1420–1427 *Nucleic Acids Research, 1996, Vol. 24, No. 8*

Effects of cell cycle dependent histone H1 phosphorylation on chromatin structure and chromatin replication

Lothar Halmer and Claudia Gruss*

Division of Biology, Universität Konstanz, D-78434 Konstanz, Germany

Received January 30, 1996; Revised and Accepted March 4, 1996

ABSTRACT

We have reconstituted salt-treated SV40 minichromosomes with differentially phosphorylated forms of histone H1 extracted from either G0-, S- or M-phase cells. Sedimentation studies revealed a clear difference between minichromosomes reconstituted with S-phase histone H1 compared with histone H1 from G0- or M-phase cells, indicating that the phosphorylation state of histone H1 has a direct effect on chromatin structure. Using reconstituted minichromosomes as substrate in the SV40 *in vitro* replication system, we measured a higher replication efficiency for SV40 minichromosomes reconstituted with S-phase histone H1 compared with G0- or M-phase histone H1. These data indicate that the chromatin structure induced by the phosphorylation of histone H1 influences the replication efficiency of SV40 minichromosomes *in vitro*.

INTRODUCTION

A major element in the control of chromatin organization is histone H1, which plays a role both in the stabilization of the nucleosomal structure and in the higher order coiling of the chromatin fiber (reviewed in 1).

Histone H1 has been shown to be differentially phosphorylated during the cell cycle (reviewed in 2–4). This has been examined in a number of different organisms and cell types, most notably with the slime mold *Physarum polycephalum* (5,6) and with synchronized Chinese hamster ovary cells (7,8). Levels of H1 phosphorylation are usually lowest in the G1 phase of the cell cycle and continuously rise during S-phase and mitosis. During mitosis, phosphorylation becomes maximal just before or at metaphase and sharply decreases thereafter (5,8,9). Serine and threonine residues are the acceptors of phosphate groups in H1 histones. The short N- and long C-terminal basic regions of the molecule appear to contain all the phosphorylation sites in conserved (K[S/T]PXK) or (K[S/T]PK) sequence motifs. These motifs are consensus sequences for the cdc kinase family and Langan *et al.* (10) could show that the growth-associated histone H1 kinase is identical with a mammalian homolog of the cdc2/CDC28 yeast kinase (reviewed in 11,12).

Conflicting data exist about the influence of this cell cycle dependent modification on chromatin structure (reviewed in 13). A correlation between histone H1 phosphorylation and chromatin condensation was demonstrated in numerous *in vitro* and *in vivo* experiments. The decreased phosphorylation of histone H1 in a temperature sensitive growth mutant of FM3A cells results in an abnormal chromosome condensation at the non-permissive temperature (14,15), and hyperphosphorylation of histone H1 in tsBN2 cells leads to premature chromatin condensation (PCC) at the non-permissive temperature (16). Treatment of cells with the topoisomerase II inhibitor VM26 (17) or the protein kinase inhibitor staurosporine (18, both induce a dephosphorylation of histone H1), leads to a concomitant decondensation of the chromatin, indicating that the phosphorylation of histone H1 is required to maintain the highly condensed state.

However, H1 phosphorylation is uncoupled from mitosis and chromosome condensation in the amitotic macronucleus of *Tetrahymena*, where H1 is hyperphosphorylated in exponentially growing cells and completely dephosphorylated in the highly condensed state (19). During sea urchin spermatogenesis histone H1 is also found to be dephosphorylated in the highly condensed chromatin of mature sperm (20). Similarily, histone H5, a homologue of H1, is dephosphorylated in avian erythrocytes at a time when compaction of chromatin occurs (21). Furthermore, treatment of a temperature-sensitive $p34^{cdc2}$ mutant cell line with phosphatase 1 and 2A inhibitors resulted in full chromosome condensation in the absence of histone H1 phosphorylation (22). Thus, the role of histone H1 phosphorylation during chromatin condensation is still a matter of controversy.

Little is known about whether the phosphorylation of histone H1 has an effect on chromatin replication. Studies on the phosphorylation sites of human histones H1A and H1B during the HeLa cell cycle have shown that phosphorylation of one site in the C-terminal domain of histone H1 precedes, while phosphorylation of a second site follows the onset of DNA replication (23). This led to the proposal that H1 phosphorylation preceding the onset of DNA replication might produce a chromatin conformation that permits DNA replication. By using antibodies generated against hyperphosphorylated histone H1, Lu *et al.* (24) observed a replication-dependent phosphorylation of histone H1 during

* To whom correspondence should be addressed

S-phase, which was accompanied by a diffuse nuclear staining. They propose that H1 phosphorylation acts as a first step mechanism to promote transient chromatin decondensation, which allows access of specific DNA binding factors during cell cycle regulated processes such as gene activation, DNA replication, as well as chromosome condensation. Furthermore *in vivo* experiments with ts mutants, defect in histone H1 phosphorylation, have shown that the decrease in H1 phosphorylation resulted in an incomplete DNA replication (15).

We have used salt-treated SV40 minichromosomes reconstituted with differentially phosphorylated forms of histone H1 to investigate the influence of H1 phosphorylation on chromatin structure and replication. We found that at a physiological ratio of one molecule histone H1 per nucleosome different chromatin structures are induced depending on the phosphorylation state of histone H1. These minichromosomes replicate with different efficiencies indicating a direct effect of H1 phosphorylation on chromatin replication.

MATERIAL AND METHODS

Cell synchronization

African green monkey kidney (CV-1) cells were grown in Dulbecco's modified Eagle medium supplemented with 5% calf serum (DMEM). Cells were synchronized for G0-arrest by incubation in isoleucine-deficient DMEM medium supplemented with 5% dialysed serum for 72 h (25). For S-phase synchronization cells were first incubated in isoleucine-deficient DMEM medium for 48 h, followed by incubation in DMEM medium containing 7.5 mM thymidine for 18 h to arrest in early S-phase (26). Release from this block was for 5.5 h by incubation in DMEM medium. As monitored by ^{3}H-thymidine pulse labelling, cells reached mid S-phase after this time. For mitotic arrest, cells were grown in DMEM medium containing 1 µg/ml nocodazole for 20 h (27). Cell cycle analysis was done by flow cytometry.

Purification of histone H1

Histone H1 was isolated from synchronized CV-1 cells by extraction with 5% perchloric acid (PCA) (28,29). All buffers contained the following phosphatase inhibitors: 50 mM NaF, 80 mM β-glycerophosphate and 0.1 mM sodium orthovanadate. Histone H1 was analysed by 15% SDS–PAGE (30) and silver staining (31). H1 concentrations were determined by comparison with commercially available calf thymus H1 (Calbiochem) by densitometric scanning of the gel (Biometra, Scanpack).

Characterization of histone H1 phosphorylation

For *in vivo* labelling of histone H1 cells were synchronized as described but for the last 4 h of synchronization the medium was changed to the corresponding phosphate-free medium containing 40 µCi/ml [^{32}P]orthophosphoric acid. Histone H1 was then extracted as described before and analysed on 15% SDS–PAGE.

HEPES–histidine cationic disc electrophoresis at neutral pH was used to control the phosphorylation of unlabelled histone H1 (32). Treatment of histones H1 with alkaline phosphatase was performed as described (38).

Preparation of SV40 DNA, SV40 T-antigen (T-Ag), cytosolic S100 extract and SV40 minichromosomes

Preparation of SV40 DNA from infected CV-1 cells was performed according to the Hirt procedure (33). The SV40 T-Ag was purified from infected insect cells (Sf9) by immunoaffinity chromatography (34). Cytosolic S100 extracts were prepared from HeLa cells exactly as described (35). The SV40 minichromosomes were isolated 38 h after infection of CV1 cells (36,37). To remove RNA particles comigrating on sucrose gradients, the minichromosome eluate was incubated with 100 µg/ml RNase A for 10 min at room temperature before loading on 5–30% sucrose gradients (29).

Reconstitution of SV40 minichromosomes with histone H1

Salt-treated SV40 minichromosomes (50 µg/ml) were incubated with increasing amounts of differentially phosphorylated forms of histone H1 (10, 15, 17.5 and 20 µg/ml). Histone H1 and minichromosomes were mixed on ice, dialysed against a linear salt gradient and centrifuged through 5–30% sucrose gradients (SW40, 39 000 r.p.m., 3 h, 4°C) (29). The DNA concentration was determined by measuring the absorbance at 260 nm and by comparison with SV40 DNA standards on agarose gels. The protein composition of the reconstituted minichromosomes was analysed by electrophoresis on 15% denaturing polyacrylamide gels (30), followed by silver staining (31) and densitometric scanning of the gel. The amount of bound H1 per nucleosome was determined by comparison with commercially available calf thymus H1. Based on the fact that the histone to DNA ratio (w/w) appears to be ~1 (1), 1.5 µg chromatin contain 258 ng histone H1 at a H1:nucleosome ratio of 1.

Electron microscopy

Minichromosomes (in 10 mM HEPES, pH 7.8; 30 mM NaCl) were fixed with 0.1% glutaraldehyde and spread with 2×10^{-4}% BAC exactly as described (38). For contrast enhancement the grids were rotary-shadowed with tungsten at an angle of 8°.

Stability of H1 phosphorylation under replication conditions

Phosphorylation of H1 during replication. Soluble histone H1 as well as chromatin bound H1 were incubated in cytosolic S100 extracts under replication conditions in the presence or absence of SV40 T-Ag. To detect phosphorylation of H1 under these conditions the reaction was done in the presence of 40 µCi [γ-^{32}P]ATP and phosphatase inhibitors. After 120 min incubation at 37°C proteins were separated on 12% SDS–PAGE, labelled proteins were visualized by autoradiography.

Dephosphorylation of histone H1 reconstituted minichromosomes. Histone H1 was phosphorylated *in vitro* with purified cdc2/cyclin B kinase (39). Salt-treated minichromosomes were reconstituted with *in vitro* labelled H1, purified as described above and incubated for increasing times (0–120 min) in cytosolic S100 extract. One half of the sample was taken for protein gel analysis (12% SDS–PAGE), the other for precipitation with 25% trichloracetic acid (TCA).

Stability of H1 phosphorylation in the presence of phosphatase inhibitors. In vivo labelled histone H1 from M-phase cells (see above) was incubated in cytosolic S100 extract at 37°C for

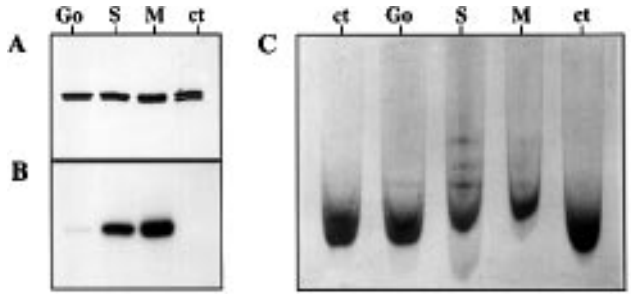

Figure 1. Phosphorylation of histone H1 during the cell cycle. Histone H1 was extracted from CV-1 cells synchronized in either G0-, S- or M-phase of the cell cycle. Proteins were labelled *in vivo* with [^{32}P]orthophosphoric acid. Equal amounts of histone H1 were separated on a 15% SDS–PAGE and visualized by silver staining (**A**) and autoradiography (**B**). 200 ng of calf thymus H1 (ct) were used as a marker. Unlabelled histone H1 purified from the different cell cycle phases (G0, S, M) was separated on a HEPES–histidine gel. (**C**) The mobility of the proteins depends on the net charge, whereby the protein is retarded with increasing phosphate content.

120 min in the presence of increasing amounts of one of the phosphatase inhibitors: 2–20 mM NaF (Merck), 5–30 mM β-glycerophosphate (Merck) or 0.01–5 µM okadaic acid (Calbiochem).

In vitro replication of SV40 minichromosomes

The S100 extracts were pretreated with immobilized RNase A (29) and 2.5 µM OA for 30 min at 37°C before adding equal amounts (500 ng) of the reconstituted minichromosomes. Replication was done for 120 min at 37°C exactly as described (29).

RESULTS

Isolation and characterization of differentially phosphorylated forms of histone H1

The effect of histone H1 phosphorylation on chromatin structure and chromatin replication was studied with SV40 minichromosomes. Minichromosomes were prepared from infected African monkey kidney (CV-1) cells at 500 mM potassium-acetate to obtain salt-treated minichromosomes lacking histone H1 and most non-histone chromatin proteins (36,37,40). These minichromosomes were reconstituted with exogenously added histone H1, prepared from CV-1 cells, arrested at different stages of the cell cycle.

Cells were arrested in G0 by isoleucine deprivation (25), S-phase cells were obtained by thymidine block (26) with subsequent release for 5 h, and mitotic cells by treatment with nocodazole (27). The cell cycle state was monitored by flow cytometry, showing that we obtained 80–90% synchronization for the individual cell cycle phases (data not shown). We have determined the phosphorylation state of histone H1 either by labelling with radioactive [^{32}P] phosphate *in vivo* or by analysis of unlabelled H1 using cationic disc electrophoresis (32).

Histone H1 was prepared from isolated nuclei of ^{32}P-labelled cells and investigated by PAGE in the presence of SDS and silver staining (Fig. 1A). The gel was further analysed by autoradiography showing that histone H1 is not phosphorylated in G0-phase, moderately phosphorylated in S-phase and hyperphosphorylated in M-phase (Fig. 1B).

The differences in the phosphorylation state of histone H1 were further investigated by cationic disc electrophoresis (32). Electrophoretic mobility in this system depends on differences in net charge. Therefore, the unphosphorylated form of histone H1 moves faster than the phosphorylated form. Accordingly, histone H1, prepared from CV-1 cells arrested at G0-phase has the highest electrophoretic mobility, S-phase H1 is retarded compared with G0-H1, and M-phase H1 has the lowest mobility (Fig. 1C). Treatment of histones H1 with alkaline phosphatase removes the differences in gel mobility, confirming that the slower migration rates are indeed due to phosphorylation (data not shown). Our data, as shown in Figure 1, demonstrate that CV-1 histone H1, prepared from the individual cell cycle states, is differentially phosphorylated.

Characterization of reconstituted SV40 minichromosomes

We used salt-gradient dialysis to reconstitute salt-treated SV40 minichromosomes with increasing amounts of histone H1 from either G0-, S- or M-phase cells (29). We use the terms G0-H1 chromatin, S-H1 chromatin or M-H1 chomatin for these reconstituted minichromosomes. Reconstituted minichromosomes were purified on sucrose gradients to remove excess histone H1. Minichromosome containing fractions were pooled and concentrated on 30% sucrose cushions. To investigate the protein composition of the reconstituted minichromosomes equal amounts of chromatin were analysed on a protein gel. The amount of chromatin bound H1 was estimated by comparison with known amounts of calf thymus H1 (Fig. 2). We found that the differentially phosphorylated forms of histone H1 bind with similar affinities to chromatin.

Reconstituted minichromosomes were further characterized by micrococcal nuclease digestion. Due to the presence or absence of histone H1 we observed different micrococcus kinetics for salt-treated and reconstituted minichromsomes, which are more resistant to digestion (data not shown). Furthermore, comparison of the nucleosomal repeat revealed that the size of the monomeric DNA increased from salt-treated minichromosomes to reconstituted minichromosomes by 20–25 bp (data not shown). The size of the MNase resistant fragment of H1-reconstituted minichromosomes was similar to that obtained with native minichromosomes (data not shown), indicating that histone H1 is properly bound to the nucleosome under the conditions used for reconstitution. The micrococcal nuclease digestion revealed no differences in the kinetic and the nucleosomal repeat pattern between chromatin reconstituted with either G0-, S- or M-phase histone H1 (data not shown).

The reconstituted minichromosomes were further analysed by electron microscopy. Based on earlier electron microscopical studies we expected that histone H1 induces a condensation of viral DNA–protein complexes (38,41), but we wanted to determine whether unphosphorylated and phosphorylated H1 have similar effects. For this purpose, we compared minichromosomes, reconstituted with one molecule H1/nucleosome with salt-treated SV40 minichromosomes (Fig. 3) and adsorbed samples by direct mounting to carbon grids (38). H1-free chromatin shows an extended beaded-string conformation, in which the linker DNA between individual nucleosomes is clearly visible. However, addition of purified histone H1 to these minichromosomes induces a distinct compaction of the chromatin,

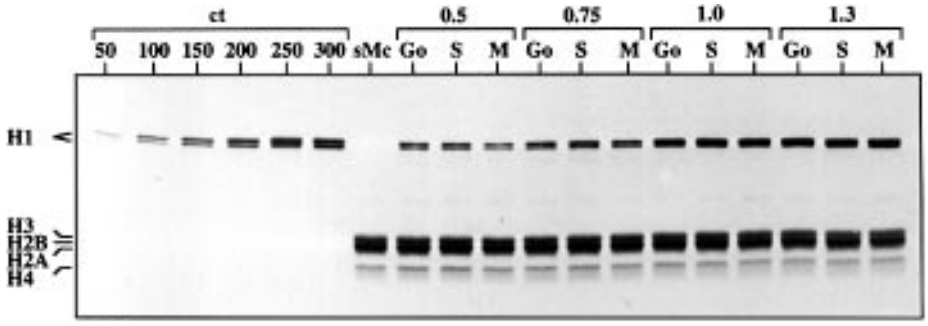

Figure 2. Protein analysis of reconstituted minichromosomes. Salt-treated SV40 minichromosomes (sMc) were reconstituted by salt gradient dialysis with H1 extracted from G0-, S and M-phase cells (G0, S, M) at different ratios of H1/nucleosome (0.5; 0.75; 1.0; 1.3). Equal amounts of sucrose gradient purified minichromosomes were separated on a 15% SDS–PAGE, the proteins were visualized by silver staining. The amount of bound histone H1 was determined by comparison with known amounts (ng) of calf thymus H1 (ct). The mobility of the core histones (H3, H2B, H2A, H4) is indicated.

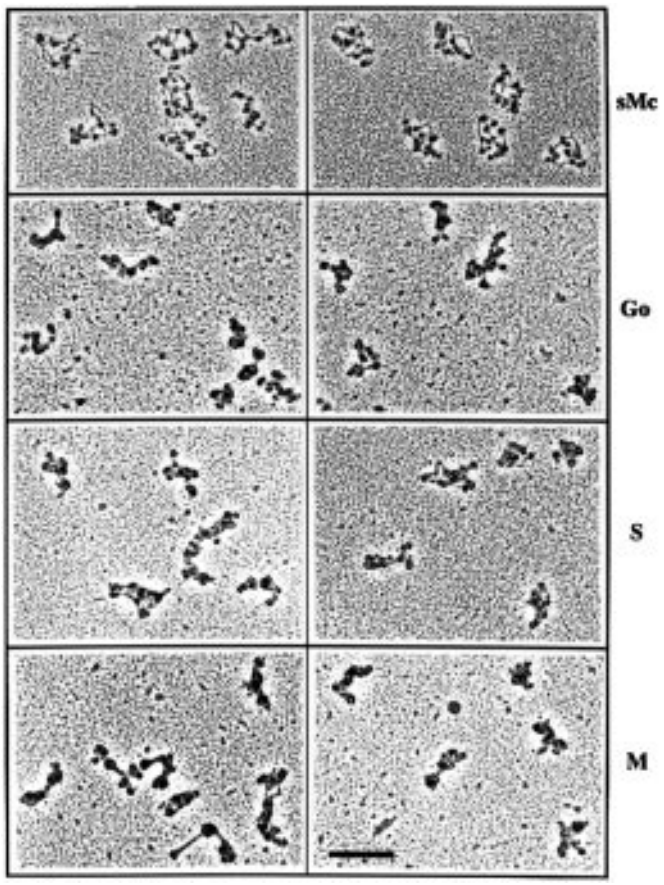

Figure 3. Electron microscopic visualization of reconstituted SV40 minichromosomes. Salt-treated (sMc) or H1 reconstituted minichromosomes (G0, S, M, one molecule H1 per nucleosome) were fixed with 0.1% glutaraldeyde and spread with 2×10^{-4}% BAC for electron microscopy. Left and right colums represent duplicates of individual reconstitutions. Nucleoprotein complexes were visualized by shadowing with tungsten at an angle of 8°. Bar represents 200 nm.

where the nucleosomes are in close contact and the linker DNA is no longer visible. At a H1/nucleosome ratio >1 minichromosomes aggregate, forming complexes of 5–10 SV40 molecules (data not shown).

Thus, under the conditions used for reconstitution, histone H1 binds to the nucleosomes and induces a condensation of the minichromosomes. Furthermore, no differences could be detected in the degree of compaction induced by the differentially phosphorylated forms of histone H1.

Sedimentation studies of reconstituted minichromosomes

Differences in chromatin structure induced by the differentially phosphorylated forms of histone H1 might be below the resolution of the electron microscope. For this reason we have investigated the hydrodynamic properties of reconstituted minichromosomes using sucrose gradient centrifugation (Fig. 4). At a ratio of 0.5 molecules histone H1 bound per nucleosome we found no difference in the sedimentation rate of G0-H1, S-H1 and M-H1 chromatin. In all cases the S-value increased from 50S for H1-depleted minichromosomes to 66S for H1-carrying chromatin. At ratios of 0.75 molecules H1 per nucleosome we detected a small reduction of 4S in the sedimentation rate of S-H1 chromatin compared with G0-H1 and M-H1 chromatin. This difference became more pronounced at the physiological ratio of one molecule H1 per nucleosome (42), resulting in an S value of 71S for S-H1 chromatin compared with 80S for G0-H1 and M-H1 chromatin. At a histone H1:nucleosome ratio >1, minichromosomes tend to aggregate and differences in the hydrodynamic properties of the differentially reconstituted minichromosomes disappear. The sedimentation profiles indicate a distributive binding of histone H1 to the chromatin for all ratios and all phosphorylation states used, resulting in a homogenous chromatin population.

We conclude that the phosphorylation state of histone H1 has a direct effect on chromatin structure. Thus, S-phase histone H1 induces a more open chromatin structure.

Replication of reconstituted minichromosomes

As histone H1 is one of the few known substrates of the cdc-kinase family (10), it may be possible that phosphorylation of histone H1 has a regulatory effect on chromatin replication. We investigated this question in the SV40 *in vitro* replication system, by using salt-treated SV40 minichromosomes, reconstituted with G0-, S- and M-phase histone H1, as template for replication in unfractionated extracts.

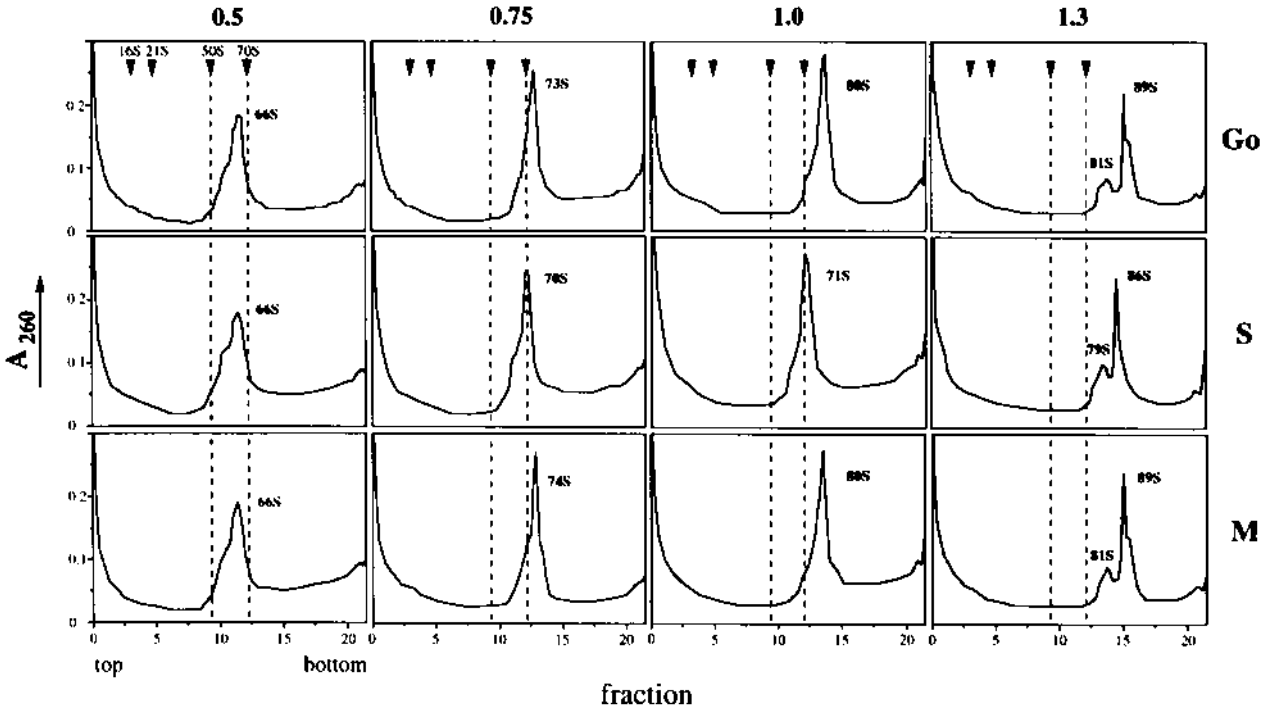

Figure 4. Sedimentation behaviour of G0-H1, S-H1 and M-H1 chromatin. Salt-treated SV40 minichromosomes were reconstituted with histone H1 as described in Figure 2. Reconstituted minichromosomes were purified on 5–30% sucrose gradients and chromatin containing fractions were identified by an UV monitor (A_{260}). Sedimentation of protein-free form II (16S) and form I (21S) SV40 DNA, salt-treated (50S) and native (70S) SV40 minichromosomes is indicated by arrowheads, and for sMc and nMC additionally by dotted lines. The sedimentation coefficient for the reconstituted chromatin is indicated (S) and was determined in three independent experiments.

Since it was not known whether the H1 phosphorylation state remains unchanged during incubation under replication conditions, we determined whether H1 is phosphorylated in the cytosolic S100 extract used for replication. For that purpose, we included [γ-^{32}P]ATP in the reaction mixture, which was incubated in the absence or presence of the SV40 T-Ag. Phosphorylation of histone H1 could not be detected under replication conditions, showing that a phosphorylation of unphosphorylated G0-H1 or hemiphosphorylated S-H1 does not occur during incubation (data not shown).

We also wanted to demonstrate that H1 is not dephosphorylated when incubated in the replication extract. We therefore reconstituted salt-treated SV40 minichromosomes with H1, either labelled *in vitro* with purified p34^{cdc}-kinase (Fig. 5A and B) or isolated from M-phase cells and phosphorylated *in vivo* (Fig. 5C and D). The reconstituted minichromosomes were purified by sucrose gradients and incubated at 37°C under replication conditions in cytosolic S100 extract. Samples, taken during incubation, were investigated on protein gels followed by autoradiography (Fig. 5A). For quantitation, the phosphate content of histone H1 was determined by TCA precipitation (Fig. 5B). We found that already after 10 min incubation 50% of the input histone H1 was dephosphorylated, and after 2 h incubation only 10–20% of histone H1 was still phosphorylated, indicating that active phosphatases are present in the S100 extract.

To stabilize phosphorylated H1, we performed the experiments with various phosphatase inhibitors. We found that Na-fluoride as well as okadaic acid inhibited the dephosphorylation of both *in vitro* labelled H1 (data not shown) and *in vivo* labelled M-phase H1 (Fig. 5C and D) to ~90%. The *in vitro* replication assays of reconstituted minichromosomes were therefore performed in the presence of 2.5 μM okadaic acid which has no effect on the replication efficiency of the extracts, in contrast to NaF which inhibits the replication to ~50% (data not shown).

Equal amounts of salt-treated (Fig. 6A) or reconstituted minichromosomes (Fig. 6B) were used as templates in the *in vitro* replication. Incubation was for 2 h in the presence of the SV40 T-Ag and [α-^{32}P]dATP, to label the newly synthesized DNA. Replication products were purified and analysed by agarose gel electrophoresis and autoradiography (Fig. 6A and B), the incorporation was determined by TCA precipitation (Fig. 6C). At low ratios of histone H1 per nucleosome (0.5; 0.75), no differences could be detected in the replication efficiency and the products between G0-H1, S-H1 or M-H1 chromatin (data not shown). However, at the physiological ratio of one molecule H1 per nucleosome we observed significant differences in the replication efficiencies between the individual templates. In this case the replication efficiency of G0-H1 or M-H1 chromatin was reduced to 70 and 40%, respectively, compared with the replication efficiency of S-phase chromatin (Fig. 6B). We observed no change in the distribution of replication products; the radioactive incorporation was reduced both in the high molecular weight DNA (HMW) and the completely replicated molecules (Fig. 6B, between form I and II). Raising the ratio of H1 per nucleosome over the physiological value of 1 resulted in an overall reduction in replication efficiency, which had been observed before (29). At this point no differences in replication efficiency and products between G0-H1, S-H1 or M-H1 chromatin were detected (data not shown).

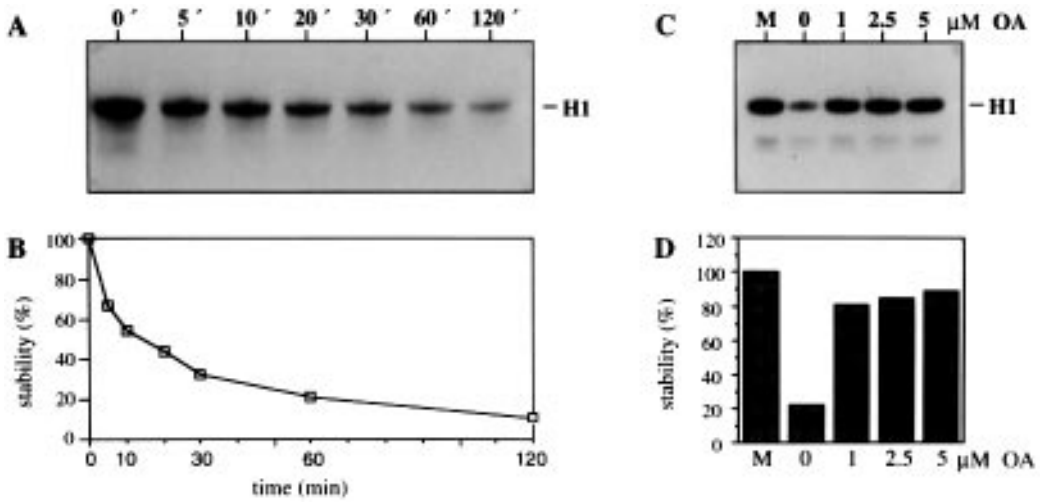

Figure 5. Stability of the H1 phosphorylation state under *in vitro* replication conditions. Chromatin-bound histone H1, labelled *in vitro* with purified cdc2/cyclin B kinase, was incubated for increasing times in cytosolic S100 replication extract. Proteins were separated on 15% SDS–PAGE and visualized by autoradiography (**A**). The ^{32}P-content of histone H1 was determined by TCA precipitation (**B**). *In vivo* labelled mitotic histone H1 was incubated in cytosolic S100 extract for 2 h at 37°C in the presence of increasing amounts of okadaic acid (OA). Proteins were separated on 15% SDS–PAGE and visualized by autoradiography (**C**). The stability of H1 phosphorylation under these conditions was determined by TCA precipitation (**D**).

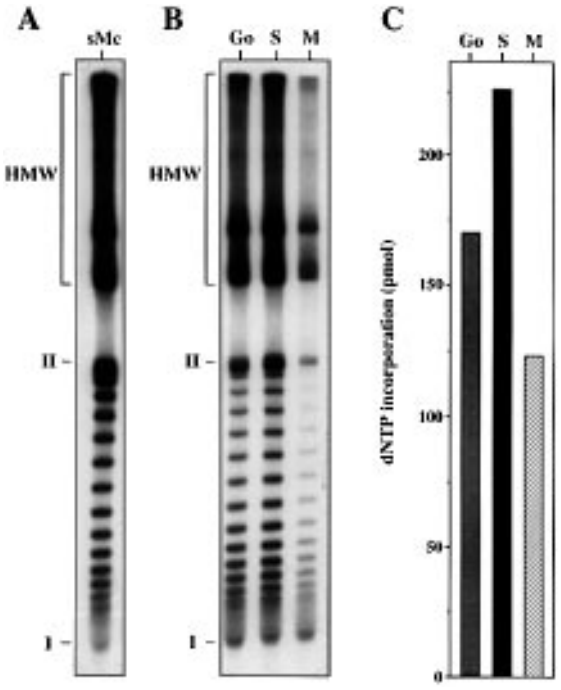

Figure 6. *In vitro* replication of reconstituted SV40 minichromosomes. Equal amounts of salt-treated minichromosomes (**A**) or G0-H1, S-H1 and M-H1 chromatin (**B**) were used as substrate in the SV40 *in vitro* replication system. Replication was for 2 h at 37°C in the presence of 2.5 µM okadaic acid. Replication products were deproteinized, separated on 0.8% agarose gels and visualized by autoradiography (I, covalently closed circular; II, relaxed circular; HMW, high molecular weight DNA). Replication efficiencies as determined by TCA precipitation are given as average pmol dNTP incorporated from three independent experiments (**C**).

Thus differences in replication efficiency between minichromosomes reconstituted with either G0-, S- or M-phase histone H1 were only observed in a narrow range, corresponding to the physiological value of one molecule H1 per nucleosome. In this case S-H1 chromatin replicates with a higher efficiency than G0-H1 or M-H1 chromatin. Interestingly, we have observed a reduced S-value of 9S for the S-H1 chromatin at this H1 ratio, compared with G0-H1 or M-H1 chromatin (Fig. 4), which is indicative for a more open chromatin structure. This structure seems to facilitate the replication of minichromosomes.

DISCUSSION

With the exception of *Saccharomyces cerevisae* (43) all eukaryotic organisms analysed so far contain a linker histone. It is now widely accepted that members of the H1/H5 family of linker histones are involved in the condensation of chromatin filaments into both 30 nm fibers and higher-order chromosomal structures (44). Nevertheless, the precise nature of the interactions of these lysine-rich histones with DNA and other chromosomal constituents during chromatin packaging remains largely elusive. One of the uncertainties surrounding histone H1 is the role(s) played by the cell cycle dependent phosphorylation of the protein. Mitotic hyperphosphorylation of histone H1 has been assumed to trigger or promote chromosome condensation (5), it is however still controversial whether H1 phosphorylation plays an active or passive role during condensation (13). So far, no special function has been linked to the moderate phosphorylation of histone H1 during S-phase. This is of particular interest, because histone H1 is a specific substrate of the cdc kinases (10), which are thought to regulate the activity of proteins involved in DNA replication by phosphorylation (reviewed in 12,45).

To get further insights into these processes we have reconstituted H1-depleted SV40 minichromosomes (40,41) with increasing amounts of histone H1 extracted from G0, S or M-phase cells. Phosphorylation and ADP-ribosylation are the only known

cell-cycle dependent post-translational modifications of histone H1. Because there is no evidence at present that poly (ADP-ribose) synthesis on nuclear proteins other than poly (ADP-ribose) polymerase is of any physiological relevance (46), we assume that the observed effects are due to the different phosphorylation state of histone H1.

The phosphorylation state of histones H1, prepared from different phases of the cell cycle, was determined by *in vivo* labelling and analysis on HEPES–histidine gels (32) (Fig. 1). Proper binding of histone H1 to the nucleosome was confirmed by electron microscopy (Fig. 3), where we observed in all cases a condensation of the minichromosomes induced by the addition of histone H1 as described earlier (41). Furthermore micrococcal nuclease digestion of reconstituted minichromosomes in comparison to salt-treated minichromosomes showed the expected increase in protection of the monomeric DNA band.

Comparison of the chromatin structure of the reconstituted minichromosomes by sedimentation studies revealed significant differences only in a narrow range, corresponding to the physiological ratio of one molecule histone H1 per nucleosome. Whereas both the completely unphosphorylated form of histone H1 prepared from G0-phase cells and the hyperphosphorylated form of histone H1 prepared from M-phase cells induce the same degree of compactness, resulting in an S-value of 80S, we determined a more retarded sedimentation for the minichromosomes reconstituted with the same amount of the moderately phosphorylated form of histone H1 prepared from S-phase cells, resulting in an S-value of only 71S (Fig. 4). As we have found that the same amount of the differentially phosphorylated forms of histone H1 is bound to the reconstituted minichromosomes, the slower sedimentation of the S-H1 chromatin is indicative for a more open chromatin structure.

The results concerning the variations in chromatin structure induced by the differentially phosphorylated forms of histone H1 do not follow a simple pattern because both unphosphorylated G0-H1 chromatin and hyperphosphorylated M-H1 chromatin have identical hydrodynamic properties, whereas only S-H1 chromatin shows a reduced sedimentation rate.

The basic N- and C-terminal tails of histone H1 in the unphosphorylated G0-H1 can neutralize negative charges of the phosphodiester backbone of linker DNA (47), which may result in a coiling or bending of the linker DNA (48,49) and in a moderate condensation of the chromatin. This situation is comparable with the condensation observed in the presence of dephosphorylated H1 (H5) in *Tetrahymena* macronuclei (19), avian erythrocytes (21) and mature sperm (20).

However, hyperphosphorylation of histone H1 during mitosis may weaken the interaction between histone tails and linker DNA (50), and this could cause a repulsion of the tails from the linker DNA and an opening of the chromatin structure. The induction of a similar chromatin structure as observed with G0-H1 reconstituted minichromosomes could be generated by a modulation of the interaction between H1 histones themselves, or between H1 and H2A (51) or H1 and H3 (52,53) bringing nucleosomes into closer contact. The degree of condensation we observe after reconstitution with mitotic histone H1 is certainly only one of the reactions required for chromatin condensation. During mitosis hyperphosphorylation of histone H1 may be required to recruit 'compaction factors' as topoisomerase II (54) or the SMC (structural maintenance of chromosomes) proteins (55) to get access to the DNA and to further condense the chromatin by an as yet unknown mechanism.

An interesting result of our studies is a clear difference in hydrodynamic properties after reconstitution of SV40 minichromosomes with histone H1 prepared from S-phase cells. As known from studies in the CHO cell cycle (8), histone H1 phosphorylation occurs during S-phase only on serine residues in the C-terminal domain of histone H1, resulting in an asymmetric charge distribution. It has been suggested that the SPKK motif, which is phosphorylated in the C-terminal domain of H1, forms β-turn secondary structures, which bind to the minor groove of DNA (56). Phosphorylation in this domain is likely to disrupt this conformation and to weaken the interaction of these sequences with DNA (57). Thus, the moderate and asymmetric phosphorylation of histone H1 may lead to a dissociation of the C-terminal histone tails from the linker DNA. The linker DNA may be more extended compared with G0-chromatin resulting in a larger distance between individual nucleosomes and in a more open chromatin structure.

Interestingly, the differences observed in chromatin structure are reflected in the replication efficiencies of the reconstituted minichromosomes. Both G0-H1 and M-H1 chromatin show a reduced replication efficiency compared with S-H1 chromatin (Fig. 6). The relaxed structure of the S-H1 chromatin could either increase the accessibility for replication factors to the DNA, or this type of H1 phosphorylation may directly affect the interaction with replication proteins. It has been shown for example that the SV40 T-Ag hexamer directly interacts with histone H1 in an ATP-dependent reaction (58). It is however not known whether this interaction is influenced by the phosphorylation of H1.

It is widely assumed that the chromatin structure is transiently disrupted during passage of the replication fork, whereby histone H1 is removed from the replication fork and reassociates with the daughter strands during maturation of the chromatin (reviewed in 59). Weakening of H1–DNA interactions could facilitate the detaching of histone H1 and by this way stimulate the movement of the replication machinery.

Thus it seems that the process of replication is not only controlled on the level of the activity of replication proteins but also on the level of the chromatin structure. These data are in agreement with *in vivo* experiments using cell mutants, which are defect in histone H1 phosphorylation (15). The decrease in H1 phosphorylation resulted in an incomplete DNA replication and a defect in chromosome condensation, indicating that histone H1 phosphorylation also plays a role during S-phase of the cell cycle.

ACKNOWLEDGEMENTS

We thank Rolf Knippers for helpful discussions and critical reading of the manuscript and Rosmarie Mettke for technical assistance. This work was supported by Sonderforschungsbereich 156 (C4).

REFERENCES

1 Thoma,F. (1988) In Kahl,G. (ed.) *Architecture of Eucaryotic Genes.* VCH Verlagsgemeinschaft mbH, Weinheim, pp. 163–185.
2 Hohmann,P. (1983) *Mol. Cell. Biochem.*, **57**, 81–92.
3 Lennox,R.W. and Cohen,L.H. (1988) In Aldolph,K.W. (ed.) *Chromosomes and Chromatin.* CRC Press, Boca Raton, Vol 1, pp. 33–56.
4 Bradbury,M. (1992) *BioEssays*, **14**, 9–16.

5 Bradbury,E.M., Inglis,R.J. and Matthews,H.R. (1974) *Nature*, **247**, 257–261.
6 Mueller,R.D., Yasuda,H. and Bradbury,E.M. (1985) *J. Biol. Chem.*, **260**, 5081–5086.
7 Gurley,L.R., Walters,R.A. and Tobey,R.A. (1975) *J. Biol. Chem.*, **250**, 3936–3944.
8 Gurley,L.R., D'Anna,J.A., Barham,S.S., Deaven,L.L. and Tobey,R.A. (1978) *Eur. J. Biochem.*, **84**, 1–15.
9 Ajiro,K., Borun,T.W. and Cohen,L.H. (1981) *Biochemistry*, **20**, 1445–1454.
10 Langan,T.A., Gautier,J., Lohka,M., Hollingsworth,R., Moreno,S., Nurse,P., Maller,J. and Sclafani,R. (1989) *Mol. Cell. Biol.*, **9**, 3860–3868.
11 Nurse,P. (1990) *Nature*, **344**, 503–508.
12 Norbury,C. and Nurse,P. (1992) *Annu. Rev. Biochem.*, **61**, 441–470.
13 Roth,S.Y. and Allis,C.D. (1992) *Trends Biochem. Sci.*, **17**, 93–98.
14 Matsumoto,Y., Yasuda,H., Mita,S., Marunouchi,T. and Yamada,M. (1980) *Nature*, **284**, 181–183.
15 Yasuda,H., Matsumoto,Y., Mita,S., Marunouchi,T. and Yamada,M. (1981) *Biochemistry*, **20**, 4414–4419.
16 Ajiro,K., Nishimoto,T. and Takahashi,T. (1983) *J. Biol. Chem.*, **258**, 4534–4538.
17 Roberge,M., Th'ng,J., Hamaguchi,J. and Bradbury,E.M. (1990) *J. Cell Biol.*, **111**, 1753–1762.
18 Th'ng,J.P., Guo,X.-W., Swank,R.A., Crissman,H.A. and Bradbury,E.M. (1994) *J. Biol. Chem.*, **269**, 9568–9573.
19 Lin,R., Cook,R.G. and Allis,C.D. (1991) *Genes Dev.*, **5**, 1601–1610.
20 Poccia,D.L. (1989) In Schatten,G. and Schatten,H. (ed.) *The Molecular Biology of Fertilization.* Academic Press, New York. Vol. l pp. 115–135.
21 Sung,M.T. (1977) *Biochemistry*, **16**, 286–290.
22 Guo,X.W., Th'ng,J.P.H., Swank,R.A., Anderson,H.J., Tudan,C., Bradbury,E.M. and Roberge,M. (1995) *EMBO J.*, **14**, 976–985.
23 Ajiro,K., Borun,T.W., Shulman,S.D., Fadden,G.M. and Cohen,L.H. (1981) *Biochemistry*, **20**, 1454–1464.
24 Lu,M.J., Dadd,C.A., Mizzen,C.A., Perry,C.A., McLachlan,D.R., Annunziato,A.T. and Allis,C.D. (1994) *Chromosoma*, **103**, 111–121.
25 Tobey,R.A. and Ley,K.D. (1971) *Cancer Res.*, **31**, 46–51.
26 Galavazi,G., Schenk,H. and Bootsma,D. (1966) *Exp. Cell Res.*, **41**, 428–437.
27 Zieve,G.W., Turnbull,D., Millis,J.M. and McIntost,J.R. (1980) *Exp. Cell Res.*, **126**, 397–405.
28 Sanders,C. and Johns,E.W. (1974) *Biochem. Soc. Trans.*, **2**, 547–550.
29 Halmer,L. and Gruss,C. (1995) *Nucleic Acids Res.*, **23**, 773–778.
30 Laemmli,U. (1970) *Nature*, **227**, 680–685.
31 Wray,W., Boulikas,T., Wray,V.P. and Hancock,R. (1981) *Anal. Biochem.*, **118**, 197–203.
32 Paulson,J.R., Mesner,P.W., Delrow,J.J., Mahmoud,N.N. and Ciesielski,W.A. (1992) *Anal. Biochem.*, **203**, 227–234.
33 Hirt,B. (1967) *J. Mol. Biol.*, **26**, 365–369.
34 Simanis,V. and Lane,D.P. (1985) *Virology*, **144**, 88–100.
35 Stillman,B.W. and Gluzman,Y. (1985) *Mol. Cell. Biol.*, **5**, 2051–2060.
36 Gruss,C., Wu,J., Koller,T. and Sogo,J.M. (1993) *EMBO J.*, **12**, 4533–4545.
37 Gruss,C. and Knippers,R. (1995) In Adolph,K.W. (ed.) *Methods in Molecular Genetics.* Academic Press, Orlando. Vol 7, pp. 101–113.
38 Thoma,F., Koller,T. and Klug,A. (1979) *J. Cell Biol.*, **83**, 402–427.
39 Pondaven,P., Meijer,L. and Beach,D. (1990) *Genes Dev.*, **4**, 9–17.
40 Krude,T. and Knippers,R. (1992) *Chromosoma*, **102**, 83–92.
41 Müller,U., Zentgraf,H., Eicken,I. and Keller,W. (1978) *Science*, **201**, 406–415.
42 Bates,D.L. and Thomas,J.D. (1981) *Nucleic Acids Res.*, **9**, 5883–5894.
43 Grunstein,M. (1990) *Annu. Rev. Cell Biol.*, **6**, 6643–678.
44 van Holde,K.E. (1988) *Chromatin.* Springer, New York.
45 Nigg,E.A. (1995) *BioEssays*, **17**, 471–480.
46 Lindahl,T., Masahiko,S.S., Poirier,G.G. and Klungland,A. (1995) *Trends Biochem Sci.*, **20**, 405–411.
47 Blank,T.A. and Becker,P.B. (1995) *J. Mol. Biol.*, **252**, 305–313.
48 Yao,J., Lowary,P.T. and Widom,J. (1990) *Proc. Natl Acad. Sci.USA*, **87**, 7603–7607.
49 Yao,J., Lowary,P.T. and Widom,J. (1990) *Biochemistry*, **30**, 8408–8414.
50 Hill,C.S., Packman,L.C. and Thomas,J.O. (1990) *EMBO J.*, **9**, 805–813.
51 Boulikas,T., Wiseman,J.M. and Garrard,W.T. (1980) *Proc. Natl Acad. Sci. USA*, **77**, 127–131.
52 Mazen,A., Hacques,M.-F. and Marion,C. (1987) *J. Mol. Biol.*, **194**, 741–745.
53 Shibata,K. and Ajiro,K. (1993) *J. Biol. Chem.*, **268**, 18431–18434.
54 Adachi,Y., Luke,M. and Laemmli,U.K. (1991) *Cell*, **64**, 137–148.
55 Strunnikov,A.V., Hogan,E. and Koshland,D. (1995) *Genes Dev.*, **9**, 587–599.
56 Churchill,M.E.A. and Suzuki,M. (1989) *EMBO J.*, **8**, 4189–4195.
57 Suzuki,M., Sohma,H., Yazawa,M., Yagi,K. and Ebashi,S. (1990) *J. Biochem.*, **108**, 356–364.
58 Ramsperger,U. and Stahl,H. (1995) *EMBO J.*, **14**, 3215–3225.
59 Gruss,C. and Knippers,R. (1996) In Cohn,W.E. and Moldave,K. (ed.) *Progress In Nucleic Acid Research and Molecular Biology.* Academic Press, San Diego. Vol 52, pp. 337–365.