Cook, 1999). The position of certain individual genes has been associated with transcriptional state. For example, the ikaros proteins are located around centromeric heterochromatin in lymphocytes, and silenced genes are recruited to the ikaros sites, whereas active genes are located away from heterochromatin (Brown et al., 1999). In yeast, the Sir-dependent telomeric gene silencing is associated with recruitment of the silenced genes to Sir protein sites at the nuclear periphery (Gasser et al., 1998; Laroche et al., 2000). Francastel et al. showed that transcriptional enhancers may suppress silencing of a transgene by preventing its localisation close to centromeric heterochromatin and recruiting it into an active compartment (Francastel et al., 1999); mutations in the enhancer that led to increased silencing resulted in localisation at the centromeric heterochromatin. Dernburg et al. showed that insertion of heterochromatin at one allele of the brown locus in *Drosophila* caused both the altered gene and the other allele to associate with centromeric heterochromatin (Dernburg et al., 1996). Lundgren et al. used a transgene integrated into pericentromeric heterochromatin to show that transcriptional activation was associated with changes in the heterochromatin structure, but the transgene was not relocated away from the heterochromatin on activation (Lundgren et al., 2000). Tumbar and Belmont used GFP in vivo labelling to show that the VP16 transcriptional activator can alter the nuclear positioning of an engineered reporter sequence (Tumbar and Belmont, 2001).

Changes in chromatin organisation are associated with transcriptional activation, DNA methylation and histone acetylation. Several chromatin remodelling complexes have been identified, in plants as in animals, of which the best known are the SNF2 type (Felsenfeld, 1996; Workman and Kingston, 1998; Travers, 1999), and these are also specifically involved in transcriptional regulation. In plants, DNA

methylation has been correlated with chromatin structure by the identification of the *ddm1* gene from *Arabidopsis thaliana*, which encodes an SNF2-family protein (Jeddeloh et al., 1999). Mutants defective in this gene gradually lose their normal methylation patterns, with repetitive regions of the genome being hypomethylated first. This suggests that chromatin conformational changes are necessary to allow access of maintenance methylases during DNA replication.

DNA methylation and histone acetylation have been clearly linked with the regulation of gene expression, with active genes tending to be under-methylated and associated with nucleosomes whose core histones show increased acetylation (Loidl, 1994; Workman and Kingston, 1998; Wolffe and Matzke, 1999; Wolffe and Guschin, 2000). Current hypotheses suggest that histone acetylation produces a more open chromatin conformation, which allows greater access for the transcriptional machinery. There is evidence going back over many years that increased histone acetylation is correlated with increased transcription (Allfrey et al., 1964; Kouzarides, 2000), and in the past few years several specific histone acetylases and deacetylases have been identified in animals, plants and fungi (Cheung et al., 2000; The Arabidopsis Genome Initiative, 2000). DNA methylation and histone deacetylation have been directly functionally linked. For example, the transcriptional repressor proteins MeCP1 and MeCP2 have methyl-CpG-binding domains and independently or together interact with other structural components of the chromatin to regulate transcription (Meehan et al., 1992; Meehan et al., 1989; Ng and Bird, 1999). MeCP2 appears to act by recruiting histone deacetylases (HDACs) (Nan et al., 1998; Jones et al., 1998), and it can displace H1 from the nucleosome (Nan et al., 1996).

There is as yet little direct evidence to link the molecular-scale changes in DNA accessibility or conformation accompanying transcriptional activation, DNA methylation and histone acetylation to the higher-order organisation seen at the level of interphase chromosomes. We have made use of the very regular organisation seen in wheat interphase chromosomes to analyse how they are modified by global changes in methylation or histone acetylation. We show that after germinating seeds either in the presence of 5-Azacytidine (5-AC), leading to DNA hypomethylation, or trichostatin A (TSA), which results in histone hyperacetylation, the architecture of the interphase chromosome territories shows significant changes. We have also used transgenic wheat lines to investigate changes in chromosome architecture at the level of individual genes. In lines carrying multiple transgene integrations at widely spaced sites, we show that the transgenes are usually colocalised during interphase, but that after 5-AC or TSA treatment the transgene activity increases, and the multiple transgene sites are dispersed. This suggests that the colocalisation/dispersion of the transgenes may be a function of specific interphase chromosome architecture.

Materials and Methods

Plant material
The following wheat (*Triticum aestivum*) genotypes were used: AABBDD, $2n=6\times=42$, cv. Chinese Spring with the addition of rye (*Secale cereale* L. cv. Imperial) chromosome pairs, 5R or 1R, and translocation lines where the long arm of wheat chromosome 1A or 1D is replaced by the short arm of rye chromosome 1R ($1A^l/1R^s$) and

$(1D^l/1R^s)$, respectively. Wheat lines (cv. Bobwhite) were transformed with the plasmid pAHC25, containing the Gus reporter gene, by particle bombardment using the Biolistics PDS 1000/He device (Abranches et al., 2000). Seeds were germinated for 4 days at 24°C on filter paper soaked in water alone or water containing 80 µM 5-azacytidine (5-AC, Sigma) or water containing 15 µM Trichostatin A (TSA – Sigma) (diluted just prior to use from a 10 mM stock solution in dimethyl sulfoxide). The 5-AC was freshly dissolved in water and changed daily. The root-tips were excised and fixed in 4% (w/v) formaldehyde freshly prepared from paraformaldehyde in PEM buffer (50 mM PIPES/KOH pH 6.9; 5 mM EGTA; 5 mM $MgSO_4$) for 1 hour at room temperature, then washed in TBS (10 mM Tris-HCl, pH 7.4; 140 mM NaCl) for 10 minutes.

Protein extraction and immunoblotting analysis
Total root proteins were extracted by homogenising roots in SDS sample buffer (Laemmli, 1970) [Sample buffer: 0.125 M TRIS/HCl pH 6.8, 4% (w/v) SDS, 20% glycerol, 10% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue]. Protein samples were resolved by SDS gel electrophoresis on 15% gels and transferred to nitrocellulose by western blotting (Towbin et al, 1979). The blots were probed with antibody AHP418 (Serotec), which is specific for acetylated histone H4, or antibody AHP416 (Serotec), which is specific for Histone H4 acetylated at lysine 12, diluted in TBS according to the manufacturer's instructions. Proteins were visualised using a secondary antibody goat anti-rabbit alkaline phosphatase, diluted 1 in 1000 in TBS.

Root sections
30 µm thick sections from root tips were sectioned using a Vibratome Series 1000 (TAAB Laboratories Equipment Ltd., Aldermarston, UK) and allowed to dry on multi-well slides (ICN Biomedicals Inc.). The slides were pre-treated by washing in 3% (v/v) Decon for 1 hour, rinsing thoroughly with distilled water. They were then coated with a freshly prepared solution of 2% (v/v) 3-aminopropyl triethoxy silane (APTES, Sigma) in acetone for 10 seconds and activated with 2.5% (v/v) glutaraldehyde in phosphate buffer for 30 minutes, rinsed in distilled water and air dried.

In situ hybridisation on wheat root sections
The tissue sections were permeabilised by incubation with 2% (w/v) cellulase (Onozuka R-10) in TBS for 1 hour at room temperature, washed in TBS for 10 minutes, dehydrated in an ethanol series of 70% and 100% and air dried. Root sections from wheat transgenic lines were additionally treated with RNAse (100 µg/ml) for 1 hour at 37°C, washed in 2×SSC (20×SSC: 3 M sodium chloride, 300 mM trisodium citrate, pH 7.0) and dehydrated as described above. Genomic in situ hybridisation and generation of total genomic probe was performed according to Schwarzacher et al. (Schwarzacher et al., 1992) and Abranches et al. (Abranches et al., 1998). The hybridisation mixture contained 50% deionised formamide, 20% dextran sulphate, 0.1% sodium dodecyl sulphate, 10% 20×SSC, 200 ng of rye genomic DNA sonicated to 10-12 kb fragments as a probe and 1 µg of sonicated salmon sperm as blocking DNA. Fluorescence in situ hybridisation was used to visualise the transgenes on wheat root sections, using pHAC25 DNA (200 ng) as a probe. Probes were labelled with digoxigenin-11-dUTP (Boehringer Mannheim Corp. Indianapolis, IN) or biotin-16-dUTP (Boehringer Mannheim) by nick translation. Denaturation of the hybridisation mixture was carried out at 95°C for 5 minutes, cooled in ice for another 5 minutes and immediately applied to the sections. Target DNA denaturation was carried out in a modified thermocycler (Omnislide; Hybaid LTD., Long Island, NY) at 78°C for subsequent hybridisation at 37°C overnight. Post-hybridisation washes were carried out using 20% formamide in 0.1SSC at 42°C.

BrUTP incorporation into tissue sections

For transcription analysis, the procedures followed are those described previously (Thompson et al., 1997; Abranches et al., 1998). Briefly, vibratome sections were cut in a Modified Physiological Buffer (MPB: 100 mM potassium acetate, 20 mM KCl, 20 mM Hepes; 1 mM MgCl$_2$; 1 mM ATP (disodium salt, Sigma) in 50 mM Tris, pH 8.1% (v/v); 1% (v/v) thiodiglycol (Sigma), 2 µg/ml aprotinin (Sigma) and 0.5 mM PMSF (Sigma). To improve nuclear transcription as opposed to nucleolar transcription, 1% BSA was added to the MPB buffer. The tissue sections were transferred to a tissue-handling device (Wells, 1985) for subsequent ease of handling. The permeabilisation was done by a very brief treatment (10 seconds) with 0.05% Tween 20 in MPB. The transcription mix consisted of 50 µM CTP (sodium salt, Pharmacia), 50 µM GTP (sodium salt, Pharmacia), 25 µM BrUTP (sodium salt, Sigma), 125 µM MgCl2, pH 7.4 with KOH); 100 U/ml RNA guard (Pharmacia) made up in MPB. The tissue was incubated with the transcription mix for 5 minutes and then fixed in 4% formaldehyde in PEM as described above. After fixation, the sections were washed in TBS, then in water and finally removed from the tissue-handling device and placed onto activated APTES-treated slides.

Immunodetection

Probes labelled with digoxigenin were detected by an anti-digoxigenin antibody conjugated to FITC (Boehringer Mannheim Corp., Indianapolis, IN), and biotin-labelled probes were detected with extravidin-cy3 (Sigma, Chemical Co.). Both antibodies were diluted in 3% BSA in 4×SSC/ 0.2% tween-20 (Sigma), and the antibody incubations were carried out in a humid chamber for 1 hour at 37°C followed by 3×5 minutes washes in 4×SSC/0.2% Tween-20 at room temperature. The detection of BrUTP incorporation involved incubation for 1 hour at room temperature with mouse anti-BrdU (Boehringer) followed by a second incubation with a secondary fluorescent anti-mouse Alexa-568 (Molecular Probes) antibody for 1 hour at room temperature. The sections were counterstained with 1 µg/ml, 4′6-diamidino-2-phenylindole (DAPI – Sigma Chemical Co) for 5 minutes and mounted in Vectashield antifade solution (Vector Laboratories Inc. Burlingame, CA).

β-Glucuronidase (Gus) assay

Gus activity was determined by testing root material by a quantitative assay as described previously (Jefferson et al., 1987), using 4-methyl umbelliferyl glucuronide (MUG) as a substrate.

Confocal fluorescence microscopy and imaging processing

Confocal optical section stacks were collected using a Leica TCS SP confocal microscope (Leica Microsystems, Heidelberg GMBh, Germany) equipped with a Krypton and an Argon laser. The microscopy data were then transferred to NIH image (a public domain program for the Macintosh by W. Rasband available via ftp from ftp://zippy. nimh.nih.gov) and composited using Adobe Photoshop 5.0 (Adobe Systems Inc., Mountain View, CA). 3D models were made from stacks of confocal sections using Object-Image [an extension to NIH image written by Vischer et al. (Vischer et al., 1994)] by drawing manually the limit of the nucleus and marking the localisation of the transgene fluorescence sites as dots. The 3D reconstruction models were visualised using Rotater (by Craig Kloeden) available from ftp://Rarn.adelaide.edu.au/rotater/rotater-3.5.cpt.hqx). Final images were printed on a Pictography P3000 printer.

Results

The arms of each chromosome are usually colocalised in interphase

Wheat lines carrying either the addition of a pair of entire rye chromosomes (1R or 5R) or the translocation of the short arm of the rye 1R chromosome into wheat (1R/1B or 1R/1A translocations) were used for genomic in situ hybridisation. Entire rye chromosomes (Fig. 1) as well as individual chromosome arms (Fig. 2) were clearly visualised during interphase. In the lines shown, a large sub-telomeric heterochromatin block, which identifies the telomeric region, can often be seen as arrowed in Fig. 1A,B,D and Fig. 2A. In control seedlings the chromosomes showed a clear and regular Rabl configuration, with the centromeres lying at one side of the nuclear envelope and the telomeres at the other side. The arms of each chromosome were very close to each other and could only occasionally be distinguished, as arrowed in Fig 1A. The individual chromosome arms had a very smooth and regular appearance (Fig. 2A-C). The rye arm translocation shown (1R) carries a nucleolar organizer region (NOR) – a series of tandem repeats of rDNA – and a gap in the chromosome arm territory is seen corresponding to this (Fig 2A), indicating that the chromatin is decondensed and dispersed into the nucleolus, which is located adjacent to this chromosomal region. This was confirmed by double-labelling with both rye genomic probe and a probe to the rDNA (data not shown).

Induced changes in DNA methylation or histone acetylation cause remodelling of interphase chromosome territory organisation

To determine whether changes in the DNA methylation or histone acetylation state caused observable large-scale changes in interphase chromosome organisation, we germinated the seedlings in the presence of either 5-azacytidine (5-AC), which reduces DNA methylation (Neves et al., 1995; Castilho et al., 1999), or Trichostatin A (TSA), which inhibits histone deacetylases, thus increasing histone acetylation levels (Yoshida et al., 1990). In pilot experiments we germinated seedlings on a range of concentrations of the two drugs, and chose for further analysis the highest concentration in each case, which allowed near normal growth and development (80 µM 5-AC, or 15 µM TSA). In both cases, higher concentrations substantially reduced growth, and still higher concentrations were lethal. The concentrations used for further study gave growth rates of at least 90% of that shown by the control seedlings germinated in water. Castilho et al. have shown directly by analysis with methylation-sensitive restriction enzymes that germination of wheat seedlings in the presence of 5-AC does indeed decrease the level of DNA methylation (Castilho et al., 1999). For the TSA treatment, western blotting using antibodies specific for acetylated histone H4 showed that germination of seedlings in the presence of TSA caused biochemically detectable increases in histone acetylation (Fig. 3). Since in animal cells TSA has been reported to cause cell cycle arrest at G1 and G2 (Yoshida and Beppu, 1988), we used flow cytometric analysis to measure the relative DNA content of wheat nuclei extracted from roots germinated in water or TSA. No significant changes were seen in the relative numbers of G1, S and G2 nuclei, showing that there was little effect on the cell cycle using this TSA concentration (data not shown). After treatment with either of these reagents, there were striking changes in the interphase chromosome organisation. The two chromosome arms, which in the controls usually lay
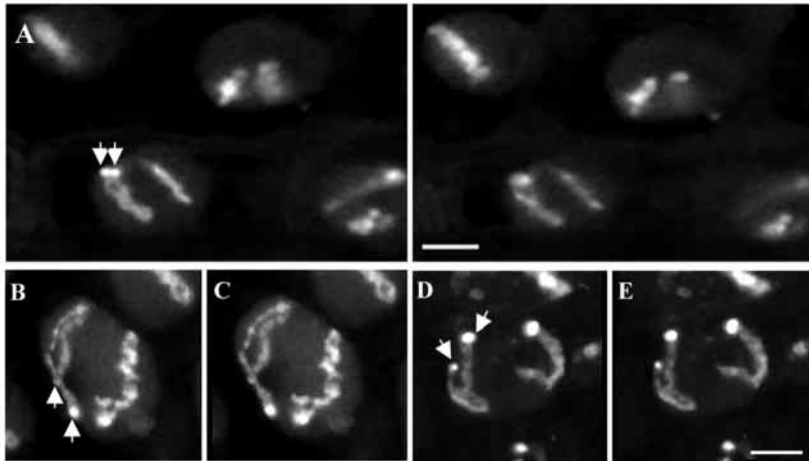
Fig. 1. Genomic in situ hybridisation using total genomic rye DNA as a probe in wheat root sections followed by confocal microscopy imaged entire chromosomes during interphase. Projections of consecutive confocal sections of entire rye chromosome pairs are shown. (A) Two consecutive confocal sections of control seedlings germinated on water. The interphase chromosomes appear as elongated domains with the two arms very close together and only rarely distinguishable (arrows). Bar, 10 µm. (B,C) Stereo pairs of nuclei after germination in the presence of 5-AC. The two chromosome arms can be seen separated and the chromosome structure appears very irregular with a more complex and broken configuration. (D,E) Stereo pairs of nuclei after germination in the presence of TSA. Similar changes to those produced by 5-AC are seen. However, the overall Rabl configuration is maintained, with the centromeres and subtelomeric heterochromatic blocks (arrows in B,D) remaining at the nuclear periphery. Bar, 10 µm (B-E).

so close together as to be indistinguishable (Fig. 1A, Table 1), became widely separated (Fig. 1B-E, Table 1). Sometimes, but not always, the two telomeres remained close to each other, whereas the rest of the chromosome arms lay apart. The overall Rabl configuration was, however, maintained, with telomeres and centromeres remaining on opposite sides of the nuclear envelope.

The results from a quantitative analysis of many nuclei from different seedling roots are shown in Table 1. The frequency of colocalisation of the arms is very high in untreated seedlings – 89% and 74% in 1R and 5R chromosomes, respectively. We also identified some cases in which the two interphase chromosome arms were of different lengths such as the chromosome 5R, which in metaphase has a sub-metacentric morphology. The complete separation of the two arms was very rare in the control seedlings (4% and 2% in 1R and 5R chromosomes, respectively). On the other hand, in seedlings treated with 5-AC and TSA, the interphase chromosome arms in each chromosome were often found separated from each other (44% and 48% in 1R and 5R chromosomes, respectively, in seedlings germinated in 5-AC; 22% and 8% in 1R and 5R, respectively when the seedlings were germinated in TSA).

To analyse the effects on individual chromosome arms in more detail we used single arm translocation lines. With both 5-AC (Fig. 2D,E) and TSA (Fig. 2F,G) the labelling pattern of the chromosome arms became irregular and showed gaps where the DNA was decondensed. The effect of 5-AC was more dramatic, and the arms were dispersed into several smaller labelled regions separated by gaps, corresponding to chromosome regions where the chromatin was decondensed. The arms showed a more extended, meandering path across the

nucleus. With TSA, the arms typically showed about five regions with intervening gaps of decondensed chromatin. However, the chromosome arms appeared to remain smoother in appearance, straighter and generally closer to their normal appearance than with 5-AC.

Induced changes in DNA methylation or histone acetylation alter the relative positions of individual genes during interphase

In order to analyse the positions of specific genes we used transgenic wheat lines made by particle bombardment with a plasmid containing the GUS reporter gene. Fluorescence in situ hybridisation on intact root tissue slices was used to visualise the transgene arrangement in interphase nuclei. Three lines were analysed in detail [line numbers refer to the original characterisation, given in (Abranches et al., 2000)]. Line 6 is homozygous, and carries five transgene copies on each homologue of chromosome 4A. In situ labelling on metaphase chromosomes shows two integration sites on opposite arms of the chromosome, one in a subtelomeric position on the short arm and the other about one-third of the arm length from the telomere of the long arm of the chromosome (Abranches et al., 2000). Line 2, which is not homozygous, contains more than 10 copies of the transgene at four distinct sites along the short arm of chromosome 6B, spanning 30% of the length of the short arm of the chromosome (Abranches et al., 2000). All the line 2 plants analysed in this paper were heterozygous, since homozygous plants have a strong tendency to silence. Finally, line 3 is homozygous and carries two transgenes per homologue at a single site at metaphase on the long arm of chromosome 6B (Abranches et al., 2000).

Despite being well separated along the metaphase chromosomes, in these lines the multiple transgene sites are brought into close physical proximity during interphase (Abranches et al., 2000). To quantify the transgene arrangements in detail, confocal section stacks were modelled using Object-Image (Vischer et al., 1994), by tracing the outline of the nuclei on each section and marking every distinguishable transgene site with a red dot, as shown in Fig. 4.

Fig. 4A shows a single confocal section of a typical group of cells from a control seedling of line 6, and Fig. 4D shows the corresponding 3D models. Only two FISH signals per nucleus are seen in these nuclei, that is, a single site per homologue, showing that the sites on opposite chromosome arms are located closer together than the optical resolution limit. When seedlings were germinated in either 5-AC or TSA, we observed a greater number of transgene sites, more closely correlating with the number of sites seen on the metaphase chromosomes. Thus line 6 germinated in 5-AC (Fig. 4B,E) showed mostly four sites per nucleus (a,b,c): the G2 nucleus (d) shows eight sites. Fig. 4C,F shows the equivalent results with TSA – again all the four nuclei show four sites. Fig 4G shows a group of nuclei from a control seedling of line 2, and in Fig. 4J the corresponding 3D models are shown. A single FISH signal is shown in nuclei a,c,d and
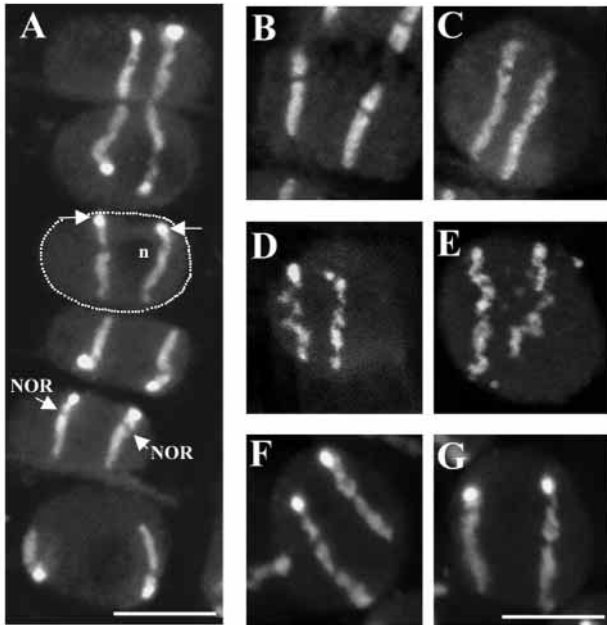
Fig. 2. Genomic in situ hybridisation in interphase nuclei from wheat root sections labelled an individual chromosome arm in the 1R wheat/rye translocation line. Projections of consecutive confocal sections are shown. The rye telomeric knobs (arrows in 2A) are clearly seen as intensely labelled regions at one end of each arm, located at the nuclear periphery (line in 2A). (A) A group of interphase nuclei from control seedlings germinated on water. A gap is usually seen at the position of the decondensed nucleolar organisers (NOR), where the rDNA genes are decondensed into the nucleolus (n). Bar, 10 μm. (B,C) Two nuclei from control seedlings. (D,E) Two nuclei after treatment with 5-AC. The chromosome arms are much more irregular, and regions of strong labelling are interrupted by several gaps of decondensed chromatin. (F,G) Two nuclei after treatment with TSA. Again the chromosome arms are interrupted by four to five gaps, but the overall configuration appears more regular than after 5-AC. Bar, 10 μm, in B-G.

two signals in nucleus b. Fig. 4H,K shows nuclei from line 2 germinated in 5AC. Four FISH signals are shown in nucleus a and three or two FISH signals are shown in nuclei b and c, respectively. Fig. 4I,L show nuclei from line 2 germinated in TSA. Four FISH signals are shown in nuclei a,d, three in nucleus c and two in nucleus b. To obtain reliable quantitative data, we modelled several hundred nuclei from the three lines using the three germination conditions. Only clearly labelled, intact nuclei were included in the analysis, and large nuclei clearly in the G2 phase of the cell cycle were excluded. The number of sites seen in the various lines is shown in Table 2. This confirms quantitatively the results shown in Fig 4. In order to be able to compare the results for the homozygous plants with those from the heterozygous line 2 plants, which carry the transgenes on only one homologue, we have presented the results in Table 2 as counts per (interphase) chromosome. In all the nuclei from the homozygous lines included in the analysis, the transgene sites were arranged in two well separated groups corresponding to the two homologous chromosomes. In line 6, the majority of interphase chromosomes (84%) showed a single site in the control seedlings, whereas only 16% showed two or more sites. Germinated on either 5-AC or TSA, only about 20% showed a single site, and the remainder showed two or more sites. The mean number of sites per homologue increased from 1.2 in the controls to 1.8 or 1.9 after 5-AC or TSA, respectively. Line 3, which show only a single transgene site at metaphase, showed a single site per homologue whether germinated on water alone or in the presence of 5-AC or TSA.

In heterozygous plants from line 2, 95% of interphase chromosomes showed one or two sites, with a mean of 1.5. This increased to means of 2.0 or 3.0 germinated on 5-AC or TSA respectively.

Induced changes in DNA methylation or histone acetylation cause an increase in transgene activity

Histochemical staining and a fluorometric assay were used to determine GUS activity in roots in control seedlings
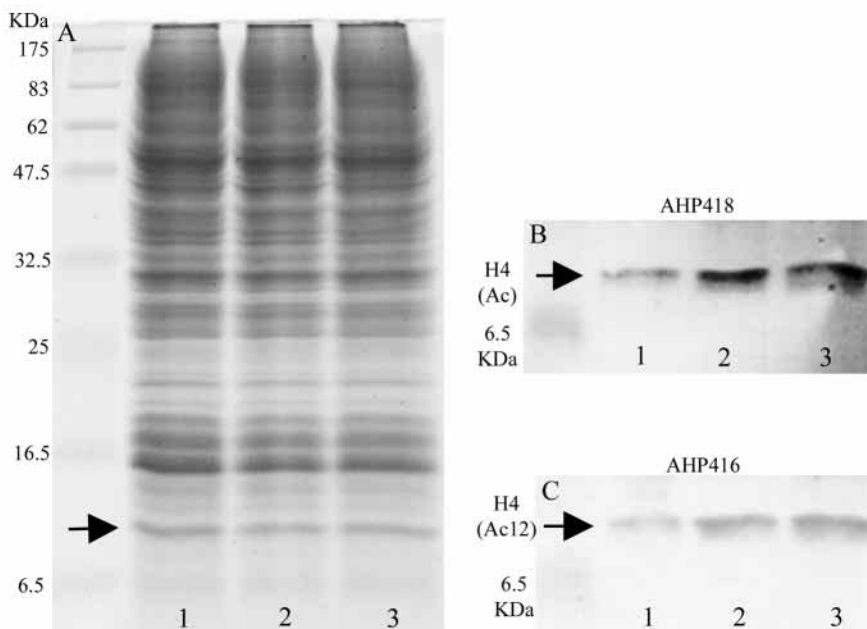

Fig. 3. Effect of TSA on histone acetylation. Total extracted proteins from roots germinated in water (lane1), in 15 μM TSA changed daily (lane 2) or in 15 μM TSA without changes (lane 3) were electrophoresed on a 15% SDS gel. (A) Total proteins stained with Coomassie, showing equivalent loading in the three lanes. (B) The equivalent gel to A was western blotted with antibody AHP 418, specific to acetylated histone H4. (C) Equivalent gel to A, western blotted with antibody AHP 416, specific to histone H4 acetylated at lysine 12. There is an increase in the labelling of the histone H4 band at ~10 kDa in lanes 2 and 3 with both antibodies (arrows in B,C), showing increases in the amount of acetylated histone H4, but no difference in intensity between daily changes (lane 2) and a single application of TSA (lane 3).

germinated in water and in seedlings germinated in the presence of either 5-AC or TSA. Table 3 shows the results from the quantitative fluorometric assay on the three transformed lines and the untransformed control. A striking result was that 5-AC treatment substantially increased activity

in all the transformed plants. TSA increased the activity in line 6 and line 2 but not to the same extent as 5-AC. A conclusion of these measurements is that these lines, which all contain multiple inserts, may all be partially transcriptionally suppressed.
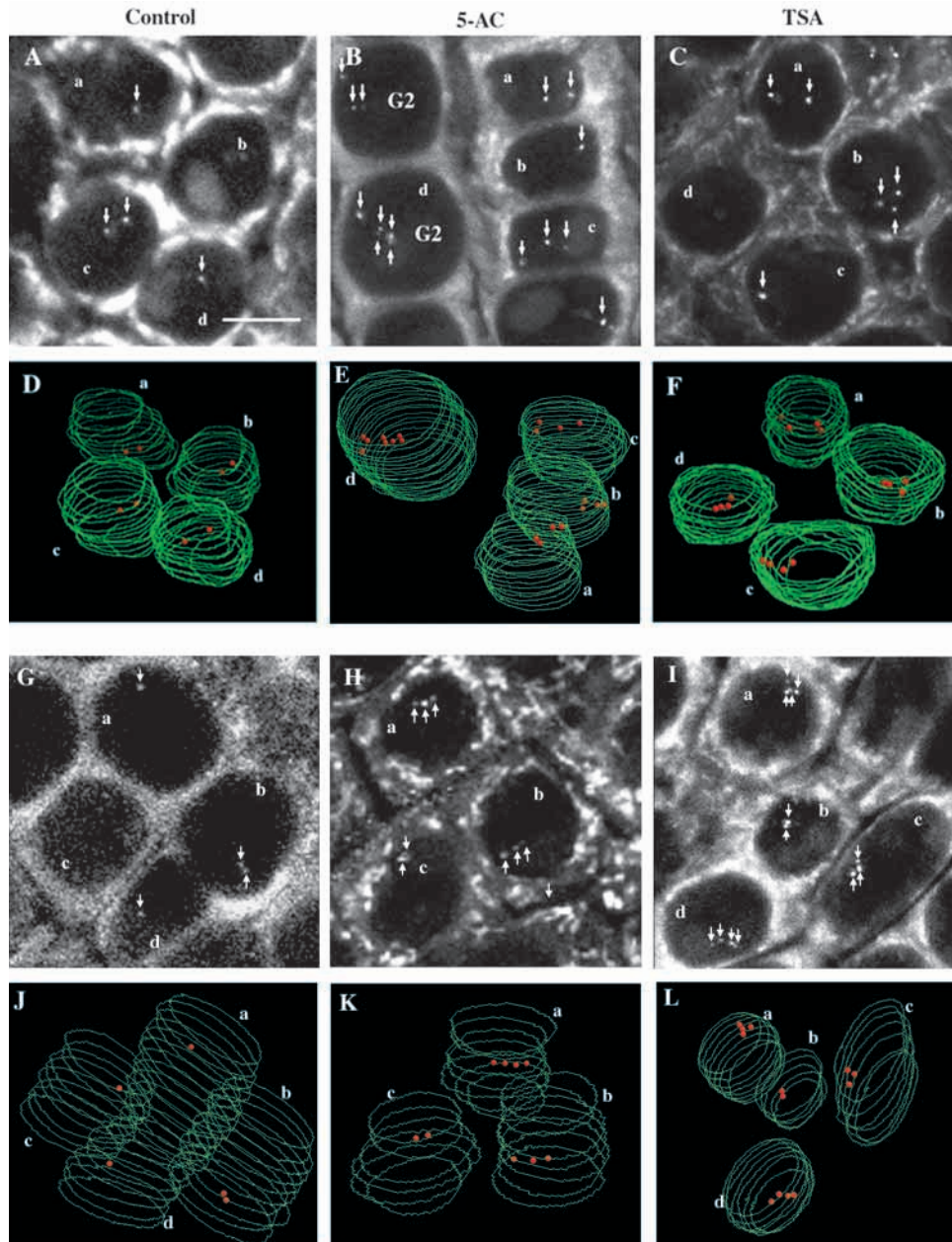

Fig. 4. Transgene sites visualised in root interphase nuclei of transgenic wheat lines after germination in water (control) or in the presence of 5-AC or TSA. Two lines are illustrated: line 6 (A-F), which carries five transgene copies at two sites on metaphase chromosomes; and line 2 (G-L), which carries more than 10 transgene copies at four sites on metaphase chromosomes (Abranches et al., 2000). The number of sites was determined by computer modelling of the entire 3D stacks as described by Abranches et al. (Abranches et al., 2000). Note that in the single confocal sections, only some of the sites present in the full 3D data from which the models were made are visible. The sites clearly visible on the single optical sections shown are indicated by arrows. (A) Single confocal section from line 6: control seedling germinated on water. (B) Single confocal section from line 6 after 5-AC. (C) Single confocal section after TSA. (D) Model from complete 3D data stack shown in A. The four nuclei each show two sites – one per homologue. (E) Model from the 3D stack shown in B. Three G1 nuclei (a,b,c) each show four sites, the G2 nucleus (d) shows eight sites. (F) Model from 3D stack shown in C. Each of the four nuclei show four sites. (G) Single confocal section from line 2 control seedlings germinated on water. (H) Single confocal section from line 2 after 5-AC. (I) Single confocal section from line 2 after TSA. (J) Model from the 3D stack shown in G. Three nuclei (a,c,d) show one site each and nucleus b shows two sites. (K) Model from the 3D stack shown in H. Four sites are shown in nucleus a and two or three sites are shown in nuclei b and c, respectively. (L) Model from the 3D stack shown in I. Two nuclei (a,d) show four sites each and the other two (b,c) show two or three sites each, respectively. Bar.10 µm (A-C,G-I).

Table 1. Disposition patterns of interphase chromosome arms in wheat/rye addition lines

Wheat line	Germination medium	Chromosomes analysed (N)			
CS+1R1R	Control	124	89%	7%	4%
	5-AC	18	39%	17%	44%
	TSA	68	49%	29%	22%
CS+5R5R	Control	46	74%	24%	2%
	5-AC	31	42%	10%	48%
	TSA	50	56%	36%	8%

Table 2. Number of transgene sites per homologue in interphase nuclei

	Line 6			Line 3			Line 2		
Number of transgene sites per metaphase chromosome									
	Control	5-AC	TSA	Control	5-AC	TSA	Control	5-AC	TSA
Number of transgene sites per interphase chromosome									
1	84%	22%	18%	100%	100%	100%	52%	24%	0%
2	15%	78%	74%	0%	0%	0%	43%	57%	26%
3	1%	0%	6%	0%	0%	0%	5%	14%	49%
4	0%	0%	2%	0%	0%	0%	0%	5%	25%
5	0%	0%	0%	0%	0%	0%	0%	0%	0%
Mean	1.17	1.78	1.91	1.0	1.0	1.0	1.5	2.0	2.99
Number of interphase chromosomes analysed									
	87	94	66	132	60	202	130	42	61

**Table 3. Fluorometric assay to measure GUS expression in roots from wheat transgenic lines***

		GUS expression in roots		
Line	Transgene (GUS) copy number‡	Control	5-AC	TSA
Line 6	5	245±51	1668±459	773±39
Line 3	2	678±48	1548±346	696±168
Line 2	>10	968±157	2146±190	1303±43
Untransformed	0	36±4	35±2	27±5

*pmole/min/mg soluble protein.
‡As determined in Abranches et al., 2000.
The measurements were done in three-day-old seedlings germinated in water (control), 5-AC (80 μM) or TSA (15 μM) solutions. For each measurement, three replicate experiments were carried out and each measurement used the roots pooled from 10 seedlings.
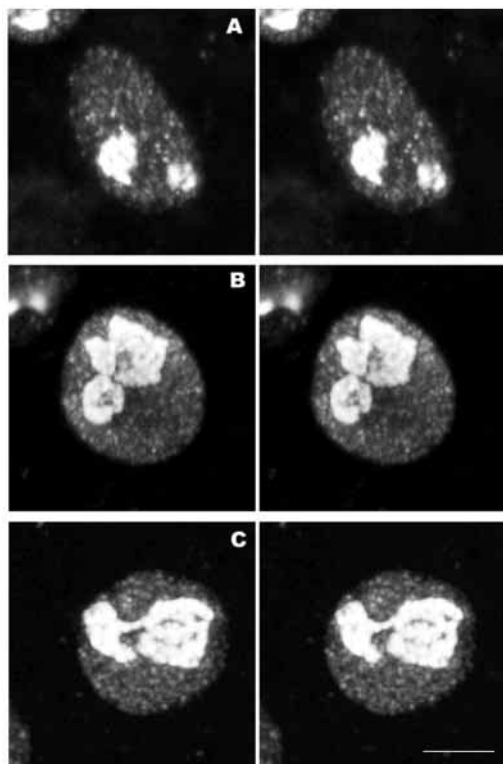
Induced changes in DNA methylation or histone acetylation do not cause obvious changes in the distribution of transcription sites in the nucleus

BrUTP incorporation into unfixed wheat root sections was used to determine the organisation of transcription sites (Fig. 5). As described previously, this labelling shows many closely spaced punctate sites in the nucleolus (Thompson et al., 1997), and more dispersed foci in the nucleoplasm (Abranches et al., 1998). This distribution was confirmed in control seedling roots germinated in water (Fig. 5A). In spite of the large-scale reorganisation of the chromosome territories caused by 5-AC or TSA treatment, there was no obvious difference in the overall organisation of transcription sites visualised by BrUTP

Fig. 5. Visualisation of transcription sites in interphase nuclei by BrUTP incorporation into unfixed wheat root seedling sections. Stereo pairs of 3D confocal stacks are shown. (A) Control seedling germinated in water. (B) Seedling germinated in 5-AC. (C) Seedlings germinated in TSA. The BrUTP labelling comprises many small punctate foci dispersed throughout the nucleus (Abranches et al., 1998), and there is no obvious difference in the overall organisation of the nuclear transcription sites after either treatment. The labelling of transcription is particularly strong in the nucleolus and comprises intranucleolar foci that are closely associated, much like closely packed beads on a string, and organised in a network of strands throughout the nucleolus. Bar, 10 μm.

after either treatment. A similar punctate labelling was seen in the nucleoplasm (Fig. 5B,C).

Discussion

We have shown previously that wheat chromosomes have a very consistent and well ordered arrangement during interphase, occupying elongated domains and with the arms of each chromosome closely associated with each other (Abranches et al., 1998). In this paper, we show that germination of wheat in the presence of 5-AC, which produces decreased DNA cytosine methylation, or TSA, which inhibits histone deacetylases and thus produces increased histone acetylation, causes large-scale reorganisation of the interphase chromosome territories. After these treatments, the chromosome arms were no longer smooth and continuous, but appeared as smaller blocks separated by gaps. This must be because of decondensation of chromatin in the gap regions, whereas the intervening chromosomal regions remain more condensed. It is possible that the decondensed chromatin regions are gene-rich DNA loops, whereas the condensed regions contain mostly repetitive sequences. This is certainly the case for the NOR region of the chromosomes, where the rDNA repeats are decondensed into the nucleolus, and a gap is generally seen in the interphase chromosome. Although there is not yet a fine-enough physical map of the rye genome to interpret these structures in detail, it is known that in cereals the chromosomes contain gene-rich islands interspersed among the vast excess of repetitive sequences (Panstruga et al., 1998). Alternatively, the DNA decondensed in the gaps may represent specific heterochromatin regions whose organisation is particularly amenable to remodelling. In either case, our results suggest that a global reduction in methylation or increase in histone acetylation produces a dispersed conformation of a limited number of DNA segments and disrupts the overall rod-like conformation of the interphase chromosome arms. Thus the chromosome arms take a meandering path across the nucleus, and the two arms of a single chromosome generally do not remain alongside one another as they do in control plants. However the overall Rabl configuration is retained, with the centromeres at one side of the nuclear envelope and the telomeres at the other side of the envelope. Others have shown that the levels of DNA methylation and histone acetylation do influence the gross architecture of human chromosome territories. For example, TSA caused an increase in the nuclear volume occupied by the gene-rich chromosome 19 (Croft et al., 1999) and 5-AC caused decondensation of constitutive heterochromatin segments and G band chromatin of human chromosomes (Kokalj-Vokac et al., 1993).

We have further characterised the changes in nuclear organisation by using transgenic wheat lines carrying multiple, widely separated transgene insertions. During interphase in these lines, the multiple transgenes show a strong tendency to colocalise during interphase (Abranches et al., 2000). Line 6 has a total of approximately five transgene copies at two sites on opposite chromosome arms, and line 2 has about 10 copies located at four sites extending along approximately one-third of the metaphase chromosome arm, and thus separated by several megabases of genomic sequence. In each case, a simple model in which the metaphase chromosome is folded in half at the centromere and extended across the nucleus in interphase

would place these sites 4-6 μm apart (Abranches et al., 2000). Thus specific aspects of the interphase chromosome structure or direct, ectopic interactions between the sequences or proteins bound to them must cause these sites to colocalise.

The GUS activity of all the transgenic lines we analysed was increased by 5-AC, and in most cases was also increased by TSA, suggesting that these lines, which all show colocalisation of more than one transgene copy, are all at least partially suppressed. It will be interesting to determine whether this is due to complete silencing of a subset of the transgenes in each nucleus or a partial suppression of all of them.

After germination in either 5-AC or TSA, the transgenes were more dispersed in the interphase nuclei and were generally seen as clusters of separate sites rather than single sites. As a control, we used a line that shows a single site at metaphase, and this line showed a single site per homologue in interphase as predicted, after germination on water or in the presence of either 5-AC or TSA. If the multiple sites are colocalised in these transgenic plants because of specific chromosome architecture, then the dispersal of the sites can be explained by the disruption of chromosome territory structure that we have shown occurs after these treatments. If this is the case, it implies that aspects of the interphase chromosome architecture are very well defined, and are maintained or reproduced through many cycles of cell division. On the other hand, if the colocalisation is a consequence of ectopic pairing, or interaction with specific nuclear structures, then such interactions must themselves be modified by the changes in DNA methylation or histone acetylation. Further work analysing the behaviour of the genomic sequences flanking the transgenes will be needed to distinguish between these possibilities.

Although we have shown extensive rearrangement of interphase chromosome territories, and a dispersal of individual transgene sites after TSA or 5-AC treatment, we saw little change in the overall organisation of the nuclear transcription sites revealed by BrUTP incorporation. This suggests that there is some other organisational principle underlying the location of the transcription sites and is consistent with the presence of specialised nuclear locations to which active genes are recruited.

There is now a great deal of evidence that genes can be sequestered to transcriptionally inactive regions of the nucleus as part of their regulation (Cockell and Gasser, 1999). Telomeric silencing has been widely studied in yeast (e.g. Gasser et al., 1998), and there are now several examples of sequestering inactive genes to centromeric heterochromatic regions in mammalian cells (Brown et al., 1999; Dernburg et al., 1996; Francastel et al., 1999; Lundgren et al., 2000). Although our results indicate that specific genes can be repositioned by large distances in molecular terms (~4-6 μm), the regular, polarised organisation of the interphase chromosomes in wheat means that a large proportion of all the chromosome arms in these nuclei must be further away than this from either the centromeres or the telomeres, which are invariably located at the nuclear periphery. It is possible that a level of transcriptional control equivalent to that already observed at telomeric or centromeric heterochromatin in mammalian and yeast nuclei may be achieved by sequestering genes to the heterochromatic regions interspersed along these large plant chromosomes.

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