The EMBO Journal vol.12 no.1 pp.115-126, 1993

A model for chromatin opening: stimulation of topoisomerase II and restriction enzyme cleavage of chromatin by distamycin

Emmanuel Käs, Leonora Poljak, Yasuhisa Adachi and Ulrich K.Laemmli

Departments of Biochemistry and Molecular Biology, University of Geneva, 30 Quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland

Communicated by U.K.Laemmli

Histone H1 preferentially and cooperatively binds scaffold-associated regions (SARs) *in vitro* via specific interactions with the numerous short A+T-rich tracts (A-tracts) contained in these sequences. Selective titration of A-tracts by the oligopeptide distamycin abolishes this interaction and results in a redistribution of H1. Similarly, treatment of intact cells and isolated nuclei with distamycin specifically enhances cleavage of inter-nucleosomal linkers of SARs by topoisomerase II and restriction enzymes. The increased accessibility of these linkers is thought to result from the unfolding (or opening) of the chromatin fiber and to be due to a reduced occupancy by histone H1. Chromatin extraction and H1 assembly experiments support this view. We discuss a model whereby open, H1-depleted chromatin regions may be generated by titration of A-tracts by putative distamycin analogues; this local opening may spread to adjacent regions assuming highly cooperative H1-H1 interactions in chromatin.

Key words: chromatin/distamycin/histone H1/SAR/satellite/topoisomerase II

Introduction

The enormous eukaryotic genome, unlike that of prokaryotes, is not readily accessible for genetic readout—its genetic information content is censored by compaction into nucleosomes and supra-nucleosomal structures. The transcriptional repertoire of a nucleus is determined both by chromatin structure and by the availability of transcription activators. The transcriptional activation of inactive, compact chromatin is preceded or accompanied by both local and long range structural alterations of the chromatin fiber (reviewed by Elgin, 1988; Gross and Garrard, 1988; Grunstein, 1990).

A wealth of data has demonstrated the association of local perturbations of the chromatin fiber, classically defined as DNase I hypersensitive sites (Wu *et al.*, 1979), with transcriptionally active genes. Nuclease hypersensitive sites are gaps in the nucleosomal array that localize to the *cis*-regulatory elements of active genes. Longer range structural perturbations of the chromatin fiber, classically defined by a general sensitivity of the chromatin to DNase I (Weintraub and Groudine, 1976), usually extend far beyond the actual boundaries of active or potentially active genes, thus delimiting chromatin domains. The mechanisms underlying the activation of such chromatin domains, which are thought to represent an early preparative step for genetic readout, are not understood.

In contrast to the thick, transcriptionally inert chromatin fiber, active chromatin is unfolded to a more extended configuration (e.g. Ericsson *et al.*, 1990). Histone H1 is thought to be primarily responsible for the higher order organization of nucleosomes into the thick chromatin fiber (Thoma *et al.*, 1979). Histone H1 interacts with chromatin primarily via the linker DNA where it enters and exits the nucleosome dyad, forming a polymer as observed in cross-linking experiments (Clark and Thomas, 1986; De Bernardin *et al.*, 1986). A number of studies have illustrated the involvement of H1 in the generalized repression of gene activity (reviewed by Weintraub, 1985; Zlatanova, 1990). Studies on the developmental regulation of the 5S RNA genes in *Xenopus* strongly implicate histone H1 in the repression of the oocyte 5S RNA genes (Schlissel and Brown, 1984; Wolffe, 1989; Shimamura *et al.*, 1989). Other biochemical studies indicate that H1 or its analogue, H5, is depleted in regions containing active or potentially active class II genes (Kamakaka and Thomas, 1990; Tazi and Bird, 1990).

Histone H1 binds to naked DNA and to chromatin in a highly cooperative manner (Lennard and Thomas, 1985). Such a mode of binding could be a key parameter in the general repression of chromatin (Renz, 1975; Alberts *et al.*, 1977; Weintraub, 1985). If the loss or alteration of H1-H1 interactions results in both the unfolding of the chromatin fiber, a process which we refer to as chromatin opening, and in a generalized de-repression of transcription, an important question arises: what is the mechanism whereby certain chromatin domains are either activated or repressed by the specific, regional loss or by the assembly of histone H1, respectively?

One attractive possibility by which specific opening or closing of chromatin regions could be achieved is to propose the existence of DNA elements which serve as nucleation sites for the cooperative assembly of histone H1. We have previously proposed that scaffold-associated regions (SARs), which are DNA regions thought to define the bases of chromatin loops (reviewed by Gasser and Laemmli, 1987a), may serve as nucleators of histone H1 assembly (Izaurralde *et al.*, 1989; Käs *et al.*, 1989). This suggestion is based on experiments which demonstrated that SARs serve as tight binding sites for histone H1 from where cooperative assembly of H1 is propagated onto flanking DNA.

Histone H1 binds preferentially to AT-rich DNA (Renz, 1975) and the SAR-dependent nucleation of H1 assembly, as well as the specific SAR-scaffold interaction, are mediated by numerous short oligo(dA)·oligo(dT) tracts, here referred to as A-tracts, that are common to all SARs (Käs *et al.*, 1989). This conclusion is based on experiments with synthetic SAR sequences and with the antibiotic distamycin A, which binds specifically to the minor groove of dA·dT base pairs (Van Dyke *et al.*, 1982; Fox and Waring, 1984).

This drug inhibits the preferential binding of histone H1 to SAR-containing DNA, leading to a non-selective redistribution of H1 between SAR and non-SAR DNA. In contrast, chromomycin A_3, which selectively binds the minor groove of GC-rich DNA (Van Dyke and Dervan, 1983; Fox and Howarth, 1985; Gao and Patel, 1989), does not interfere with SAR–protein interactions *in vitro* (Käs *et al.*, 1989). Our current view is that A-tracts interspersed over several hundred base pairs of DNA constitute an SAR. If SARs or other DNA regions rich in A-tracts also serve as nucleators for H1 assembly when they are compacted into chromatin, then titration of A-tracts by distamycin should induce chromatin opening due to a redistribution of histone H1. In support of this prediction, treatment of living mouse cells with distamycin A dramatically reduces the condensation of centromeric heterochromatin (Radic *et al.*, 1987).

In the present study the mechanism of chromatin opening and closing was analyzed, both *in vivo* and *in vitro*, by using topoisomerase II as a probe to assess the occupancy of the inter-nucleosomal linkers of both the *Drosophila* histone SAR and the heterochromatic satellite III repeats by linker-bound proteins. We find that topoisomerase II cleavage at these linkers is most sensitive to steric hindrance by histone H1. The results of our experiments support the view that SAR (or SAR-like) sequences are implicated in the regional opening or closing of chromatin, presumably through their ability to serve as regulators for the histone H1-induced condensation of chromatin.

Results

We have studied the phenomenon of chromatin opening and closing in *Drosophila* Kc cells and nuclei at two genetic loci. The 1.688 g/cm^3 (satellite III) repeat serves as the prototype of a transcriptionally inert heterochromatic region, and the histone gene cluster represents a transcriptionally active domain whose chromatin structure has been well characterized (Worcel *et al.*, 1983). The chromatin opening events observed at these loci are thought to be representative of the initial events along the pathway to transcriptional activation. It is difficult to monitor the initial steps of chromatin activation by relying solely on a transcriptional assay, since a multitude of factors which interact consecutively with chromatin are involved. We have therefore used topoisomerase II, as well as restriction enzymes, as convenient probes to monitor the unfolding of chromatin. This chromatin opening process is operationally accompanied by a significant enhancement of cleavage in the nucleosomal linker DNA.

Distamycin induces chromatin opening of the heterochromatic satellite III repeat

The *Drosophila* 1.688 g/cm^3 satellite III characterized by Brutlag and collaborators (for a review, see Brutlag, 1980) is a prominent target for topoisomerase II cleavage *in vivo* (Käs and Laemmli, 1992). Each 359 bp monomer unit of the SAR-like satellite III sequence is associated with two nucleosomes that are phased relative to a strong sequence-specific topoisomerase II cleavage site located in one of the two nucleosome linkers (referred to as linker sites, see diagram below Figure 1A). The phasing of the cleavage site results in a topoisomerase II-induced DNA ladder with a precise repeat length of 359 bp (see lane 2 of Figure 1A).

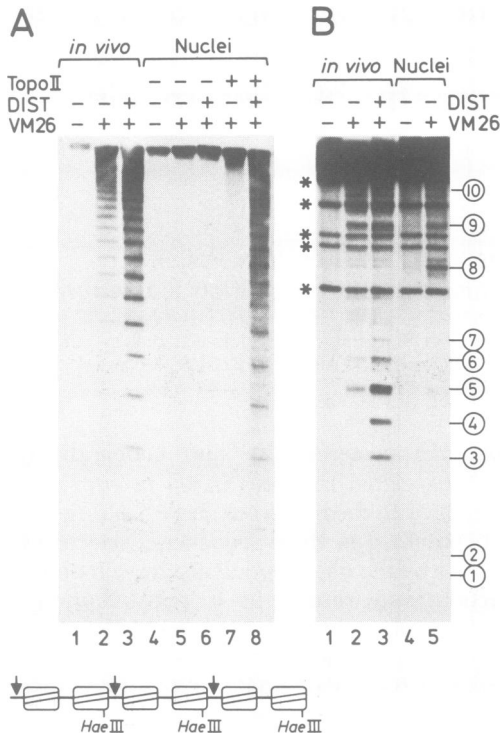

Fig. 1. *In vivo* and *in vitro* topoisomerase II cleavage in satellite III and histone repeats. Panel **A**: Topoisomerase II cleavage in satellite III repeats was analyzed in Kc cells (lanes 1–3) and nuclei (lanes 4–8) as described in Materials and methods. Lanes 1–3 contain DNA from Kc cells treated with no drugs, VM26 alone or VM26 plus 25 μM distamycin ('DIST') as indicated by plus and minus signs above each lane. DNA samples in lanes 4–8 were purified from isolated Kc nuclei incubated with VM26, distamycin (3.5 μM) and 1.5 μg of purified topoisomerase II ('Topo II', approximately 1 dimer per 2000 bp DNA) as indicated above each lane. The diagram shown below the gel represents six nucleosomes spanning three adjacent *Hae*III 359 bp satellite III monomers. The location of each unique topoisomerase II cleavage site is indicated by the arrows above the diagram; the actual cleavage sequence determined at this site is 5′-GATGAC↓CCCCC-TCCTTAC with cleavage occurring six bases downstream on the lower strand (Käs and Laemmli, 1992). Panel **B** shows topoisomerase II cleavage products detected in the histone gene cluster *in vivo* and in nuclei; lanes 1–5 contain DNA samples identical to those in lanes 1–5 of panel A. Samples were analyzed as described in Materials and methods. Cleavage sites detected in the histone repeat are numbered according to the map shown in Figure 2C. Note that bands marked with asterisks below the main 5 kb genomic fragment are minor variants of the histone repeat detected by the probe and do not correspond to topoisomerase II cleavage products.

Addition of distamycin to the cell dramatically stimulates cleavage at this linker site as is evident from the increased intensity of the cleavage products and the extension of the digestion ladder to lower repeat units including the 359 bp monomer (Figure 1A, lane 3). We estimate that distamycin stimulates cleavage by a factor of ~10.

Interestingly, isolated nuclei are very poor substrates for cleavage at the satellite III linker site (lane 5) even after addition of purified topoisomerase II (lane 7). However, cleavage is completely restored by addition of an optimal concentration of distamycin (compare lanes 7 and 8 in Figure 1A). The distamycin-induced cleavage pattern in nuclei is very similar to the ladder observed *in vivo*, although minor differences can be noted. Both patterns show a major repeat ladder of 359 bp, but the cleavage induced by

distamycin in nuclei reveals an additional minor ladder with half this repeat length. This indicates that in isolated nuclei topoisomerase II is able to cleave, at least partially, in both of the nucleosome linkers found in each repeat. As will be demonstrated below, the loss of cleavage activity observed in isolated nuclei is not due to a significant loss of the endogenous topoisomerase II. Rather, this phenomenon appears to result from the redistribution of a linker protein, presumably histone H1, during the procedure used to isolate nuclei.

The striking stimulation of topoisomerase II cleavage induced by distamycin in cells and in nuclei was initially somewhat surprising since topoisomerase II cleaves naked DNA at an *in vitro* consensus sequence that has a relatively AT-rich core (Sander and Hsieh, 1985). As a consequence, cleavage at such sequences *in vitro* is quite sensitive to inhibition by distamycin (Adachi *et al.*, 1989). The *in vitro* consensus sequence, although abundantly represented in SARs (Cockerill and Garrard, 1986; Gasser and Laemmli, 1986), is not used in the cell. Cleavage in chromatin occurs instead at an *in vivo* 'consensus' that is characterized by a prominent GC-rich core flanked by A-tracts (Käs and Laemmli, 1992). These relatively GC-rich cleavage sites are not titrated by the dose of distamycin that is optimal for cleavage enhancement in chromatin. The actual sequence of the satellite topoisomerase II cleavage site is composed of a core sequence with a six-base stagger (CCCCCT) embedded in A-tract DNA (see Käs and Laemmli, 1992 and legend to Figure 1A).

Distamycin strongly stimulates topoisomerase II cleavage at inter-nucleosomal linker sites but not at 'open' sites

The topoisomerase II cleavage pattern in the histone gene cluster is more complex than that of the satellite III repeat. Our detailed analysis of topoisomerase II cleavage *in vivo* in the *Drosophila* histone gene and satellite III repeats identified two distinct classes of major topoisomerase II cleavage sites (summarized in Figure 2C; see also Käs and Laemmli, 1992). One class consists of 'open' sites (sites 1, 8, 9 and 10, Figure 1B) which co-localize with DNase I hypersensitive regions. These sites fall in regions of chromatin that display a normal G+C sequence content. The other class of cleavage sites maps to a select number of consecutive nucleosome linker sites (sites 2 and 3–7, Figure 1B) in the AT-rich SAR region, much like the one described above for the SAR-like satellite III repeat (for details see Käs and Laemmli, 1992). These linker sites fall between the regularly spaced nucleosomes that were previously mapped in the histone SAR region (Worcel *et al.*, 1983). Note that bands marked with asterisks in this and subsequent figures correspond to minor variants of the histone gene repeat detected by the probe and are not topoisomerase II cleavage products (Figure 1B, lane 1).

Again, isolated nuclei were poor substrates for cleavage at the nucleosome linker sites of the histone gene repeat, although cleavage at open sites was normal. This is evident from a comparison of the topoisomerase II cleavage patterns observed *in vivo* and *in vitro* (compare lanes 2 and 5, Figure 1B). In contrast to the behavior of the open sites, cleavage at the linker sites is not detectable in nuclei. Note that the cleavage behavior at site 8 is unusual: strong cleavage is observed at site 8 in nuclei but not in intact cells.

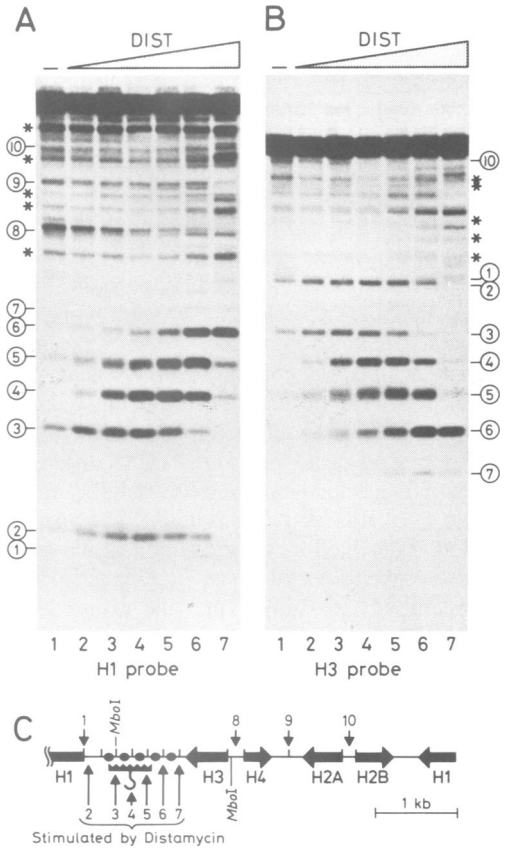

Fig. 2. Dose-dependent reactivation of linker-site cleavage by distamycin in isolated nuclei. Panel **A**: nuclei were incubated with VM26 and 3.5 μg of purified topoisomerase II (lane 1) plus 1, 2, 3.5, 5, 7.5 or 15 μM distamycin (lanes 2–7). Topoisomerase II cleavage products were detected as in Figure 1B, using a probe spanning the 5′ coding region of the H1 gene and proximal to site 1 (H1 probe). Panel **B** shows identical DNA samples digested with *Ava*I (which cuts in the H3–H4 intergenic spacer and in the H2A gene) and hybridized to a probe spanning the 5′ flank and coding region of the H3 gene (H3 probe). The H3 probe is proximal to site 7. The use of higher amounts of topoisomerase II results in cleavage at additional minor sites which were not studied further. Panel **C** shows the location of *in vivo* topoisomerase II cleavage sites in the major *Drosophila* histone gene repeat, which is represented as a 5 kb *Hin*dIII fragment. Arrows above the line represent cleavage sites located in open regions, arrows below the line indicate the location of nucleosomal linker sites (Käs and Laemmli, 1992). The minimal 657 bp SAR located between the H1 and H3 genes is represented by the hooked bar. The *Mbo*I restriction sites shown are discussed in Figure 7.

As reported previously, distamycin selectively stimulates cleavage *in vivo* by a factor of ~3- to 7-fold at the nucleosome linker sites, although the intensity of cleavage at the open sites remains unaltered (compare lanes 2 and 3 in Figure 1B; see also Käs and Laemmli, 1992). Again, as was the case for satellite III, distamycin restores linker-site cleavage that is lost in isolated nuclei.

In order to determine the amount of distamycin required to activate cleavage at linker sites, we incubated isolated nuclei with a fixed amount of purified topoisomerase II and different drug concentrations (Figure 2). Addition of topoisomerase II in the absence of distamycin does not significantly increase cleavage at the linker sites (lane 1 of Figure 2A). In the presence of distamycin, however, a marked dose-dependent activation of cleavage at the linker sites is observed (Figure 2A, lanes 2–7). This activation

is maximal at intermediate levels (2−5 μM, lanes 3−5) and is eventually quenched by higher concentrations of the drug. The stimulation by distamycin is remarkably selective for linker-site cleavage; no enhancement of cleavage at the major open sites is observed at intermediate drug concentrations. We observed a similar distamycin-dependent activation of cleavage by the endogenous topoisomerase II in the absence of additional purified enzyme (data not shown).

Activation of linker-site cleavage is likely to be a consequence of the selective interaction of distamycin with the A-tracts flanking GC-rich cleavage sites, while quenching of cleavage is due to the eventual titration of the actual DNA cleavage site at high drug concentrations (Adachi *et al.*, 1989 and Discussion). The progressive activation and quenching of cleavage at sites 3−7 detected by the site 1-proximal H1 probe (Figure 2A) occur in a remarkably ordered fashion that closely parallels the increasing GC-richness of these sites (see Table I in Käs and Laemmli, 1992). An identical progression is detected by hybridization of the same DNA samples to a probe proximal to site 7 (H3 probe; Figure 2B). This rules out the possibility that the ordered activation observed is due to the polarity of the probe relative to the different SAR cleavage sites, and also indicates that the cleavage products do not originate from a small subpopulation of multiply cleaved templates. Moreover, addition of higher amounts of topoisomerase II leads to correspondingly more efficient cleavage at the histone linker sites in the presence of distamycin (data not shown).

In summary, topoisomerase II cleavage at the linker sites associated with the histone SAR and satellite III repeats is lost upon isolation of nuclei, in contrast to cleavage at the open chromatin regions. The addition of distamycin re-opens the inter-nucleosomal linker sites, allowing prominent topoisomerase II cleavage. The inaccessibility of the SAR sites to topoisomerase II in nuclei indicates that the structure of these chromatin regions is altered in the course of nuclear isolation. The increase in accessibility induced by distamycin, which restores the *in vivo* cleavage pattern, suggests that the drug might reverse perturbations introduced during nuclear isolation, which in turn might reflect the loss or inactivation of protein factors that kept these regions accessible to topoisomerase II cleavage *in vivo*. The loss of linker-site cleavage in nuclei illustrates the potential shortcomings of *in vitro* approaches, such as crosslinking and immunological techniques, to study the distribution of proteins that interact with nucleosome linkers. The lack of cleavage at nucleosomal linker sites observed in isolated nuclei is not due to a significant loss of topoisomerase II activity, since cleavage at the open sites occurs at about the same level as that observed *in vivo*, or even higher in the case of site 8. Moreover, addition of purified topoisomerase II results in greatly increased cleavage at the open sites but not at the nucleosome linker sites; in the presence of distamycin, however, cleavage occurs predominantly at the linker sites (Figure 2 and data not shown).

Histone H1 is the predominant protein found in nucleosome linkers and accessibility of the linker DNA, as assayed here by enzymatic cleavage, is likely to be sensitive to the presence of this histone. The loss of topoisomerase II cleavage at nucleosome linker sites might reflect a tight association of SARs with histone H1 in nuclei. Previous studies have shown that H1 specifically binds to SARs *in vitro* and that this preferential interaction is inhibited by distamycin (Izaurralde *et al.*, 1989; Käs *et al.*, 1989). The data shown above thus suggest that histone H1 might impede topoisomerase II cleavage of the nucleosome linker sites and could be selectively displaced from these sites by distamycin.

A sub-fraction of histone H1 binds tightly to chromatin via A-tracts

If histone H1 preferentially interacts with the A-tracts of SARs in chromatin, thereby hindering topoisomerase II cleavage at the adjacent GC-rich sites, then a more tightly bound sub-fraction of H1 might exist, which could be selectively displaced from these binding sites by distamycin.

We tested this idea by extracting nuclei and chromosomes with the polymer poly-glutamic acid. Poly-glutamic acid increases the solubility of chromatin in the presence of histone H1 and can promote the ordered assembly of H1-containing nucleosomes *in vitro* (Künzler and Stein, 1983; Stein and Künzler, 1983). This polymer is also an effective agent for the selective extraction of H1 from chromatin (Gasser *et al.*, 1986). The equilibrium between assembly or extraction of histone H1 is simply governed by the ratio of H1 to poly-glutamic acid.

Figure 3 shows the protein pattern of HeLa chromosomes that were extracted with poly-glutamic acid under a variety of conditions. Incubation with poly-glutamic acid results in the solubilization of >90% of the histone H1 protein (lanes 5 and 11). The extraction is quite selective for H1, in keeping with previous observations (Gasser *et al.*, 1986). When chromosomes were incubated with poly-glutamic acid and 2.5 μM chromomycin, no more H1 was released than by poly-glutamic acid alone (lanes 6 and 12). Incubation with poly-glutamic acid and 2.5 μM distamycin, however, resulted in the quantitative removal of the remaining tightly bound H1 sub-fraction (lanes 7 and 13). Indeed, the additional extraction induced by distamycin is quite specific for H1. The only other proteins that appear to be released are minor amounts of the core histones, probably due to a slight dissociation of the extracted pellet. The extent of core histone extraction is variable and, most importantly, stimulation of topoisomerase II cleavage activity is observed in the absence of poly-glutamic acid (see Figure 1A, lane 8 and Figure 2, lanes 3−5 of panels A and B), under conditions where core histones are not disturbed (Figure 3, lanes 4 and 10). Identical extraction profiles were obtained using interphase nuclei isolated from HeLa or *Drosophila* cells (data not shown).

Extraction of chromosomes with 2.5 μM chromomycin or distamycin in the absence of poly-glutamic acid did not result in the release of any proteins other than those observed after incubation in buffer alone (compare supernatant lanes 8−10). The drug concentrations used in these experiments represent a molar ratio of drug to DNA base pairs of ~0.1 (r = 0.1); this represents the same ratio at which near optimal activation of topoisomerase II linker-site cleavage is observed in nuclei (see Figure 2B, lane 5).

In summary, poly-glutamic acid serves as an effective competitor for the binding of H1 to the majority of its sites in intact chromatin. A small amount of H1 remains bound to chromatin under these conditions, most probably reflecting association with tighter binding sites. This residual fraction of H1 (~5−10% of total) is selectively solubilized by distamycin but not chromomycin, suggesting that it is associated with A-tracts such as those found in SARs. This

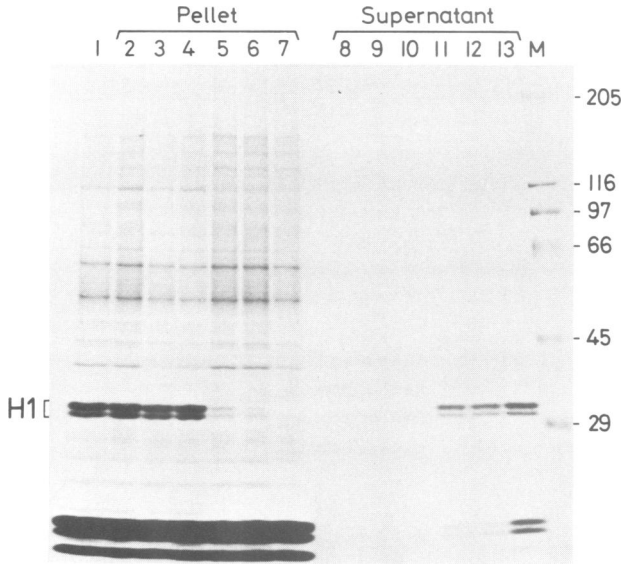

Fig. 3. Extraction of histone H1 from metaphase chromosomes by poly-glutamic acid. The extraction of chromosomal proteins by poly-glutamic acid and DNA minor groove binders was analyzed by SDS−PAGE. HeLa metaphase chromosome clusters were extracted with buffer alone (lanes 2 and 8), 2.5 μM chromomycin (lanes 3 and 9), 2.5 μM distamycin (lanes 4 and 10), 1 mg/ml poly-glutamic acid (lanes 5 and 11), 1 mg/ml poly-glutamic acid plus 2.5 μM chromomycin (lanes 6 and 12), or 1 mg/ml poly-glutamic acid plus 2.5 μM distamycin (lanes 7 and 13). The proteins remaining in the pellet fraction (lanes 2−7) or those released in the supernatant (lanes 8−13) were separated on a 7.5−15% SDS−polyacrylamide gradient gel and stained with Coomassie blue. Lane 1 shows the total protein pattern of unextracted chromosomes. The position of histone H1 and the size of molecular weight standards (lane M) are indicated next to the outermost lanes. It should be noted that the extent of extraction of histone H1 by poly-glutamic acid alone is strongly dependent on the composition of the buffer and particularly on the concentration of divalent cations (L.Poljak, unpublished observations).

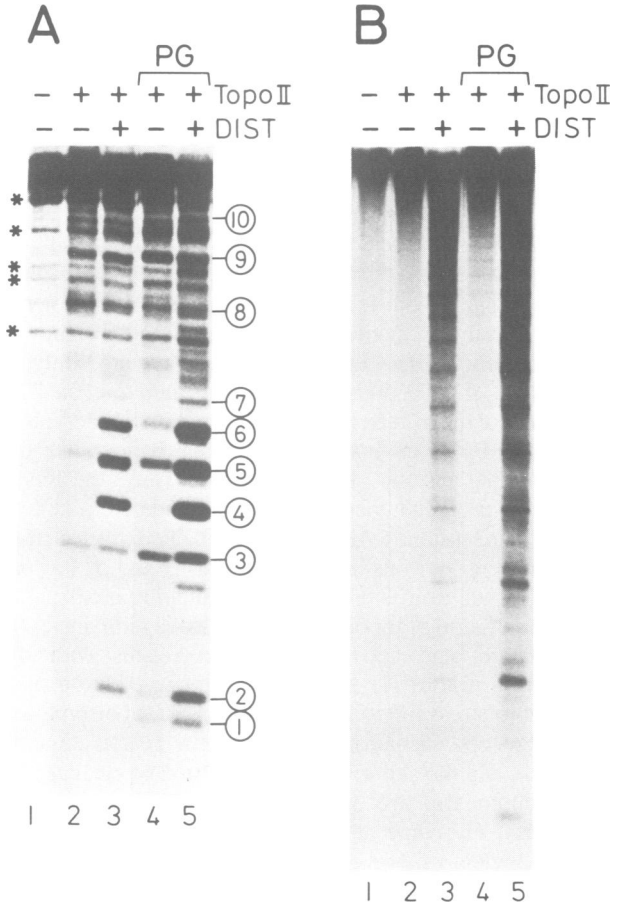

Fig. 4. Selective removal of histone H1 activates topoisomerase II cleavage. Topoisomerase II cleavage was analyzed in nuclei extracted with poly-glutamic acid and distamycin. DNA samples were analyzed as described in Figure 1 and to detect VM26-induced topoisomerase II cleavage products in the histone (panel **A**) or satellite III (panel **B**) repeats. Topoisomerase II (2.5 μg) and distamycin (2.5 μM) were also added to some of the samples as indicated by plus and minus signs above each lane. Samples were extracted with buffer alone (lanes 1−3) or with buffer containing 1 mg/ml poly-glutamic acid ('PG', lanes 4 and 5). Cleavage sites in the histone repeat are numbered as in Figure 2C.

interpretation is consistent with our observation that histone H1 still preferentially binds SARs *in vitro* in the presence of poly-glutamic acid and is selectively dissociated by distamycin, but not chromomycin (L.Poljak, unpublished results).

Poly-glutamic acid stimulates linker-site cleavage by topoisomerase II

If loss of topoisomerase II cleavage at linker sites in isolated nuclei is due to the selective binding of histone H1, then poly-glutamic acid should at least partially activate cleavage at these sites. Partial activation is expected as H1 might bind tightly but with different affinities to the different nucleosome linkers of the SAR. Important for the feasibility of the following experiments was the observation that the presence of a large excess of poly-glutamic acid does not interfere negatively with topoisomerase II activity.

Figure 4A compares the activation of topoisomerase II cleavage by distamycin and poly-glutamic acid in isolated nuclei. When nuclei are incubated with distamycin and purified topoisomerase II, cleavage in the nucleosome linker sites of the histone SAR is stimulated ~10-fold relative to the levels observed in the absence of the drug (compare lanes 2 and 3). A comparison of lanes 2, 3 and 4 in Figure 4A demonstrates that poly-glutamic acid can partially mimic the effect of distamycin. Incubation of nuclei with poly-glutamic acid results in a strong stimulation of topoisomerase II cleavage at sites 3 and 5, a lesser stimulation at sites 1, 2 and 6, and no stimulation at site 4.

When nuclei are incubated with both poly-glutamic acid and distamycin, an even greater activation of topoisomerase II cleavage is observed than with either reagent alone (compare lanes 3 and 5 of Figure 4A). The additive enhancement of cleavage resulting from the combination of distamycin and poly-glutamic acid occurs mostly in the nucleosome linker sites of the SAR region. Note that cleavage at site 4, which is resistant to activation by poly-glutamic acid alone, is impressively enhanced if distamycin is included (>150-fold, compare lanes 4 and 5). Under these conditions, addition of more topoisomerase II leads to the quantitative cleavage of the 5 kb histone repeat, predominantly at sites 3−6 in the SAR region (data not shown).

Figure 4B shows DNA samples identical to those shown in Figure 4A, but probed for the satellite III repeat. Again, topoisomerase II cleavage at the satellite III nucleosome linker sites is suppressed in isolated nuclei (lanes 1 and 2)

but can be activated by distamycin (lane 3). In this case as well, poly-glutamic acid can partially mimic the distamycin effect. The addition of this polymer results in a more developed cleavage ladder (compare lanes 2 and 4). Incubation with both poly-glutamic acid and distamycin results in a dramatic activation of topoisomerase II cleavage, presumably due to the loss of linker-bound proteins in this heterochromatic region (lane 5).

The significant and selective activation of cleavage induced by poly-glutamic acid at some but not all nucleosome linker sites is consistent with the behavior of the majority of the histone H1 in the extraction experiments shown in Figure 3. In contrast, those cleavage sites that are activated only in the presence of poly-glutamic acid and distamycin, such as linker-site 4 of the histone SAR and the satellite III site, might be occupied by linker-bound proteins that behave like the tightly binding histone H1 sub-fraction. Thus, the linker protein (or proteins) that presumably hinders linker-site cleavage in the histone SAR region is partially extracted from certain nucleosome linkers, such as sites 3 and 5, but not extracted at all from site 4, by poly-glutamic acid.

The stimulation of topoisomerase II cleavage in nuclei by poly-glutamic acid and distamycin correlates with the extraction profile of H1 from chromatin. In addition, it is important to stress the following: the loss of topoisomerase II cleavage upon isolation of nuclei, the reactivation by distamycin and the site-selective stimulation of cleavage by poly-glutamic acid are only observed at the nucleosome linker sites. Cleavage at the open sites (DNase I hypersensitive regions) shows a different pattern of behavior; no significant inhibition or activation of cleavage by distamycin and/or poly-glutamic acid is observed.

Linker-site cleavage is inhibited by histone H1 assembly

The spacing between the topoisomerase II cleavage sites observed at nucleosome linkers in cells and in nuclei is similar and is not altered by the presence of distamycin and/or poly-glutamic acid. This indicates that significant nucleosome sliding does not occur during these experimental manipulations. In addition, the ionic strength of the buffer chosen for these *in vitro* cleavage experiments ($\sim$0.12 M), as well as the poly-glutamic acid concentration used for the selective linker-site activation (1 mg/ml), correspond closely to the experimental conditions that allow assembly of histone H1 onto chromatin (Künzler and Stein, 1983; Stein and Künzler, 1983; Stein and Mitchell, 1988). These authors have demonstrated that poly-glutamic acid can mediate H1-dependent assembly of native chromatin with the correct inter-nucleosomal spacing. Thus, if linker-site activation is due to extraction of histone H1 by poly-glutamic acid, then addition of purified H1 to such samples should drive the reverse reaction—H1 assembly. The addition of H1 should not, however, affect topoisomerase II cleavage at the open sites.

In the following experiments, nuclei were treated with poly-glutamic acid to increase basal levels of topoisomerase II cleavage at the histone SAR sites and the topoisomerase II cleavage pattern was analyzed after addition of increasing amounts of histone H1. The selective induction by poly-glutamic acid of linker-site cleavage at SAR sites 3, 5 and 6 (but not at site 4) is shown again in lane 1 of Figure 5A. The progressive addition of histone H1 results in a strong dose-dependent inhibition of cleavage at these nucleosome

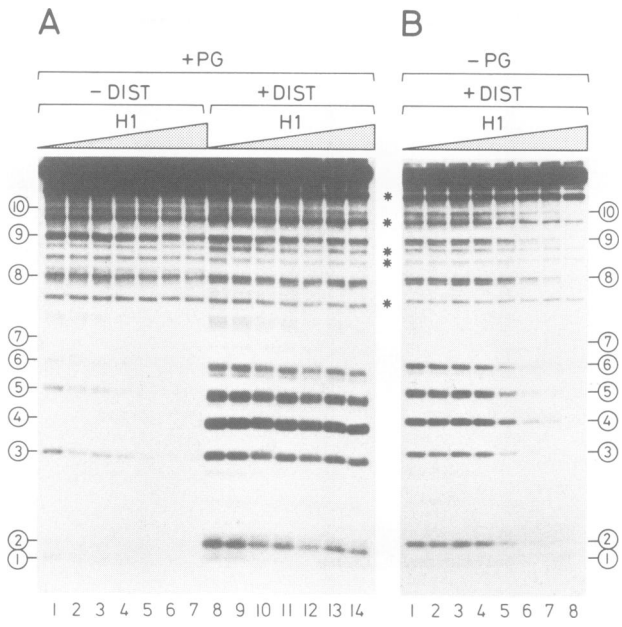

Fig. 5. Topoisomerase II cleavage in the histone SAR is repressed by H1 assembly. Kc nuclei were extracted with poly-glutamic acid and incubated with increasing amounts of histone H1. Topoisomerase II cleavage products in the histone gene cluster were analyzed as in Figure 1B. Panel **A**: DNA samples were purified from nuclei extracted with poly-glutamic acid (0.5 mg/ml) and incubated with 2 μg of purified topoisomerase II (corresponding to one dimer per 1500 bp DNA). In addition, nuclei were incubated with increasing amounts of purified histone H1 in the absence (lanes 1−7) or presence (lanes 8−14) of 2.5 μM distamycin. The amounts of histone H1 added were 0.25, 0.5, 1, 2.5, 5 and 10 μg (lanes 2−7 and lanes 9−14), corresponding approximately to a 0.5- to 20-fold excess over endogenous levels. Cleavage sites 1−10 are numbered according to the map in Figure 2C. Panel **B**: Samples in lanes 1−8 were purified from nuclei incubated with 1.5 μg purified topoisomerase II in the presence of 3.5 μM distamycin. Samples in lanes 2−8 were additionally incubated with 0.025, 0.05, 0.1, 0.25, 0.5, 1 or 2.5 μg purified histone H1, corresponding approximately to a 0.05- to 5-fold excess over endogenous levels.

linker sites (lanes 2−7). From a densitometric analysis of the gel, we estimate that cleavage is inhibited by a factor of $\sim$10 at the highest H1 concentration. No inhibition was observed following addition of an equivalent amount of bovine serum albumin (data not shown). This H1-dependent inhibition is specific for the linker sites; cleavage at open sites (sites 8, 9 and 10) is only marginally inhibited by a factor of $\sim$1.5. Thus, under these conditions, addition of histone H1 mimics the selective loss of accessibility of linker sites observed in isolated nuclei.

As an important control, we also examined the H1 assembly reaction in the presence of distamycin. Lanes 9−14 demonstrate that the addition of increasing amounts of H1 does not lead to significant inhibition of cleavage in the presence of this drug. The fact that distamycin renders topoisomerase II cleavage at linker sites refractory to H1 assembly suggests that the A-tracts in the SAR might be directly involved in H1 assembly in chromatin, especially since H1 appears to have no effect on topoisomerase II cleavage at the open sites, which do not contain A-tracts. We observed qualitatively similar results (selective H1-dependent closing which can be inhibited by distamycin) when we analyzed identical DNA samples for cleavage at the satellite III repeats (data not shown).

Free histone H1 binding sites exist in nuclei

The distamycin-stimulated topoisomerase II cleavage at linker sites in the absence of poly-glutamic acid is thought to be due to a redistribution of H1 from its preferred binding sites in SARs to free binding sites. This would be a simple extension of our previous observations with naked DNA: *in vitro*, distamycin induces a non-selective redistribution of histone H1 between SAR and non-SAR DNA sequences alike (Käs *et al.*, 1989). In accordance with the notion that free H1 binding sites exist in nuclei, we observed that titration of nuclei with H1 (in the absence of poly-glutamic acid) progressively inhibits distamycin-induced cleavage by topoisomerase II, albeit in a non-selective manner at all sites (Figure 5B). This experiment also serves as an important control; the selective inhibition of linker-site cleavage by H1 assembly described in Figure 5A is observed only in the presence of poly-glutamic acid. This polymer serves as a competitor and mediator for the preferential interaction of H1 in chromatin with the linker sites as opposed to the open sites. By titrating the excess H1, poly-glutamic acid thus provides a means to distinguish experimentally between the two classes of binding sites. In the absence of poly-glutamic acid, addition of excess H1 to nuclei results in the inhibition of topoisomerase II cleavage at all sites.

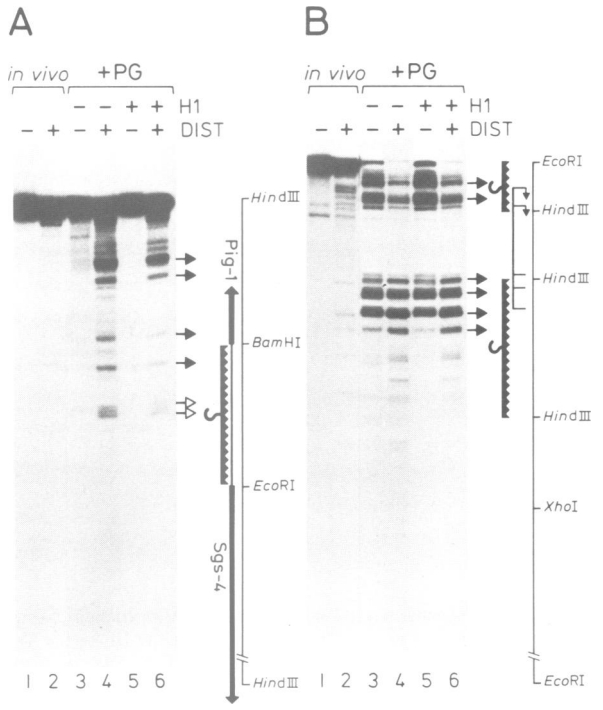

Fig. 6. Topoisomerase II cleavage in inactive and active chromatin. DNA samples were analyzed as described in Materials and methods to visualize topoisomerase II cleavage products in the *Sgs-4* (panel **A**) and actin 5C (panel **B**) loci. In each panel, identical DNA samples were purified from cells treated with no drugs (lane 1) or with VM26 and 25 μM distamycin (lane 2), or from nuclei extracted with 1 mg/ml poly-glutamic acid and incubated with 10 μg purified topoisomerase II. Distamycin (2.5 μM) and purified histone H1 (5 μg) were also added to the samples as indicated above each panel. A map of the 3.4 kb *Sgs-4* and 9.2 kb actin 5C genomic fragments detected is shown to the right of each panel. Horizontal dark arrows indicate the location of major topoisomerase II cleavage sites. Panel **A** shows the location of the divergent *Sgs-4* and *Pig-1* genes (Saumweber *et al.*, 1990). The two clear arrows correspond to topoisomerase II cleavage sites at positions −405 and −480 that co-map with DNase I hypersensitive sites in active *Sgs-4* chromatin (Shermoen and Beckendorf, 1982). Panel **B** shows the actin 5C gene with its two transcription start sites (arrows) and three polyadenylation sites indicated by horizontal lines (Bond and Davidson, 1986). The upstream SAR associated with the *Sgs-4* gene (Gasser and Laemmli, 1986) and the 5′ and 3′ SARs associated with the actin 5C gene (Mirkovitch *et al.*, 1988) are represented by wavy lines with a hook.

Activation of topoisomerase II cleavage in inactive chromatin

Does the extent of topoisomerase II cleavage reflect the transcriptional activity (or potential) of the chromatin region probed? We studied topoisomerase II cleavage at the glue protein gene, *Sgs-4*, which is transcriptionally inert in *Drosophila* Kc cells. *In vivo*, no cleavage products are detectable in the upstream region of *Sgs-4*. This region contains a SAR which co-localizes with the *Sgs-4* promoter and enhancer elements (see diagram in Figure 6A; see also Gasser and Laemmli, 1986). The absence of cleavage upstream of the *Sgs-4* gene reveals that SARs are not constitutively cleaved by topoisomerase II. We made similar observations for the transcriptionally inert alcohol dehydrogenase (*Adh*) and *fushi tarazu* (*ftz*) genes (data not shown). Thus, transcriptionally inactive genes are not significantly cleaved by topoisomerase II *in vivo*. Upon addition of distamycin to the cells, however, we observe a low but reproducible induction of topoisomerase II cleavage in flanking regions of the *Sgs-4* (Figure 6A, lane 2), as well as of the *Adh* and *ftz* genes (E.Käs, unpublished results).

It is operationally possible to distinguish between topoisomerase II cleavage at nucleosomal linkers and at open sites as shown above for the histone gene cluster: while cleavage at the linker sites is sensitive to stimulation by distamycin and repression by poly-glutamic acid-mediated histone H1 assembly, cleavage at open sites is not. We studied cleavage at the *Sgs-4* and actin 5C genes in isolated nuclei to classify cleavage sites by these criteria. These experiments were carried out with isolated nuclei incubated with poly-glutamic acid and a fixed amount of purified topoisomerase II. Figure 6A demonstrates that, in the presence of poly-glutamic acid, the cleavage pattern of *Sgs-4* is strongly stimulated by distamycin (compare lanes 3 and 4) while it is completely repressed by histone H1 assembly in the absence (lane 5) but not in the presence of distamycin (lane 6). Thus, all cleavage sites associated with the *Sgs-4* gene behave like those observed in nucleosomal linkers, as might be expected for sites near a transcriptionally silent gene.

We also examined the actin 5C gene which is transcribed at high levels in *Drosophila* Kc cells. In contrast to *Sgs-4*, the actin 5C gene displays a topoisomerase II cleavage pattern with a number of very prominent open sites flanking the gene (Figure 6B, lane 3). Cleavage at these open sites is only slightly stimulated by distamycin (lane 4) and is not repressed by histone H1 assembly (lanes 5 and 6). Thus the transcriptionally active domain of actin 5C is already 'wide open' for topoisomerase II cleavage and distamycin does not result in significant further chromatin unfolding. Some cleavage sites which behave like nucleosome linker sites are also observed in the 3′ region of the gene; cleavage at these sites is stimulated by distamycin (lane 4) and repressed by histone H1 assembly in the absence (lane 5), but not in the presence, of distamycin (lane 6).

In summary, topoisomerase II cleavage correlates well with transcriptional activity, and in this respect behaves

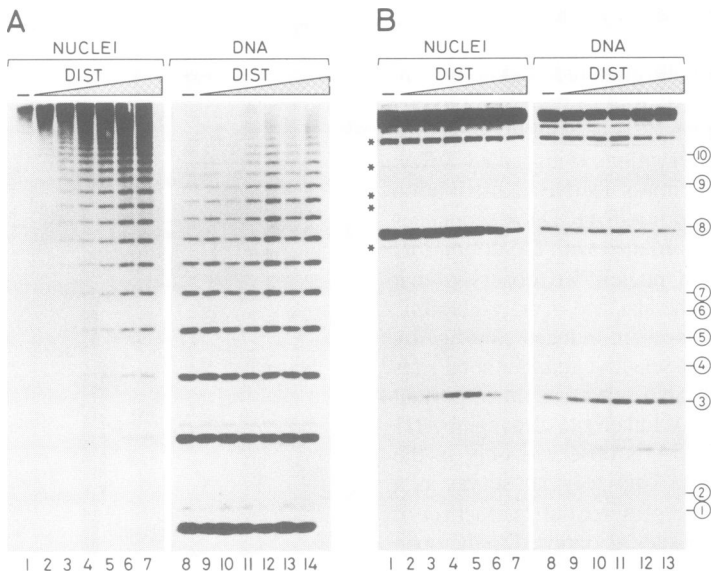

Fig. 7. Distamycin enhances linker site cleavage by restriction enzymes. The dose-dependent activation of restriction enzyme cleavage of linker-sites by distamycin is shown for *Hae*III digestion of satellite III (panel **A**) and *Mbo*I digestion of histone gene repeats (panel **B**). The locations of the relevant restriction sites are shown in the maps of Figure 1A (satellite III) and Figure 2C (histone repeat). Panel **A**: nuclei (lanes 1–7) and purified DNA samples (lanes 8–14) were digested with *Hae*III in the presence of 0, 1, 2, 3.5, 5, 10 and 25 μM distamycin. Panel **B**, nuclei (lanes 1–7) were treated as in panel A and digested with *Mbo*I, while DNA samples (lanes 8–13) were digested in the presence of 0, 2, 3.5, 5, 10 and 25 μM distamycin. As evident from comparison of nuclei and DNA digests, additional *Mbo*I sites (not shown in the map of Figure 2C) are located in the histone gene repeat but are not accessible in chromatin. Topoisomerase II cleavage sites are numbered as in Figure 2C.

similarly to DNase I. The major advantages of the topoisomerase II cleavage assay are that it can be performed *in vivo* and that it can distinguish between nucleosomal linker and open sites. Cleavage at the nucleosomal linker sites, such as those found near inactive genes (e.g. *Sgs-4*), is sensitive to occupancy by linker proteins such as histone H1. In contrast, cleavage at sites within open chromatin domains, such as those flanking active genes (e.g. actin 5C), is not sensitive to repression by histone H1 assembly.

Chromatin opening is also observed using restriction enzymes

If the stimulation of topoisomerase II cleavage by distamycin is due to a reduced occupancy of nucleosomal linkers by histone H1 and not to a bias in its enzymatic behavior, then it should be possible to observe the phenomenon of linker-site opening using other enzymes. We demonstrate next that in nuclei, distamycin selectively stimulates restriction enzyme cleavage at sites which map to nucleosomal linker DNA but not to open chromatin regions. The restriction enzymes used were chosen on the basis of their ability to cut DNA within linker or open sites, respectively.

The satellite III repeat contains a single *Hae*III cleavage site which is localized at the presumed edge of the nucleosome linker DNA (see diagram in Figure 1A). *Hae*III cleavage at this site in isolated nuclei is strongly stimulated by distamycin in a dosage dependent fashion as evidenced by the development of an extensive satellite III cleavage ladder (Figure 7A, lanes 1–7). Further stimulation is observed in the presence of both distamycin and poly-glutamic acid, most likely due to the complete extraction of histone H1 (data not shown). As a control, cleavage of naked DNA by *Hae*III is barely altered by distamycin (lanes 8–14).

Similarly, the histone gene repeat contains several *Mbo*I cleavage sites, one of which maps very near the linker site 3, while another is associated with the open site 8 (see diagram in Figure 2C). Digestion of nuclei with *Mbo*I results in poor cleavage at the linker site in contrast to the strong cleavage at the open site (Figure 7B, lane 1). Addition of an optimal concentration of distamycin, however, significantly stimulates cleavage at the linker but not at the open site (lanes 4 and 5). At higher distamycin concentrations, inhibition at both *Mbo*I sites is noted, due to the titration of the cleavage site by distamycin. In the case of the *Hae*III digest of satellite III, whose recognition site contains only G or C residues, there is no inhibition and stimulation is observed even at the highest distamycin concentration tested (Figure 7A, lane 7). We obtained similar results with *Cla*I, *Hin*fI and *Rsa*I, which cleave additional sites near both linker and open sites of the histone repeat (data not shown). These experiments strongly support the main conclusion developed in this report: the titration of A-tracts with distamycin leads to a reduction in the occupancy of nucleosome linker sites by histone H1 (opening of chromatin) thereby allowing enhanced cleavage by topoisomerase II as well as restriction enzymes.

Discussion

The transcriptionally silent chromatin fiber is thought to be compacted by histone H1 via its association with the inter-nucleosomal linker DNA. The unfolding of this fiber, which we refer to as chromatin opening, is likely to represent an early preparative step leading to transcriptional activation. To study the phenomenon of chromatin opening we have used topoisomerase II and restriction enzymes as probes to assess the occupancy, presumably by histone H1, of nucleosome linker DNA. In addition, we have used the oligopeptide distamycin A, which interferes with the preferential interaction of histone H1 with A-tract DNA. Our experiments suggest an attractive model whereby chromatin

opening is achieved by interference with the sub-fraction of cooperatively interacting histone H1 molecules that are most tightly bound via A-tracts.

Distamycin increases accessibility of nucleosome linker DNA

Addition of the dA·dT minor-groove binder distamycin to living cells significantly stimulates topoisomerase II cleavage in the nucleosome linker sites of the SAR associated with the histone gene cluster and in the satellite III repeats. This selective stimulation of cleavage is experimentally even more impressive in isolated nuclei. Without the drug, cleavage at the linker-sites is virtually absent in nuclei, and is stimulated at least 10-fold upon addition of an optimal concentration of distamycin. This observation holds true for the nucleosome linker-sites but not for the open sites which fall within DNase hypersensitive regions.

An identical observation is made using restriction enzymes instead of topoisomerase II (Figure 7). Addition of distamycin selectively stimulates cleavage by both *Mbo*I and *Hae*III at sites which map to nucleosome linkers. In contrast, *Mbo*I cleavage in an open site of the histone gene repeat is not stimulated. This important result shows that distamycin generally facilitates cleavage at nucleosomal linker DNA and confirms the reliability of using topoisomerase II cleavage to assess DNA accessibility both *in vivo* and *in vitro*.

Linker-site cleavage by topoisomerase II appears to be associated with transcriptional activity. While strong cleavage (predominantly at open sites but also at linker sites) is observed in the actin 5C gene and to a lesser extent in the active histone gene cluster, no cleavage is noted in regions spanning the *Sgs-4*, *Adh* and *ftz* genes, which are silent in *Drosophila* Kc cells. But addition of distamycin to the cells unfolds chromatin at these silent loci as assayed by topoisomerase II linker-site cleavage. Thus, cleavage studies with topoisomerase II suggest that the inter-nucleosomal linkers tested here are inaccessible near inactive genes but are partially or fully open in association with active genes.

Previous *in vitro* data demonstrated that histone H1 and topoisomerase II interact in a highly selective manner with SAR DNA via A-tracts. Addition of distamycin (25 μM) inhibits this selective interaction for both proteins, leading to a suppression of topoisomerase II cleavage in the SAR (Adachi *et al.*, 1989; Käs *et al.*, 1989). Therefore, it was initially surprising to observe that distamycin stimulated topoisomerase II cleavage in chromatin. This observation can be rationalized as follows: in chromatin, the dose of distamycin which is optimal for cleavage ($\sim 2-5$ μM) is $5-10$ times lower than the dose used previously. This optimal concentration of distamycin is sufficient to displace the tightly bound histone H1 subfraction. However, above this concentration, quenching of cleavage occurs in chromatin, as observed previously on naked DNA (Figure 2). Moreover, in chromatin, topoisomerase II cleavage occurs at a loose 'consensus' sequence which contains a GC-rich core. On naked DNA, however, topoisomerase II prefers to cleave at an *in vitro* 'consensus' which contains an AT-rich core localized within the A-tracts (for details see Käs and Laemmli, 1992). Thus, the optimal concentration for cleavage stimulation arises from two competing reactions: chromatin opening versus titration of the cleavage sequence. At low concentrations of distamycin, titration of the A-tracts results in chromatin opening, which in turn allows cleavage at sequences that are relatively GC-rich and not yet titrated by the drug. At higher concentrations of distamycin even these cleavage sites are titrated and cleavage is quenched. In accordance with this rationale, the concentration of distamycin required for an optimal stimulation of cleavage depends both on the enzymes involved and on the sequence of the actual cleavage site (see Figures 2 and 7). For this reason, different cleavage sites are titrated at slightly different drug concentrations.

The phenomenon of chromatin opening by distamycin, as assayed here by linker-site occupancy, is in accordance with previous reports. The chromatin fiber appears substantially less compact in the presence of distamycin (Sen and Crothers, 1986) and treatment of living cells with this drug results in a marked decondensation of centromeric heterochromatin (Radic *et al.*, 1987). DNA bending is known to occur at the junction between A-tracts and adjacent B-DNA (Koo *et al.*, 1986) and distamycin straightens bent DNA (Griffith *et al.*, 1986). Although the physical straightening of bent A-tract sequences by distamycin could contribute to chromatin unfolding (Radic *et al.*, 1987), our results suggest that the ensuing displacement of H1 from A-tracts might be the dominant cause of chromatin decondensation. Cleavage stimulation by topoisomerase II and restriction enzymes is observed selectively only at nucleosome linker but not at open sites. If chromatin opening was due primarily to an alteration of the DNA structure, rather than to an increased accessibility of the nucleosome linker sites, one would expect to observe cleavage stimulation at both types of site. Moreover, the observation that extraction with poly-glutamic acid also leads to a strong stimulation of cleavage supports the notion that steric de-blocking of linker DNA is responsible for stimulation of linker-site cleavage and for chromatin opening.

Histone H1 extraction stimulates cleavage in nucleosome linkers

Histone H1 is the predominant protein found in nucleosome linkers and accessibility to the linker DNA, as assayed by enzymatic cleavage, appears to be sterically blocked by histone H1. Support for this hypothesis comes from our histone H1 extraction and assembly experiments. Addition of poly-glutamic acid to nuclei induces a partial and selective activation of topoisomerase II cleavage at the linker sites; cleavage at the open sites, however, is not altered by this poly-anion (Figure 4). The partial and selective activation of cleavage observed in the histone SAR region is thought to be due to the different affinities of histone H1 (or other linker proteins) for the various linker regions studied; thus, cleavage activation by poly-glutamic acid is poor at site 4 but much better at site 5. This result is in perfect agreement with the protein extraction data that identify a minor fraction of the histone H1 that is resistant to extraction by poly-glutamic acid. Binding of this residual fraction must be stabilized by preferential interactions with A-tracts such as those found in SARs since addition of distamycin leads to the quantitative extraction of this tightly bound fraction from chromatin (Figure 3).

Removal of histone H1 would be synergistically beneficial for cleavage, due to the general unfolding of the chromatin fiber as well as steric de-blocking of linker sites that are substrates for cleavage. Indeed, extraction of nuclei with poly-glutamic acid plus distamycin, which results in the quantitative extraction of histone H1, is accompanied by the highest activation of linker site cleavage by topoisomerase

II as well as by restriction enzymes. In contrast, cleavage activation by distamycin alone (histone H1 redistributes but remains chromatin-bound) is lower by ~2- to 3-fold.

In chromatin, H1 redistribution would result in lower linker-site occupancy as manifested by enhanced topoisomerase II cleavage. Such a distamycin-induced redistribution of histone H1 necessitates free binding sites for this protein in the nucleus. This condition is experimentally met, as titration of free binding sites (both specific and non-specific) by addition of purified histone H1 to nuclei results in the loss of distamycin-stimulated topoisomerase II cleavage once the added histone H1 reaches 50–100% of endogenous levels (Figure 5B). Upon titration of the free histone H1 binding sites, redistribution is no longer possible.

Cleavage activation by poly-glutamic acid can be reversed by histone H1 assembly. This results in the loss of cleavage at linker sites but not in open chromatin regions as observed at the histone gene repeat as well as at the silent *Sgs-4* locus. The selective inhibition of linker-site cleavage by histone H1 assembly and the reactivation by distamycin strongly suggest that histone H1, through its tight interaction with the A-tracts of SARs, causally interferes with topoisomerase II activity at the linker sites. Although the histone H1 extraction and assembly experiments mimic the phenomena of chromatin opening and closing in a distamycin-dependent manner, we cannot exclude the possibility that proteins other than histone H1 might have a similar effect—the linker proteins that inhibit cleavage at site 4 of the histone SAR and in satellite III repeats are very resistant to poly-glutamic acid extraction and could possibly be non-histone in nature.

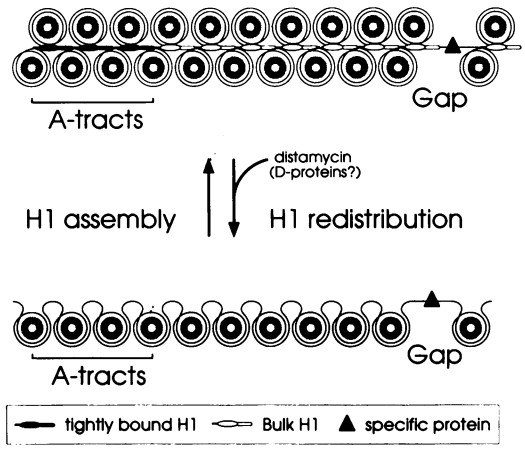

Fig. 8. A model for a chromatin opening switch. The top diagram represents nucleosomes (graphically adapted from Tazi and Bird, 1990) with histone H1 molecules assembled in a polar head-to-tail fashion (Lennard and Thomas, 1985). A subfraction of histone H1 is tightly bound via A-tracts (filled symbols) which nucleate cooperative assembly onto adjacent nucleosomes (open symbols). A-tracts distributed over several hundred base pairs of DNA are thought to constitute an SAR. Histone H1 assembly is proposed to continue until a gap occurs in the nucleosomal array. Such gaps may correspond to DNase I hypersensitive sites generated by interaction with specific factors (triangles) and constitute barriers against further spreading of histone H1. Redistribution of histone H1 mediated by specific titration of the A-tracts with distamycin or putative D-proteins results in chromatin opening (bottom diagram). The unfolded fiber depicted represents the most extreme situation—complete removal of histone H1. Intermediate levels of histone H1 depletion may be sufficient to generate active chromatin.

A model for a chromatin opening switch

The chromatin fiber is thought to be stabilized by cooperatively interacting histone H1 molecules, presumably via head-to-tail interactions (Thomas and Khabaza, 1980; Lennard and Thomas, 1985). In such a histone H1 homopolymer, the DNA-bound members facilitate, via cooperative protein–protein interactions, the binding of adjacent members. In this array of proteins interacting cooperatively along the DNA, the most tightly bound members can 'control' the interaction of their neighbors. By way of illustration, the λ repressor, interacting as a dimer with the high affinity DNA target O_{R1}, facilitates the binding of another dimer, via a cooperative interaction, at the adjacent lower affinity site O_{R2}. Interference with the binding of the repressor to O_{R1} by mutation (we use the competitive inhibitor distamycin) results in a strongly reduced occupancy of the O_{R2} (Ptashne, 1986).

Figure 8 diagrams a simple model depicting SARs as regions containing a number of clustered high affinity sites for histone H1 molecules spanning the inter-nucleosomal linker DNA. The high affinity interaction of histone H1 is mediated by the numerous A-tracts of SAR (or SAR-like) sequences. The tightly bound histone H1 molecules (or possibly other linker proteins) are proposed to nucleate histone H1 assembly along the chromatin fiber up to a gap (or open site) in the nucleosomal array. The open site prevents further spreading of the histone H1 polymer by impeding cooperative interactions. Distamycin analogues ('D-proteins') are proposed to exist in the cell, which, upon binding to A-tracts, neutralize the ability of SARs to serve as nucleators for histone H1 assembly. As a consequence, the equilibrium of histone H1 association is shifted towards a reduction in the occupancy of the nucleosomal linkers, resulting in chromatin opening. Note that our cleavage experiments do not at present establish the distance over which chromatin opening can occur. Even a partial loss of histone H1 might be sufficient to induce unfolding if the chromatin fiber is stabilized by histone H1–H1 interactions (Kamakaka and Thomas, 1990). Following this initial unfolding step, additional events necessary for transcriptional activation such as acetylation of histones, binding of transcription factors, or removal of nucleosomes, could stabilize the active chromatin state.

Specificity in the opening of chromatin domains might be brought about by the selective interaction of the putative D-proteins with A-tracts (nucleation control) and by the formation of flanking DNase I hypersensitive sites that serve as propagation barriers to histone H1 assembly (propagation control). A number of nuclear proteins have been described which bind specifically to A-tracts of DNA; these include the *Drosophila* D1 protein (Levinger and Varshavsky, 1982) and yeast datin (Winter and Varshavsky, 1989). Proteins belonging to the HMG-I family, which are preferentially expressed in rapidly proliferating cells (Johnson *et al.*, 1989), have also been shown to bind the minor groove of AT-rich regions (Strauss and Varshavsky, 1984). Their DNA-binding domain is proposed to have a secondary structure similar to that of distamycin (Reeves and Nissen, 1990). These AT-binding proteins, most of which contain prominent acidic domains, might possibly serve to destabilize the preferential interaction of histone H1 with A-tract DNA and play a role in the early steps required for chromatin opening.

Materials and methods

In vivo and in vitro topoisomerase II cleavage assays

VM26-induced *in vivo* topoisomerase II cleavage assays using *Drosophila melanogaster* Kc cells and treatment with distamycin were performed exactly as described in Käs and Laemmli (1992). For *in vitro* assays, nuclei were isolated from Kc cells as described by Mirkovitch *et al.* (1984) and stored at −20°C in buffer A containing 50% glycerol. Buffer A is 15 mM Tris−HCl, pH 7.4, 80 mM KCl, 2 mM EDTA, 0.05 mM spermine, 0.125 mM spermidine, 0.5 mM DTT, 0.1 mM PMSF, 0.1% digitonin and 1% Trasylol (Bayer). Nuclei were washed in 10 ml of buffer A and nuclear pellets (0.25 A_{260} units each) were resuspended in buffer B (20 mM Tris−HCl, pH 7.4, 20 mM KCl, 70 mM NaCl, 10 mM $MgCl_2$, 0.05 mM spermine, 0.125 mM spermidine, 0.1 mM PMSF, 0.5 mM DTT, 0.1% digitonin, 1% Trasylol). Histone H1 (0.1−10 μg) purified from rat liver nuclei (Izaurralde *et al.*, 1989) was added at that time. Samples were incubated for 15 min at 30°C before addition of 1−10 μg of purified *Saccharomyces cerevisiae* topoisomerase II (Adachi *et al.*, 1991) and, where indicated, of distamycin (1−15 μM, corresponding to molar ratios of drug to DNA base pairs ranging from 0.02 to 0.3) in 200 μl final reaction volumes. Incubations were continued for an additional 15 min. The order of addition of histone H1, topoisomerase II and distamycin has no effect on the outcome of the results reported here. ATP and VM26 were then added to final concentrations of 1.5 mM and 50 μM, respectively, and after a further incubation for 5 min, reactions were terminated by addition of SDS, EDTA and proteinase K to final concentrations of 1%, 15 mM and 250 μg/ml, respectively. Samples were incubated for 3−4 h at 45°C and the DNAs were then purified by repeated organic extractions and ethanol precipitation.

For the experiments shown in Figure 7, nuclei in buffer B were incubated with distamycin (1−25 μM) for 15 min at 30°C before addition of 5 U of *Hae*III or *Mbo*I. Incubations were continued for an additional 30 min and the DNA samples were purified as described above. Control reactions were performed using purified genomic DNA digested under identical conditions.

Topoisomerase II cleavage sites were analyzed by Southern blotting exactly as described in Käs and Laemmli (1992). For analysis of the histone repeat, purified DNA samples were digested with *Hin*dIII, which releases each major repeat as a 5 kb fragment, and hybridized to a 300 bp *Hin*dIII−*Pst*I probe spanning the 5′ coding region of the H1 gene (H1 probe). For the experiment shown in Figure 2B, DNA samples were digested with *Ava*I and hybridized to a 450 bp *Ava*I−*Sst*I probe spanning the 5′ flank and coding region of the histone H3 gene (H3 probe). The 4 kb fragment detected spans the H3−H1 spacer, the H1 and H2B genes and part of the H2A gene. For analysis of satellite III sequences, DNA samples were electrophoresed without prior restriction enzyme digestion and hybridized to a purified *Hae*III 359 bp monomer fragment (Hsieh and Brutlag, 1979). For analysis of *Sgs-4* sequences, samples were digested with *Hin*dIII and hybridized to the *Hin*dIII−*Eco*RI probes shown in Figure 6A. Actin 5C sequences were analyzed by digestion with *Eco*RI and hybridization to the *Eco*RI−*Xho*I probe shown in Figure 6B.

Poly-glutamic acid extraction of chromosomes and nuclei

Metaphase chromosome clusters were isolated from HeLa cells blocked in mitosis with 0.06 μg/ml demecolcine for 16 h (Gasser and Laemmli, 1987b). Similar results were obtained for selective extraction of histone H1 from chromosomes, rat liver nuclei and *Drosophila* nuclei. Chromosomes were rinsed once with an excess volume of A/4 buffer (3.75 mM Tris−HCl, pH 7.4, 0.05 mM spermine, 0.125 mM spermidine, 20 mM KCl, 0.5 mM K-EDTA, 0.1% digitonin, 100 U/ml Trasylol) and resuspended at a concentration of 20 A_{260} units/ml in fresh A/4 buffer. 50 μl (1 A_{260} unit) of rinsed chromosomes were slowly added to 3 ml of cold extraction buffer in an SW50.1 centrifuge tube. The tubes were gently inverted four times and the samples were incubated on ice for 30 min. Extracted chromosomes were pelleted at 20 000 r.p.m. in an SW50.1 rotor and the supernatant was decanted.

The extraction buffer was either TEN (10 mM Tris−HCl, pH 9.0, 1 mM Na-EDTA, 100 mM NaCl, 0.1% digitonin, 100 U/ml Trasylol) or TEN plus 1 mg/ml poly-L-glutamic acid (average molecular weight 102 kDa, ICN). Similar results were obtained with NaCl concentrations ranging from 20 to 100 mM or at neutral pH. Extractions in which chromomycin was included were performed using TNM buffer (TEN buffer in which the EDTA is replaced by 0.1 mM $MgCl_2$). Chromomycin or distamycin were added to the extraction buffer to a final concentration of 2.5 μM.

Pelleted chromosomes were digested with 5 μg/ml DNase I for 90 min at 37°C. SDS and β-mercaptoethanol were added to 2% and 10%, respectively, and the pellets were dispersed by sonication. The supernatants were precipitated with 20% trichloroacetic acid for 1 h on ice and the precipitated proteins were recovered by centrifugation at 10 000 *g* for 30 min. The precipitates were washed once with cold (−20°C) 70% acetone, 20% ethanol, 20 mM Tris−HCl, pH 8.0, pelleted again by centrifugation and resuspended in SDS dye mix. The pellet and supernatant fractions of extracted chromosomes were fractionated on 7.5−15% polyacrylamide−SDS gradient gels (Lewis and Laemmli, 1982).

For analysis of topoisomerase II cleavage of poly-glutamic acid-extracted nuclei, Kc nuclei prepared as above were extracted as follows before addition of purified topoisomerase II. Aliquots of 0.25 A_{260} units of nuclei washed in buffer A were resuspended in 10 μl buffer A and mixed with 190 μl of buffer B* (buffer B without magnesium) or buffer B* containing poly-glutamic acid. Final concentrations of poly-glutamic acid were in the 0.5−1 mg/ml range and extractions were carried out for 20 min on ice after gentle mixing. Where indicated, histone H1 was added directly to the extraction buffer. Samples were then incubated with distamycin and topoisomerase II after addition of $MgCl_2$ (10 mM). Cleavage reactions were performed as described above.

Acknowledgements

We thank O.Jenni, F.Bujard-Ebener and E.Boy de la Tour for preparation of the figures. This work was supported by the Swiss National Fund and by the Canton of Geneva.

References

Adachi,Y., Käs,E. and Laemmli,U.K. (1989) *EMBO J.*, **8**, 3997−4006.
Adachi,Y., Luke,M. and Laemmli,U.K. (1991) *Cell*, **64**, 137−148.
Alberts,B., Worcel,A. and Weintraub,H. (1977) In Bradbury,M. (ed.), *Proceedings of the International Symposium on the Eukaryotic Genome*. Academic Press, New York, pp. 165−191.
Bond,B.J. and Davidson,N. (1986) *Mol. Cell. Biol.*, **6**, 2080−2088.
Brutlag,D.L. (1980) *Annu. Rev. Genet.*, **14**, 121−144.
Clark,D.J. and Thomas,J.O. (1986) *J. Mol. Biol.*, **187**, 569−580.
Cockerill,P.N. and Garrard,W.T. (1986) *Cell*, **44**, 273−282.
De Bernardin,W., Losa,L. and Koller,T. (1986) *J. Mol. Biol.*, **189**, 503−517.
Elgin,S.C.R. (1988) *J. Biol. Chem.*, **263**, 19259−19262.
Ericsson,C., Grossbach,U., Björkroth,B. and Daneholt,B. (1990) *Cell*, **60**, 73−83.
Fox,K.R. and Howarth,N.R. (1985) *Nucleic Acids Res.*, **13**, 8695−8714.
Fox,K.R. and Waring,M.J. (1984) *Nucleic Acids Res.*, **12**, 9271−9285.
Gao,X, and Patel,D.J. (1989) *Biochemistry*, **28**, 751−762.
Gasser,S.M. and Laemmli,U.K. (1986) *Cell*, **46**, 521−530.
Gasser,S.M. and Laemmli,U.K. (1987a) *Trends Genet.*, **3**, 16−21.
Gasser,S.M. and Laemmli,U.K. (1987b) *Exp. Cell Res.*, **173**, 85−98.
Gasser,S.M., Laroche,T., Falquet,J., Boy de la Tour,E. and Laemmli,U.K. (1986) *J. Mol. Biol.*, **188**, 613−629.
Griffith,J., Bleyman,M., Rauch,C.A., Kitchin,P.A. and Englund,P.T. (1986) *Cell*, **46**, 717−724.
Gross,D.S. and Garrard,W.T. (1988) *Annu. Rev. Biochem.*, **57**, 159−197.
Grunstein,M. (1990) *Trends Genet.*, **6**, 395−400.
Hsieh,T.-s. and Brutlag,D. (1979) *J. Mol. Biol.*, **135**, 465−481.
Johnson,K.R., Len,D.A. and Reeves,R. (1989) *Mol. Cell. Biol.*, **9**, 2114−2123.
Izaurralde,E., Käs,E. and Laemmli,U.K. (1989) *J. Mol. Biol.*, **210**, 573−585.
Kamakaka,R.T. and Thomas,J.O. (1990) *EMBO J.*, **9**, 3997−4006.
Käs,E., Izaurralde,E. and Laemmli,U.K. (1989) *J. Mol. Biol.*, **210**, 587−599.
Käs,E. and Laemmli,U.K. (1992) *EMBO J.*, **11**, 705−716.
Koo,H.-S., Wu,H.-M. and Crothers,D.M. (1986) *Nature*, **320**, 501−506.
Künzler,P and Stein,A. (1983) *Biochemistry*, **22**, 1783−1789.
Lennard,A.C. and Thomas,J.O. (1985) *EMBO J.*, **4**, 3455−3462.
Levinger,L. and Varshavsky,A. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7152−7156.
Lewis,C.D. and Laemmli,U.K. (1982) *Cell*, **29**, 171−181.
Mirkovitch,J., Mirault,M.-E. and Laemmli,U.K. (1984) *Cell*, **39**, 223−232.
Mirkovitch,J., Gasser,S.M. and Laemmli,U.K. (1988) *J. Mol. Biol.*, **200**, 101−109.
Ptashne,M. (1986) *A Genetic Switch*. Cell Press, Cambridge and Blackwell Scientific Publications, Palo Alto.
Radic,M.Z., Lundgren,K. and Hamkalo,B.A. (1987) *Cell*, **50**, 1101−1108.
Reeves,R. and Nissen,M.S. (1990) *J. Biol. Chem.*, **265**, 8573−8582.
Renz,M. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 733−736.
Sander,M. and Hsieh,T.-s. (1985) *Nucleic Acids Res.*, **13**, 1057−1072.

Saumweber,H., Frasch,M. and Korge,G. (1990) *Chromosoma*, **99**, 52–60.
Schlissel,M.S. and Brown,D.D. (1984) *Cell*, **37**, 903–913.
Sen,D. and Crothers,D.M. (1986) *Biochemistry*, **25**, 1503–1509.
Shermoen,A.W. and Beckendorf,S.K. (1982) *Cell*, **29**, 601–607.
Shimamura,A., Sapp,M., Rodriguez-Campos,A. and Worcel,A. (1989) *Mol. Cell. Biol.*, **9**, 5573–5584.
Stein,A. and Künzler,P. (1983) *Nature*, **302**, 548–550.
Stein,A. and Mitchell,M. (1988) *J. Mol. Biol.*, **203**, 1029–1043.
Strauss,F. and Varshavsky,A. (1984) *Cell*, **37**, 889–901.
Tazi,J. and Bird,A. (1990) *Cell*, **60**, 909–920.
Thoma,F., Koller,T. and Klug,A. (1979) *J. Cell Biol.*, **83**, 403–427.
Thomas,J.O. and Khabaza,A.J.A. (1980) *Eur. J. Biochem.*, **112**, 501–511.
Van Dyke,M.W. and Dervan,P.B. (1983) *Biochemistry*, **22**, 2373–2377.
Van Dyke,M.W., Hertzberg,R.P. and Dervan,P.B. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5470–5474.
Weintraub,H. (1985) *Cell*, **42**, 705–711.
Weintraub,H. and Groudine,M. (1976) *Science*, **93**, 848–858.
Winter,E. and Varshavsky,A. (1989) *EMBO J.*, **8**, 1867–1877.
Wolffe,A.P. (1989) *EMBO J.*, **8**, 527–537.
Worcel,A., Gargiulo,G., Jessee,B., Udvardy,A., Louis,C. and Schedl,P. (1983) *Nucleic Acids Res.*, **11**, 421–439.
Wu,C., Bingham,P.M., Livak,K.J., Holmgren,R. and Elgin,S.C.R. (1979) *Cell*, **16**, 797–806.
Zlatanova,J. (1990) *Trends Biochem. Sci.*, **15**, 273–276.

Received on July 20, 1992; revised on October 15, 1992