Nucleosome positioning at the replication fork

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In order to determine the time required for nucleosomes assembled on the daughter strands of replication forks to assume favoured positions with respect to DNA sequence, psoralen cross-linked replication intermediates purified from preparative two-dimensional agarose gels were analysed by exonuclease digestion or primer extension. Analysis of sites of psoralen intercalation revealed that nucleosomes in the yeast *Saccharomyces cerevisiae* rDNA intergenic spacer are positioned shortly after passage of the replication machinery. Therefore, both the 'old' randomly segregated nucleosomes as well as the 'new' assembled histone octamers rapidly position themselves (within seconds) on the newly replicated DNA strands, suggesting that the positioning of nucleosomes is an initial step in the chromatin maturation process.

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Introduction

Replication of eukaryotic chromosomes requires both the duplication of the parental DNA and its assembly into chromatin. Nascent DNA strands are reorganized into chromatin in a very rapid process (Gasser *et al.*, 1996) characterized by the presence of nucleosomes in the vicinity of the elongation point (Cusick *et al.*, 1984; Sogo *et al.*, 1986). In order to provide the full complement of histone octamers on the nascent DNA strands, transfer of 'old' parental histones to the daughter strands must occur in coordination with the assembly of newly synthesized histones. In the first step of this process, the advance of the replication machinery destabilizes the nucleosomal organization of the chromatin fibre (Gasser *et al.*, 1996) and the parental histone tetramers (H3/H4)₂ are directly assembled on the newly synthesized daughter strands (Gruss *et al.*, 1993) in a random fashion (for reviews see Krude, 1995; Sogo and Laskey, 1995). Re-association of either old or new H2A/H2B dimers to parental (H3/H4)₂ tetramers (Jackson, 1990) completes the assembly of the so-called segregated parental nucleosomes. Newly synthesized histones H3 and H4 form *de novo* tetramers and associate with the nascent DNA strands (Jackson, 1990; Svaren and Chalkley, 1990). Chromatin assembly factor 1 (CAF-1)-

mediated assembly of newly synthesized histones H3 and H4 into nucleosomes has been well documented by Stillman and co-workers (Smith and Stillman, 1989, 1991; Kaufman *et al.*, 1995; Verreault *et al.*, 1996; for reviews see Krude, 1995; Kaufman, 1996).

Newly replicated chromatin appears to be less stable (Gasser *et al.*, 1996) and more susceptible to degradation by nucleases than the corresponding sequences within bulk chromatin (Klempnauer *et al.*, 1980; Cusick *et al.*, 1984). Factors that could account for the behaviour of this so-called immature chromatin (Cusick *et al.*, 1984) include histone composition (presence or absence of histone H1), histone modifications (reversible acetylation of the histones; for reviews see Annunziato, 1995; Wade *et al.*, 1997), as well as the newly replicated DNA sequence itself. At least the assembly of the first nucleosome immediately behind the replication fork occurs asymmetrically with respect to DNA sequence on the nascent leading and lagging strands (Sogo *et al.*, 1986). Thus, an altered distribution of nucleosomes on the different arms of a single replication fork was suggested (DePamphilis and Bradley, 1986). In this context, several intriguing questions remain to be answered, e.g. do nucleosomes of the sibling molecules find the same DNA sequence and if so when? Does this process occur on post-replicated chromosomes or are nucleosome positioning and replication elongation simultaneous processes?

Since the main obstacle to investigating these questions is the lack of an available procedure to purify replicating chromatin, alternative techniques that allow an analysis of chromatin at the replication forks are required. An opportunity to examine replication in chromatin is provided by the psoralen technique routinely used in our group (Gruss and Sogo, 1992). However, analysis of individual replicating molecules by psoralen cross-linking followed by electron microscopy (Sogo *et al.*, 1986; Avemann *et al.*, 1988; Lucchini and Sogo, 1994, 1995; Gasser *et al.*, 1996) does not provide the resolution necessary to accurately determine translational positioning of newly assembled nucleosomes. To overcome this limitation, we developed two complementary assays that allow precise mapping of nucleosomes along defined regions of living cells.

Focusing on nucleosomes immediately behind the replication fork, we reveal that nucleosomes on the nascent strands are rapidly positioned after passage of the replication machinery.

Results

***In vivo* mapping of nucleosomes in the ribosomal spacer region using psoralen–DNA cross-linking and λ -exonuclease digestion**

Psoralens are small DNA-intercalating molecules which readily diffuse through cellular membranes (Cole, 1970).

When chromatin is photoreacted with psoralen, cross-links are formed preferentially in the linker DNA between adjacent nucleosomes (Hanson *et al.*, 1976; Conconi *et al.*, 1984). The position of these cross-links can be determined by digestion of DNA fragments with exonuclease since psoralen–DNA cross-links serve as a roadblock for this enzyme (Widmer *et al.*, 1988). Further digestion with S1 nuclease leads to DNA fragments of nucleosomal and polynucleosomal size, which are expected to carry psoralen cross-links at both ends. As summarized in Figure 1A, this approach was modified by substituting S1 nuclease with mung bean nuclease, since the latter enzyme does not cleave nicked duplex DNA (see Materials and methods). Using the indirect end-labelling technique (Nedospasov *et al.*, 1989; Wu, 1989), the position of nucleosomes along a given DNA sequence can then be determined unambiguously (for details see Materials and methods). When the technique was applied to the intergenic spacer of the yeast rRNA gene locus, two defined domains with respect to chromatin organization were found. In a region that spans the 5S gene and the 5' end of the 35S transcription unit, four positioned nucleosomes were mapped, as indicated by the empty boxes in Figure 1D (middle panel). To test the fidelity of the technique described, we prepared crude nuclei in a parallel experiment, digested them with micrococcal nuclease (MNase) and mapped the nuclease cleavage sites by indirect end-labelling (Livingstone-Zatchej and Thoma, 1999). MNase digestion of isolated nuclei confirmed the result obtained by psoralen cross-linking. Four nucleosomes were mapped at the same locations between the 5S gene and the 5' end of the 35S transcription unit (Figure 1D, left panel). In contrast, nucleosome mapping of the region between the 5S gene and the 3' end of the 35S coding region revealed the same pattern of cleavage in chromatin as in control deproteinized DNA (Figure 2C; see also Fritze *et al.*, 1997). It seems that in this second domain, nucleosomes are randomly distributed. These results are confirmed by data of Vogelauer *et al.* (1998). In their study, a similar nucleosomal organization of the rRNA gene intergenic spacer was described. An important difference was detected in the nucleosomal organization of the rDNA-ARS (rARS) region. In contrast to the results of Vogelauer *et al.* (1998), where the rARS *cis*-element is placed within the linker of two adjacent nucleosomes, in the yeast strain used for this study the rARS element spanning 107 bp determined by Miller and Kowalski (1993) was located within a region that was modestly accessible to MNase digestion and to psoralen cross-linking (grey box in Figure 1D, see also Figure 4C; M. Muller, unpublished data). Although a putative nucleosome could fit into this less protected region [approximate map units (MU) –499 to MU –679 in Figure 1C], the absence of a classical nucleosomal footprint might be explained by another possibility. It is also conceivable that the ORC proteins, which are likely to cover the rARS throughout the cell cycle (Santocanale and Diffly, 1996), render the rARS element more accessible to MNase and psoralen (Lipford and Bell, 2001). We decided to maintain the nomenclature of Vogelauer *et al.* for nucleosomes 1, 3, 4 and 5, and leave out a designation for nucleosome 2.

Fig. 1. Mapping of nucleosome positions in the ribosomal intergenic spacer by the psoralen–exonuclease assay. (A) Schematic representation of the method. Crosses represent sites of psoralen cross-links in duplex DNA corresponding to the linker regions between nucleosomes (the preferential sites of psoralen cross-linking). (B) Structural organization of the spacer region. Restriction sites (upright and downright arrows), functional elements (boxes) and the transcription start site (+1) are indicated. A *BstEII*–*AvaII* fragment used as a probe for indirect end-labelling is shown (striped box). (C) Positioned nucleosomes (numbered boxes 1–5) and the corresponding linkers (roman numerals) are indicated. Positions of the linkers are given with respect to the transcriptional start site. The grey box represents the ORC binding site and adjacent sequences. The large empty box denotes randomly distributed nucleosomes towards the 3' end of the rDNA coding unit. (D) Nucleosome mapping between the 5S gene and the transcription start site by indirect end-labelling from the *BstEII* site close to the 5S gene. In the left panel, the untreated *BstEII* fragment (N, lane 1), MNase cutting sites in chromatin (C, lanes 3 and 4) or in deproteinized DNA (D, lane 2) are shown. Chromatin (lanes C) was digested for 10 min with 25 U/μl (lane 3) or 500 U/μl (lane 4) MNase. In the right panel, psoralen cross-linking sites in chromatin (C, lanes 5 and 6) and deproteinized DNA (D, lane 7) were determined by the psoralen–exonuclease assay. λ-exonuclease entry sites were created by digesting purified cross-linked DNA for 2 min with 2 U/μl (lane 6) or 10 U/μl (lane 5) MNase [see (A)]. The pBR328 DNA fragments (*BglII*–*HinfI*) used as size markers are shown.

Fig. 2. Chromatin structure of the ribosomal intergenic spacer in replicating molecules with arrested forks. (A) Structural organization of the 4.5 kb *Bgl/II* fragment containing the rRNA intergenic spacer. The y-shaped molecule represents replication intermediates blocked at the RFB. (B) Preparative 2D gel of *Bgl/II*-digested rDNA from psoralen cross-linked S-phase cells. Linear fragments (L) and replication intermediates arrested at the RFB have been excised from the gel. The expected migration pattern (left) and Southern blot analysis of the gel are shown. (C) Nucleosome mapping towards the 3' site. λ -exonuclease entry sites were created by digesting the DNA with *HpaI*, and mapping of the psoralen cross-links was achieved by indirect end-labelling from the *AvaII* site close to the 5S gene. DNA eluted from the gel plug (RFB) was subjected to the psoralen-exonuclease assay. As controls, DNA eluted from the gel plug L (lane 2) or cross-linked deproteinized DNA (lane 1) were used. Note that the random position of nucleosomes in this region is inferred from the very similar cross-linking pattern of chromatin and deproteinized DNA. The asterisk indicates the position immediately behind the branch point in RIs with arrested forks. (D) Nucleosome mapping towards the 5' site. λ -exonuclease entry sites were created by digesting the DNA with *CfoI*, whereas mapping was performed from the *BstEII* site close to the 5S gene. Gel lanes are as in (C). The λ -DNA fragments (*EcoRI*-*HindIII*) used as size markers are shown.

Nucleosome mapping of replicative intermediates (RIs) shows intact nucleosome positioning in long-lived replicative molecules

The efficacy of the psoralen-exonuclease technique for chromatin mapping encouraged us to examine the nucleosomal organization of RIs. We first concentrated on molecules where leftward moving forks were stalled near the 3' end of the 35S coding region at the

replication fork barrier ('RFB' molecules; Figure 2A bottom; Gruber *et al.*, 2000). In these long-lived molecules, newly replicated intergenic spacers are visualized by electron microscopy as rows of single-stranded (ss) bubbles, consistent with their packaging into nucleosomes (for details see Lucchini and Sogo, 1994). We therefore attempted to map the translational positions of nucleosomes on these RIs using the psoralen-exonuclease technique.

The analysis of a specific class of replicative chromatin is a two-step process. First, the positions of nucleosomes are marked at the DNA level by psoralen cross-linking of chromatin in living cells. Then, the purified DNA molecules are separated according to their mass and shape on two-dimensional (2D) agarose gels (Brewer and Fangman, 1987). In such 2D gels, the location of a replication fork relative to the DNA sequence in a particular fragment is defined by its mobility in the gel. In brief, S-phase cells were psoralen photoreacted, the isolated rDNA was enriched, digested with *Bgl/II* and fractionated in preparative 2D agarose gels (Figure 2A and B). DNA corresponding to linear fragments and RIs with arrested forks were excised from the 2D gel (Figure 2B, right panel), subjected to the exonuclease assay, and the mono- and polynucleosomal fragments generated were detected in a native agarose gel by indirect end-labelling (Figure 2C and D). Upstream of the 5S gene, no difference in the banding pattern of control deproteinized DNA and chromatin from non-replicated monomers could be detected (Figure 2C, compare lanes 1 and 2). This is consistent with MNase mapping studies, which suggest that nucleosomes are randomly distributed in this area. The banding pattern of linear non-replicating molecules was also compared with that of the homogeneous class of partially replicated molecules that accumulate at the RFB (Figure 2C, lane 3). In these molecules, the fork is arrested in a defined sequence (RFB; Figure 2A) which coincides with the *HpaI* cutting site (Gruber *et al.*, 2000). Interestingly, the two patterns vary only in the region immediately behind the elongation point (the pre-nucleosomal DNA), which exhibited hypersensitivity to psoralen (Figure 2C, asterisk). When the 5S gene downstream region was analysed in RFB molecules, four positioned nucleosomes were detected (Figure 2D, lane 3). The positions of these nucleosomes correlate strictly with those determined for the non-replicated intergenic spacers (Figure 2D, lane 2, see also Figure 1D, lanes 5 and 6). This suggests that the nucleosomes re-assembled after the passage of the replication fork rapidly adopt the translational positions observed in non-replicated chromatin.

The nucleosomal pattern of the four positioned nucleosomes between the 5S gene and 35S promoter is established immediately after passage of the replication machinery

In the RIs stalled at the RFB, both left and rightward moving forks have already passed the region between the 5S gene and the 35S promoter (Figure 2A, bottom; for more details see Figure 3A, fork 5). We designate them 'long-lived' RIs since the fork persists at the RFB for up to several minutes. As was shown above, the positioning pattern of the four nucleosomes between the 5S gene and the 35S promoter in the RFB molecules (Figure 2D, lane 3)

Fig. 3. Chromatin structure of the intergenic spacer during replication. (A) Replication mode of the yeast rDNA. The top line represents the 35S coding region (thick line) flanked by intergenic spacers. Origin of replication (Ori; active initiation of replication), RFB, ARS element and *NheI* cutting sites are indicated. Left- and rightward moving forks (1–6) are localized at different positions along the 4.7 kb *NheI* restriction fragment containing the intergenic spacer (note that the ARS element is passively replicated). Replicating molecules with leftward moving forks arrested at the RFB are shown (5). The hatched zone marks the region of chromatin analysed. (B) Schematic representation of the expected mobility for the 4.7 kb *NheI* replicating fragments in an analytical 2D gel (left). Numbers indicate the positions predicted for the replication intermediates indicated in (A). In the right panel, *NheI*-digested rDNA from psoralen cross-linked S-phase cells was digested with *NheI* and separated on a preparative 2D gel (the arrow represents partially digested replicative intermediates). Small plugs containing a variety of replication intermediates were excised. Southern blot analysis of the gel is shown. (C) Exonuclease mapping of DNA eluted from the gel plugs. The analysis has been performed as in Figure 2D.

and control non-replicated chromatin (Figure 2D, lane 2) is indistinguishable. However, analysis of the positioning pattern of long-lived RIs does not reveal what the situation was immediately before and during the passage of the replication machinery. We therefore examined the passively replicating 4.7 kb *NheI* rDNA restriction fragment, in which the timing of nucleosome formation can be

Fig. 4. Nucleosome mapping by the psoralen–primer extension assay. (A) Schematic representation of the method. Enzymatic digestion steps and linear amplification are indicated. Note that the density of cross-links is very low. (B) Structural organization of the intergenic spacer region between the 5S gene and the 5' end of the 35S coding region. Relevant restriction sites, primer 1317 (straight arrow) and strand-specific probe 1496–1317 (striped box) are indicated. (C) Schematic representation of the mapped nucleosomes. (D) Total DNA from cross-linked exponentially growing cells (C, lanes 3, 4 and 5) or deproteinized cross-linked DNA (D, lane 2) was digested with *AvaI* and *AvaII* and subjected to the psoralen–primer extension assay. As a control for the primer extension reaction, the non-treated *AvaI* fragment was used (N, lane 1). Note that only nucleosomes N1–N4 were mapped. Deproteinized DNA (D) was psoralen cross-linked with 0.08 kJ/m² (lane 2), while the chromatin (C) was cross-linked with 0.9, 1.8 and 5.4 kJ/m² (lanes 3, 4 and 5).

followed (Figure 3A, forks 1–4). These molecules containing the intergenic spacer were expected to generate the characteristic Y-arc in a 2D agarose gel (Figure 3B, left

side). In principle, DNA fragments present in the Y-arc result from rightward moving forks entering an intergenic spacer where the upstream ARS element was not activated as an origin (Figure 3A, forks 1–4). The 4.7 kb fragments containing an active origin of replication (left intergenic spacer in Figure 3A) generate either a bubble-arc (Figure 3B) or a prominent spot on the Y-arc (Figure 3B, plug 5). As expected, Southern blot analysis of a 2D agarose gel shows a classical Y- and bubble-arc (Figure 3B, right side). Except for the RFB molecules, the DNA fragments migrating in the Y-arc mainly contain rightward moving forks, since in yeast most of the rRNA genes replicate in the same direction as transcription (Brewer and Fangman, 1988; Linskens and Huberman, 1988; Muller *et al.*, 2000). In order to isolate pre- and post-replicative chromatin in the region spanning the 5S gene to the 35S promoter element (Figure 3A, slashed region), replicative molecules were isolated from preparative 2D gels at distinct sites within the Y- and bubble-arc. At the inflection point of the Y-arc (Figure 3B, plug 3), Y-shaped molecules containing arms with the same length accumulate. In these molecules, the expected location of the fork is within the 5S gene and ARS. Therefore, this region is most likely to be involved in the process of replication. In contrast, molecules isolated from the ascending (Figure 3B, plugs 1 and 2) or descending (Figure 3B, plugs 4 and 5) part of the Y-arc are pre- or post-replicative molecules with respect to the hatched region in Figure 3A (right side). Following the same criteria, another class of post-replicative molecules is found in the most retarded part of the bubble-arc (Figure 3B, plug 6).

The nucleosome positioning of the RIs described was analysed by λ -exonuclease digestion of the psoralen cross-linked DNA (Figure 3C). To our surprise, in all samples isolated from the Y-arc (Figure 3C, lanes 1–4) the nucleosomal banding pattern was indistinguishable. In addition, the pattern resembles those obtained either from control non-replicated or long-lived replicative molecules (Figure 3C, lanes L and RFB, respectively). Since nucleosome assembly of the leading and lagging strands is asymmetrical (Cusick *et al.*, 1984; Sogo *et al.*, 1986), this result is consistent with an extremely rapid positioning of nucleosomes after passage of the replication machinery. The nucleosomal organization on bubble-shaped molecules in which the intergenic spacer has just been replicated (Figure 3C, lane 6) was expected to confirm this interpretation. However, in lane 6, the banding pattern is practically absent. Most likely, the amount of purified material was not sufficient to allow the detection of the nucleosomal pattern. In order to improve the sensitivity of the analysis and to confirm the present result, we decided to analyse RIs by a similar exonuclease assay coupled to a primer extension reaction.

Primer extension confirms the nucleosomal pattern obtained in replicating chromatin

An outline of the approach is summarized in Figure 4A. Yeast cells were weakly psoralen cross-linked, then the DNA was isolated and digested with an appropriate restriction enzyme. Further treatment of the DNA with λ -exonuclease generated templates for primer extension reactions. Psoralen cross-links act as a roadblock for the *Taq* polymerase; therefore, the reaction products define the

length between the priming site of the oligonucleotide and the psoralen cross-link. As shown previously, the linkers between nucleosomes can be mapped by this method (for details see Wellinger and Sogo, 1998). In contrast to the assay described in Figure 1, linear amplification of the reaction products can be achieved by repeated cycles of primer extension. Thus, the sensitivity of the mapping procedure is significantly enhanced.

Nucleosome mapping by this method within the ribosomal spacer is shown in Figure 4D. Note that due to the position of the primer used (Figure 4B), only three nucleosomes were mapped. The calculated positions of these three nucleosomes (Figure 4C) were compared with those obtained previously (Figure 1C). With both techniques (Figures 1A and 4A), nucleosomes were mapped to the same positions within the resolution of the two techniques.

In order to confirm the results obtained by the λ -exonuclease digestion of RIs, weakly psoralen cross-linked RIs were prepared as outlined in Figure 3. The replication intermediates were isolated from preparative 2D agarose gels and subjected to a primer extension reaction. Again, in all samples analysed, the banding pattern remained very similar, independent of whether the material was eluted from areas of the 2D gel containing non-replicated, early, mid or late replicated molecules (Figure 5A). Importantly, the amount of material excised from the bubble-arc was then sufficient for mapping nucleosomes along nascent strands (Figure 5A, lane 6). In summary, we could clearly confirm that nucleosomes rapidly occupy their translational position after the corresponding DNA sequence is duplicated.

At first sight, our results showing positioned nucleosomes during or immediately after the replication process (lane 3 in Figures 3C and 5A) conflict with previous observations demonstrating non-nucleosomal DNA immediately behind the elongation point (Cusick *et al.*, 1984). In addition, we showed in an earlier study that at least the first nucleosome close to the elongation point re-associates asymmetrically on the newly replicated DNA branches (Sogo *et al.*, 1986). However, this apparent discrepancy can be explained by the somewhat heterogeneous position of the replication fork within the various classes of intermediates purified from the preparative 2D gel. In fact, a single gel plug contains a subpopulation of replication intermediates representing a pool of individual molecules where the replication-elongation point is localized at any position along a DNA stretch of ~750 bp (see Figure 5B). Even if the replication machinery transiently disrupts up to three nucleosomes in the vicinity of the fork (Gasser *et al.*, 1996), chromatin mapping of this heterogeneous population of molecules will never detect a region completely devoid of nucleosomes.

Discussion

A combination of DNA cross-linking by psoralen and subsequent preparative 2D gel analysis allowed the first direct assessment of the timing and extent of nucleosome positioning after the passage of the replication fork *in vivo*. This was achieved by selectively isolating rDNA replicative molecules from psoralen cross-linked yeast

Fig. 5. Mapping of nucleosomes in the replicating ribosomal intergenic spacer. **(A)** Replication intermediates of the 4.7 kb *NheI* fragment were isolated as described in Figure 3B. The eluted samples were subjected to the psoralen–primer extension assay and the cross-links were mapped as described in Figure 4A. As a control for the primer extension reaction, the non-treated *AvaII* fragment was used (N). The diagram to the right represents positioned nucleosomes behind the elongation point. Marker (M) represents multiples of 256 bp. **(B)** Interpretation of the results. Nucleosomes are depicted as short cylinders. The schematic drawing represents rightwards moving forks in a population of 4.7 kb replicating molecules isolated from a gel plug. As a single gel plug corresponds to approximately one-sixth of the total Y-arc (see the autoradiograph in Figure 3B), the branch points of the replication forks are uniformly distributed along a region of ~750 bp (between dashed lines). The black–grey box at the bottom gives an estimate of the nucleosomal density along the replicating region.

chromatin. Our analysis revealed a rapid repositioning of nucleosomes after passage of the replication fork.

Psoralen as a tool to investigate nucleosome positioning *in vivo*

Previous work established psoralen–DNA cross-linking as a useful tool to study the nucleosomal organization of chromatin. These studies included the investigation of bulk inactive (Hanson *et al.*, 1976; Conconi *et al.*, 1984; Widmer *et al.*, 1988; Sogo and Thoma, 1989), transcriptionally active (Conconi *et al.*, 1989; Lucchini and Sogo, 1997) and replicating chromatin (Gruss and Sogo, 1992; Sogo and Laskey, 1995). An intrinsic property of psoralen is its ability to mark the structural information of the *in vivo* organized chromatin at the level of the DNA. After UV irradiation of psoralen-treated cells, intercalated psoralen remains fixed on the isolated deproteinized DNA. However, although the DNA molecule is (reversibly) altered, its basic helical structure remains intact (Sinden and Hagerman, 1984). Remarkably, psoralen cross-links are restricted to the linker DNA between nucleosomes. This is best visualized when DNA is purified from cross-linked chromatin, spread, and mounted for electron microscopy under denaturing conditions. The nucleosomal DNA repeat can then be recognized as rows of ss-bubbles connected by small double-stranded regions. Thereby, the chromatin organization of a given domain can be deduced by direct visualization of the corresponding psoralen-marked DNA sequence. However, mapping of the resulting ss-bubbles does not allow the translational position of the nucleosomes to be determined. In order to improve the resolution of linker detection and to analyse the

nucleosome positioning in a whole population of molecules, we developed a novel technique based on a biochemical approach. This psoralen–exonuclease assay was successfully tested on the rDNA locus in yeast. Additionally, we have shown that the sensitivity of linker detection can be significantly improved when combined with a primer extension reaction (Wellinger and Sogo, 1998). The present study was carried out on a multicopy gene cluster. However, we predict that the sensitivity of the technique will be sufficient to map the nucleosome positions along long stretches of DNA at the level of single-copy genes (see also Komura *et al.*, 2001). Since psoralens have been shown to diffuse through cellular membranes (Cole, 1970), it should be possible to extend the nucleosome mapping to other cell types.

Since regulatory regions such as promoters and origins of replication are frequently associated with positioned nucleosomes (Simpson *et al.*, 1991; Thoma, 1992; Lipford and Bell, 2001), one might expect positioned nucleosomes in the yeast ribosomal intergenic spacer. Indeed, a previous report identified positioned nucleosomes between the 5S gene and the 5' end of the 35S transcription unit (Vogelauer *et al.*, 1998). The present study confirms this result by using the psoralen technique. Whether the nucleosome positioning along the right half of the intergenic spacer is determined by the DNA sequence alone or by protein boundaries enclosing the regulatory elements of this region remains to be elucidated. The fact that nucleosomes are randomly distributed in the left half of the intergenic spacer, which is also flanked by boundaries, suggests that DNA sequence (alone or in combination with boundary effects) plays a role in nucleosome positioning along the right side of the spacer.

Nucleosome positioning during replication

Despite the extraordinary amount of information accumulated on the structural organization of the eukaryotic replication fork (DePamphilis and Bradley, 1986; Annunziato, 1995; DePamphilis, 1995; Krude, 1995; Sogo and Laskey, 1995; Kaufman, 1996), nucleosome positioning along the newly replicated branches has yet to be analysed. Owing to the length variation of pre-nucleosomal DNA on the leading and lagging strands (Cusick *et al.*, 1984; Sogo *et al.*, 1986) and MNase hypersensitivity of nascent chromatin (for reviews see DePamphilis and Bradley, 1986; Annunziato, 1995), it was hypothesized that nucleosomes in newly replicated chromatin have little (if any) phase relationship with respect to the underlying DNA sequence (DePamphilis and Bradley, 1986). In contrast to this model, we show here that nucleosomes on nascent chains are quickly and efficiently positioned. Our observation is in line with the finding that, *in vivo*, DNA replication and chromatin assembly are tightly coupled processes (Gasser *et al.*, 1996). This implies that the 'old', grouped and randomly segregated nucleosomes (Sogo *et al.*, 1986; Sogo and Laskey, 1995), as well as the 'new' CAF-1-dependent assembled histone octamers (Smith and Stillman, 1989; Kaufman *et al.*, 1995; Verreault *et al.*, 1996), rapidly position themselves on their most favourable DNA sequences along the newly replicated DNA strands. Whether the same positioning mechanism is used for the 'old' and *de novo* assembled nucleosomes remains unanswered.

A previous study in our laboratory revealed that parental nucleosomes are partially disassembled, while parental (H3/H4)₂ tetramers remain in loose contact with DNA during the passage of the replication fork (Gruss *et al.*, 1993). It is conceivable that this loose contact between the core histones and the replication fork is sufficient for the rapid reformation and repositioning of nucleosomes containing the parental (H3/H4)₂ tetramers onto newly synthesized DNA. Indeed, it has been shown that the tetramer complex is able to recognize the same nucleosome positioning signals as the intact nucleosome (Dong and van Holde, 1991; Hayes *et al.*, 1991). Correspondingly, *de novo* CAF-1-dependent assembled nucleosomes may have limited access to certain DNA sequences, and consequently the assembly of the 'new' nucleosome at the most appropriate position is favoured. Recently, it has been shown that the proliferating cell nuclear antigen (PCNA) interacts with CAF-1, mediating chromatin assembly on post-replicated DNA (Shibahara and Stillman, 1999; Zhang *et al.*, 2000; see also Krude, 1999). As the PCNA loading clamp is involved in DNA synthesis on both leading and lagging strands (Hübscher *et al.*, 1996; Hübscher and Sogo, 1997; Waga and Stillman, 1998), the PCNA-CAF-1 interaction could contribute to the rapid repositioning of nucleosomes on both nascent strands.

Reversible histone acetylation might also play a crucial role in chromatin replication (for reviews see Grunstein, 1997; Kuo and Allis, 1998). Histone H4 is reversibly acetylated during chromatin assembly (Ruiz-Carrillo *et al.*, 1975; Jackson *et al.*, 1976; Allis *et al.*, 1985) and the maturation of newly replicated chromatin involves the deacetylation of histone H4 after deposition on the

replicated daughter strands (Jackson *et al.*, 1976). Moreover, the *de novo* synthesized histones H3, H2A and H2B preferentially associate with acetylated H4 (Perry *et al.*, 1993). Nevertheless, the hyperacetylated state of the newly synthesized histones is not incompatible with the rapid positioning of nucleosomes at the replication fork. Indeed, it has been shown that nucleosome positioning does not change as a consequence of histone acetylation (Bresnick *et al.*, 1991; Ura *et al.*, 1997).

A characteristic feature of newly replicated (immature) chromatin is its hypersensitivity to non-specific endonucleases (Klempnauer *et al.*, 1980; Cusick *et al.*, 1981, 1983). We demonstrated previously a quick and efficient assembly of nucleosomes, including the rapid association of histone H1 after replication (Gasser *et al.*, 1996). Yet, the full maturation of newly replicated chromatin is a delayed, post-replicative process. The results reported in this study, indicating that nucleosome positioning is determined immediately behind the replication fork, suggest that chromatin maturation and nucleosome positioning are not obligatory coordinated events. Chromatin maturation is more likely to be accomplished by deacetylation of newly formed nucleosomes, and chromatin folding into higher order structures (Annunziato, 1995).

The replication fork as a window of opportunity

For the first time, our analysis clearly established a relationship between nucleosome assembly and nucleosome positioning at the replication fork. The replication fork has been visualized as a key event both for establishing and maintaining programmes of eukaryotic gene activity (Wolffe, 1991; Wolffe and Hayes, 1999). Thus, replication-coupled chromatin assembly may be the primary mechanism for the repression of basal transcription (Almouzni and Wolffe, 1993) or, alternatively, may help to generate transcriptionally active chromatin (reviewed in Annunziato, 1995). Previous studies in our group demonstrated that immediately after the passage of the replication fork, potentially active rRNA gene promoters were found to be open (nucleosome free) and probably associated with transcription factors (Lucchini and Sogo, 1995). Taken together, we propose that a window of opportunity starts after the passage of the replication fork and persists until the deposition of the first positioned nucleosome (at a distance of ~600 bp behind the fork). This corresponds to 10–12 s in time, assuming that the speed of fork movement is of the order of 50 bp/s. Within this window of opportunity, a modulation of the active or inactive state of the newly replicated chromatin could occur. The chromatin assembly process might, therefore, provide an opportunity for transcription factors to gain access to key regulatory elements mediating gene activity, as proposed earlier (Wolffe and Brown, 1986; Svaren and Chalkley, 1990). The appearance of positioned nucleosomes on nascent DNA suggests that regulatory and structural proteins become rapidly complexed with the newly replicated *cis*-acting elements.

In addition, alternative mechanism(s) for a PCNA-dependent epigenetic inheritance of chromatin states can exist in determined loci, as recently proposed by Shibahara and Stillman (1999) and Zhang *et al.* (2000). The psoralen cross-linking and primer extension techniques provide an excellent tool to examine asymmetric chromatin assembly

at the replication fork. By choosing an amplification primer that binds to the non-replicated parental lagging strand, the nucleosome positioning on the sister chromatid corresponding to the lagging strand can be mapped selectively. Alternatively, separation of leading and lagging strand could be achieved by digestion of isolated replication forks with T4 endonuclease VII. Cleavage by this enzyme results in a break of the leading arm at the elongation point and, therefore, permits selective removal of newly replicated leading strands from replicative intermediates (Gruber *et al.*, 2000).

Materials and methods

Strains and culture conditions

Saccharomyces cerevisiae A1 (MATa *ade2-101 ura3-52 his3 Δ200 lys2-801 Δbar1::LYS2*) was used for all sets of experiments. Yeast cells were grown in complex medium at 30°C to a density of $\sim 6 \times 10^6$ cells/ml. In order to increase the amount of replicative molecules, the cells were synchronized in G₁/S phase using α -factor (Sigma), and released into S phase for a further 35–45 min prior to rDNA isolation.

Psoralen cross-linking

Yeast cells were irradiated in the presence of 4,5',8-trimethylpsoralen with a 366 nm UV lamp as described previously (Lucchini and Sogo, 1994). For the psoralen–exonuclease assay based on MNase digestion, psoralen was added five times at intervals of 15 min for a total irradiation time of 75 min. For the exonuclease mapping assay based on restriction enzyme digestion, psoralen was added only twice for a total irradiation time of 30 min.

rDNA isolation and purification of RIs by preparative 2D gel electrophoresis

Chromosomal DNA of early log phase yeast cells was isolated (Wu and Gilbert, 1995) and the rDNA enriched on CsCl gradients (Lucchini and Sogo, 1994). The enriched rDNA was digested with *NheI* or *BglII* (Roche) and subjected to neutral/neutral 2D gel electrophoresis (Brewer and Fangman, 1988). Electrophoresis and DNA isolation were performed as described elsewhere (Lucchini and Sogo, 1994; for more details see Gruber *et al.*, 2000).

Nucleosome mapping by MNase assay

Chromatin was digested for 10 min at 37°C with 25 or 500 U/μl MNase; the DNA was purified and cut with the appropriate restriction enzyme. MNase cleavage sites were mapped by indirect end-labelling as described by Livingstone-Zatchej and Thoma (1999).

Nucleosome mapping by the psoralen–exonuclease assay

Psoralen cross-linked DNA was mildly digested with MNase (or limit digested with an appropriate restriction enzyme) and subsequently incubated for 30 min at 37°C with 5 U of mung bean nuclease (Roche; reaction buffer: 30 mM sodium acetate pH 4.6, 50 mM NaCl, 1 mM zinc acetate, 0.001% Triton X-100). This treatment produces DNA fragments with blunt ends, which represent an optimal substrate for the exonuclease. The DNA was then treated for 45 min at 37°C with 4 U of λ exonuclease (BRL; reaction buffer: 67 mM glycine–KOH pH 9.4, 2.5 mM MgCl₂, 50 μg/ml bovine serum albumin), followed by a final 10 min incubation with mung bean nuclease. After each treatment, the DNA was purified by phenol–chloroform extraction and ethanol precipitation. In the experiments with DNA eluted from the gel, 1.5 μg of slightly cross-linked λ DNA were added as a carrier. Mapping of psoralen cross-links was then performed by indirect end-labelling after separation of the DNA fragments on a 1.2% native agarose gel in the presence of 0.5 μg/ml ethidium bromide.

Nucleosome mapping by the psoralen primer extension assay

Primers were purchased from Microsynth. The sequences of the primers used for primer extension and the preparation of a strand-specific probe are as follows: primer 1317, 5'-AGTAGGTGGGAGTGAGAGGTGTT-3' (nucleotides 1317–1339); primer 1496, 5'-TGCCACCTACCGACC-CAATTTCA-3' (nucleotides 1496–1474). The isolated RIs were digested

with *AvaII* and primer extension templates were prepared as described (Wellinger and Sogo, 1998).

Iterative primer extension with primer 1317 was performed using 35 amplification cycles consisting of three steps: 94°C for 45 s, 55°C for 4 min, 72°C for 2 min. The primer extension products were separated on a 1.5% alkaline agarose gel. After electrophoresis, the DNA was alkaline transferred onto a Biodyne B membrane (Pall) and hybridized to a radioactively labelled, single-stranded probe matching map units 1496–1317.

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