

## SAR-dependent mobilization of histone H1 by HMG-I/Y *in vitro*: HMG-I/Y is enriched in H1-depleted chromatin

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An experimental assay was developed to search for proteins capable of antagonizing histone H1-mediated general repression of transcription. T7 RNA polymerase templates containing an upstream scaffold-associated region (SAR) were highly selectively repressed by H1 relative to non-SAR control templates. This is due to the nucleation of H1 assembly into flanking DNA brought about by the numerous A-tracts (AT-rich sequences containing short homopolymeric runs of dA·dT base pairs) of the SAR. Partial, selective titration of these A-tracts by the high mobility group (HMG) protein HMG-I/Y led to the complete derepression of transcription from the SAR template by inducing the redistribution of H1 on to non-SAR templates. SARs are associated with many highly transcribed regulated genes where they may serve to facilitate the HMG-I/Y-mediated displacement of histone H1 in chromatin. Indeed, HMG-I/Y was found to be strongly enriched in the H1-depleted subfraction which can be isolated from chromatin.

**Key words:** chromatin/distamycin/histone H1/HMG-I/Y/SAR

### Introduction

Histone H1 plays a pivotal role in the compaction of chromatin into the transcriptionally silent fiber via its association with the inter-nucleosomal linker DNA (reviewed by Garrard, 1991). H1 appears to be a generalized repressor restricting genetic readout by folding the chromatin fiber into higher-order structures (Weintraub, 1985; Laybourn and Kadonaga, 1991). A number of observations demonstrate that transcription activation is associated with the unfolding of the chromatin fiber (chromatin opening) mediated by a reduced histone H1 complement (Ericsson *et al.*, 1990; Kamakaka and Thomas, 1990). What is the mechanism whereby the regional depletion of histone H1 is induced and maintained?

Scaffold associated regions (SARs) are highly A+T-rich (>70%) sequences of several hundred base pairs that specifically bind *in vitro* to the nuclear scaffold; these DNA elements appear to be implicated in structurally delimiting chromatin loops. Recent experiments extend this notion, suggesting a functional role of SARs in gene expression (reviewed by Laemmli *et al.*, 1992). The specificity of the SAR–scaffold interaction is mediated by proteins that recognize certain non-B structural features of DNA rather than a precise base sequence. Such structural features might

include the narrow minor groove of the A-tracts (AT-rich sequences containing short homopolymeric runs of dA·dT base pairs) which are embedded in SARs (Käs *et al.*, 1989) and/or possibly DNA bends (Homburger, 1989). These conclusions are supported by experiments using artificial SAR constructs and the antibiotic distamycin, which is an inhibitor of most (if not all) SAR–protein interactions *in vitro* (Adachi *et al.*, 1989; Käs *et al.*, 1989). Distamycin binds highly sequence-selectively to short A-tracts, making use of direct amide–base hydrogen bonds and van der Waals interactions with the floor and sides of the narrower minor groove of A-tracts (reviewed by Churchill and Travers, 1991).

A number of proteins have been reported to bind SARs selectively (Hofmann *et al.*, 1989; von Kries *et al.*, 1991; Dickinson *et al.*, 1992; Ludérus *et al.*, 1992; Romig *et al.*, 1992). We have focused our attention on topoisomerase II and histone H1 (Adachi *et al.*, 1989; Izaurralde *et al.*, 1989). Purely on theoretical grounds, topoisomerase II and histone H1 are prime candidates for playing major roles in the long-range organization of chromosomes and of the chromatin fiber. Indeed, genetic and biochemical evidence has established a direct and essential role for topoisomerase II in late stages of chromosome condensation (Uemura *et al.*, 1987; Adachi *et al.*, 1991), possibly via interactions with SARs (Käs and Laemmli, 1992).

SARs have been shown to serve *in vitro* as tight binding sites for several histone H1 molecules that bind cooperatively. The tightly bound H1 nucleates in turn the further assembly of 'bulk' H1 on to flanking DNA (Izaurralde *et al.*, 1989). Selective titration of the A-tracts of SARs by distamycin inhibits the preferential binding of H1 to SAR-containing DNA, leading to a non-selective redistribution of H1 between SAR and non-SAR DNA alike (Käs *et al.*, 1989; Izaurralde *et al.*, 1989). That is, the occupancy of the SAR by H1 is strongly reduced in the presence of distamycin and this loss of H1 also extends into flanking non-SAR sequences due to the highly cooperative binding of H1 to DNA.

Are SARs involved in chromatin opening? If histone H1 binds SARs (or SAR-like DNA) packaged into chromatin similarly tightly, then titration of A-tracts by distamycin should result in a reduced occupancy of the nucleosomal linker (chromatin opening) due to a redistribution of histone H1. Recent experiments using chromatin and whole cells support this notion (Käs and Laemmli, 1992; Käs *et al.*, 1993). Accessibility studies using either topoisomerase II (in cells and in chromatin) or restriction enzymes as probes demonstrate that addition of distamycin results in a strongly reduced occupancy of nucleosomal linkers at or near SARs, presumably due to the displacement of histone H1. The phenomenon of distamycin-induced chromatin opening, as assayed by nucleosome linker accessibility, 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). These observations encouraged us to formulate a working model for chromatin opening: SARs are proposed to be regions of chromatin with a particularly high affinity for histone H1. This is due to the clustering of high affinity sites—spanning several nucleosomes—and to cooperative interactions between histone H1 molecules. Distamycin analogs ('D-proteins') are proposed to exist in the cell which, upon binding to A-tracts, dislodge the tightly SAR-bound histone H1. As a consequence, the equilibrium of histone H1 association is shifted towards a reduction in the occupancy of the nucleosomal linkers, resulting in local chromatin opening. This local opening could spread if the interaction of histone H1 in chromatin were as cooperative as it is on naked DNA (Käs *et al.*, 1993).

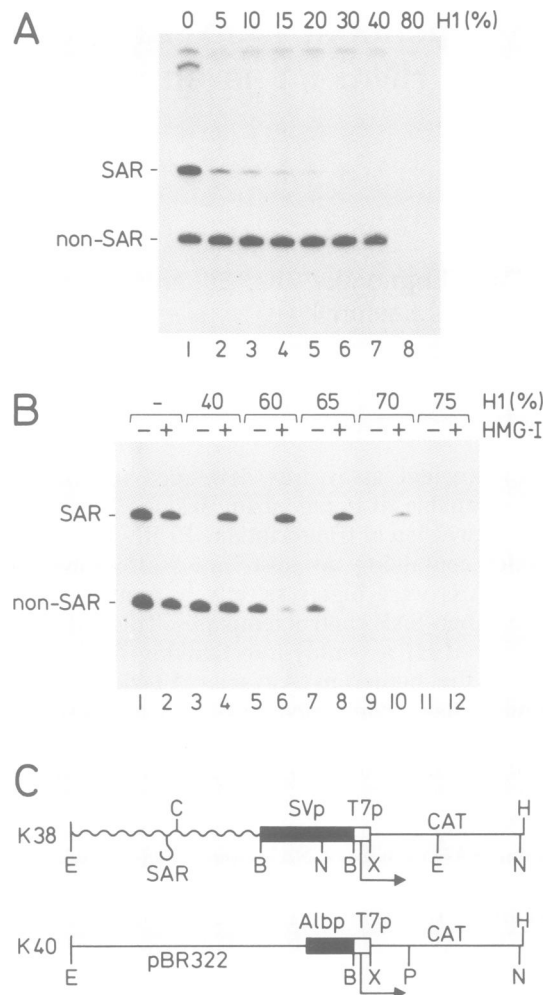
The high mobility group (HMG) protein HMG-I together with its isoform HMG-Y (collectively called HMG-I/Y) was first reported as a protein associated with centromeric heterochromatin and has been proposed to play a role in nucleosome phasing by binding to discrete sites in African green monkey  $\alpha$ -satellite repeats (Strauss and Varshavsky, 1984). HMG-I/Y has since been shown to bind to multiple sites in mouse satellite repeats leading to the suggestion that it might be involved in the condensation of heterochromatin (Radic *et al.*, 1992). HMG-I/Y also appears to play a role in DNA replication and transcription (reviewed by Reeves, 1992). How HMG-I/Y might mediate these diverse functions is not known. HMG-I/Y preferentially binds to A-tracts in DNA (Solomon *et al.*, 1986; Reeves *et al.*, 1987). This selective interaction has been shown to be mediated by three independent consensus binding domains which contact the minor groove of DNA. The backbone of this consensus peptide has a predicted planar crescent shape that shares structural characteristics with the AT-binding drugs netropsin, distamycin and Hoechst 33258 (Reeves and Nissen, 1990). This similarity prompted us to test HMG-I/Y as a candidate D-protein.

## Results

### Highly selective repression by histone H1 of T7 transcription of a SAR-containing template

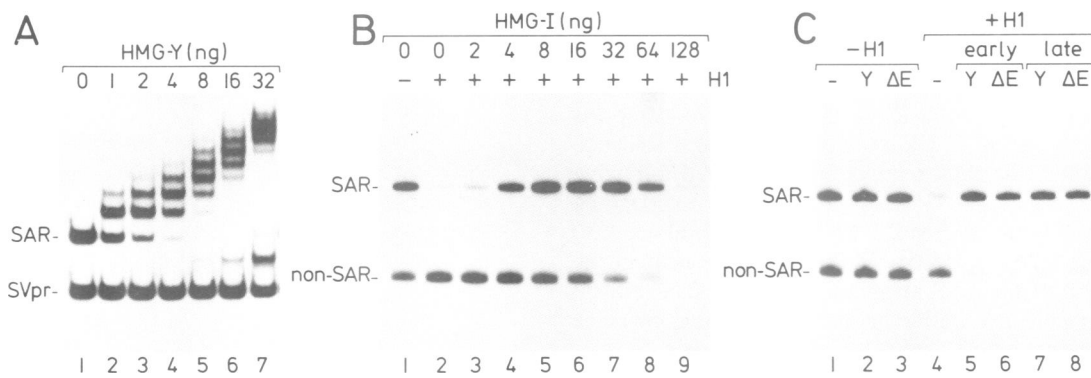
We have described SARs as DNA elements which serve *in vitro* as nucleation sites for the preferential assembly of histone H1 on to flanking non-SAR DNA (Izaurralde *et al.*, 1989); we use the term non-SAR to describe bulk DNA, which is not preferentially titrated by histone H1 and/or scaffold-bound. The preferential binding of H1 to SARs is due to the clustering of numerous A-tracts in SARs; selective titration of the minor groove of these A-tracts with the antibiotic distamycin inhibits this preferential association as well as SAR interactions with the nuclear scaffold *in vitro* (Käs *et al.*, 1989).

We used the following assay system to search for proteins capable of antagonizing the H1–SAR interaction: plasmids were appropriately digested to yield two fragments containing distinguishable T7 RNA polymerase run-off transcripts. One of these DNA fragments, called the SAR template, contains the 657 bp SAR derived from the *Drosophila* histone gene cluster (Mirkovitch *et al.*, 1984) 5' of the T7 RNA polymerase promoter. The control



**Fig. 1.** Highly selective SAR-dependent repression by histone H1 of T7 RNA polymerase transcription and derepression by HMG-I. (A) DNA samples were incubated with histone H1 at the different H1:DNA weight ratios indicated above the gel. The samples were then transcribed with T7 RNA polymerase and the transcripts were analyzed by gel electrophoresis. The SAR and the non-SAR transcripts are 252 and 150 nucleotides in length, respectively. Each DNA sample contained 1  $\mu$ g of sonicated salmon sperm DNA, 166 ng of *Eco*RI-digested K38 (SAR) plasmid and 230 ng of *Eco*RI- and *Pvu*II-digested K40 (non-SAR) plasmid in a final volume of 6  $\mu$ l. (B) The DNA samples are similar to those in panel A except that *Bgl*II-digested pUC18 DNA (200 ng) was used as competitor and 20 ng each of K38 and K40 digested as above were used as templates. Selective repression was achieved by incubation with histone H1 (40–75% weight ratios) and purified HMG-I (0 or 20 ng) was then added as indicated by minus and plus signs above the gel. The transcription potential of the templates was then tested as described above. Note that the precise amount of H1 required for SAR-specific repression varies according to the relative abundance of the SAR-containing DNA in the sample as a result of the stoichiometric titration of this DNA by about one H1 molecule per 40 bp (Izaurralde *et al.*, 1989). Maps of the SAR (K38) and non-SAR (K40) DNA templates used are shown in panel C (see Materials and methods for details): the 657 bp histone SAR is represented by a wavy line with a hook. Only relevant restriction enzyme sites are shown. B: *Bam*HI; C: *Clal*; E: *Eco*RI; H: *Hind*III; N: *Nco*I; P: *Pvu*II; X: *Xba*I.

template contains instead at the same position a non-SAR fragment of similar size to that depicted in Figure 1C. To demonstrate the highly selective repression by histone H1 of T7 transcription from the SAR template we proceeded as follows: linearized templates were incubated with histone H1 before addition of T7 RNA polymerase (Figure 1A).



**Fig. 2.** HMG-Y and HMG-Y $\Delta$ E both derepress transcription: multiple specific binding sites on the histone SAR. (A) Plasmid K38 was digested with *Bam*HI, end-labeled and digested with *Cl*aI and *N*coI to generate a 310 bp SAR DNA fragment and a 223 bp non-SAR DNA fragment containing the SV40 promoter region (see Figure 1C). The fragments together with competitor DNA were incubated with increasing amounts of HMG-Y as indicated above the gel and the samples were electrophoresed through a low ionic strength acrylamide gel. HMG-I was similarly tested and gave identical results (data not shown). (B) DNA samples identical to those in Figure 1B were incubated with histone H1 (40% weight ratio) before addition of increasing amounts of HMG-I as shown above the gel. Transcription of the templates was assayed as described in Figure 1B. (C) DNA samples identical to those in panel B were incubated in the absence or presence of histone H1 (H1:DNA weight ratio of 40%). 20 ng of HMG-Y ('Y') or of HMG-Y $\Delta$ E (' $\Delta$ E') were then added 'late' and incubation was continued for an additional 15 min. Alternatively, samples were first incubated with HMG-Y or HMG-Y $\Delta$ E ('early') before addition of histone H1. T7 transcription reactions were then performed as described above.

While the signal strength from the SAR and non-SAR templates is about the same without added H1 (lane 1), a selective and quantitative inhibition of the SAR transcript is observed following titration of the samples with histone H1 (lanes 2–7). This inhibition is complete at a weight ratio of H1 to total DNA of ~20% (lane 5). We observed no inhibition of the non-SAR transcript up to 40% weight ratios of added H1; above this level (up to 80% was tested) transcription from both templates was completely repressed due to the complete titration of all the DNA in the system (lane 8). This transcription assay, which confirms our previous observations regarding the selective interaction of H1 with SAR-containing DNA, was then used to identify proteins capable of rescuing SAR templates from H1-mediated repression.

#### **HMG-I/Y derepresses the histone H1-mediated inhibition of SAR transcription**

We focused our attention on the proteins HMG-I and Y as possible biological analogs of distamycin that might antagonize histone H1-mediated repression (see Introduction). Indeed, highly purified bacterially expressed HMG-I protein was found to derepress transcription from the SAR template inhibited by H1 with remarkable efficiency and specificity (Figure 1B). In this experiment, we titrated H1 through and above the concentration range which achieves selective inhibition of the SAR transcript and the templates were then challenged with a fixed amount of HMG-I protein. Addition of 20 ng of HMG-I alone (corresponding to a weight ratio of HMG-I to DNA of 1:12) did not stimulate transcription of the SAR and non-SAR templates; on the contrary, a slight inhibition was noted (compare lanes 1 and 2). Addition of purified HMG-I following incubation with histone H1 led to derepression of transcription from the SAR template. As shown below, the order of addition of histone H1 and HMG-I/Y is unimportant. In the range of selective H1 repression, we routinely observed quantitative derepression of SAR transcription up to the level of the control containing HMG-I but no H1 (compare lanes 2 with lanes 4, 6 and 8).

Interestingly, we also observed that derepression of transcription from the SAR template was accompanied by a concomitant repression of the non-SAR transcript. This is particularly evident in the samples containing 60 and 65% of H1, respectively (lanes 6 and 8). As shown below, this phenomenon of repression/derepression is due to the HMG-I/Y-mediated shift of the cooperatively interacting H1 from the SAR to the non-SAR templates.

#### **HMG-I/Y mediates SAR-dependent derepression by binding specifically to the histone SAR**

The results shown in Figure 1B strongly suggest that HMG-I/Y binds SARs selectively. We examined this question more closely using the band-shift technique. In the experiments shown next, we used the smaller HMG-Y isoform of HMG-I which lacks an internal 11 amino acid stretch and arises most likely from alternative splicing (Johnson *et al.*, 1989). HMG-Y behaves identically to HMG-I in the derepression assay (see below). The band-shift experiment shown in Figure 2A was carried out with two end-labeled fragments: a 310 bp fragment derived from the histone SAR and a 223 bp non-SAR control fragment derived from the SV40 promoter (see figure legend and the diagram in Figure 1C). Multiple protein–DNA complexes were formed on the SAR probe as a function of HMG-Y dose as detected by a clear ladder of shifted products (lanes 2–7). We interpret this ladder as reflecting the gradual titration of the multiple clustered A-tracts located in the SAR. About five or six bound sites can be counted in the specific range; no significant shift of the non-SAR probe is observed except at the highest HMG-Y concentration tested (lane 7), where addition of more HMG-I/Y led to some binding to the non-SAR fragment. From additional band-shift experiments with the remaining SAR fragment, we estimate a minimum of 11–13 tight HMG-I/Y binding sites on the 657 bp histone SAR which correspond to A-tracts as shown by footprinting experiments (data not shown).

This band-shift experiment demonstrates that the selective titration of SAR binding sites occurs at weight ratios of HMG-I/Y to DNA ranging from 1:250 to ~1:10. Above, we demonstrated nearly complete displacement of histone

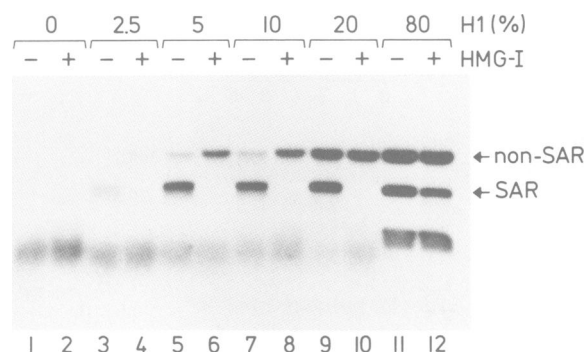
H1 from SARs to non-SARs using an HMG-I/Y:DNA weight ratio of  $\sim 1:12$  (Figure 1B), which corresponds to the high end of the specific titration of a SAR fragment by HMG-I/Y (Figure 2A, lanes 6–7). We asked next whether partial titration by HMG-I/Y might suffice to derepress transcription of a SAR template. Indeed, selective derepression of the SAR template was observed following addition of as little as 4–8 ng of HMG-I, corresponding to a weight ratio of HMG-I to DNA of 1:60 to 1:30 (Figure 2B, lanes 4 and 5). The ratios that result in the derepression of the SAR template and in the concomitant repression of the non-SAR template thus are within the range of selective titration of A-tracts by HMG-I/Y (compare lanes 3–7 of panels A and B). Full derepression is observed with 8 ng of HMG-I/Y added or one molecule of protein per 450 bp of total DNA (Figure 2B, lane 5). As is evident from a comparison of the band-shift and derepression experiments, this amount corresponds to an average of two to four filled sites in the SAR fragment shown in Figure 2A (lanes 4 and 5). Clearly, the displacement of H1 by HMG-I/Y occurs following the selective titration (by competitive interaction) of some—but not necessarily all—of the A-tracts of the histone SAR. In the titration experiment of Figure 2B, we again observed that addition of increasing amounts of HMG-I (16–64 ng) led to the progressive repression of the non-SAR template (lanes 6–8). Under these conditions, selective derepression of the SAR template occurs at weight ratios of HMG-I/Y to DNA ranging from as low as 1:60 up to 1:3.75. Higher HMG-I/Y:DNA ratios (1:1.875, lane 9) result in overtitration and inhibition of transcription from both templates.

#### **Derepression occurs independently of order of addition and does not require the acidic C-terminal region of HMG-I/Y**

Does HMG-I/Y-mediated derepression occur by simple competition for binding sites with histone H1? We expressed and purified a mutant HMG-Y protein (called HMG-Y $\Delta$ E) containing a 16 amino acid deletion of the highly acidic C-terminal region found in these proteins (Lund *et al.*, 1987; Eckner and Birnstiel, 1989; Johnson *et al.*, 1989). This negatively charged terminus, we argued, might possibly be required to dislodge H1 from the SAR. Interestingly, the HMG-Y $\Delta$ E protein was equally effective in derepressing transcription of the SAR template, demonstrating that the acidic C-terminus is dispensable for derepression (Figure 2C, compare lanes 7 and 8). In addition, the order of addition of H1 and of the HMG protein is of no importance, demonstrating the equilibrium situation of the system. Late or early addition of HMG-I/Y both result in derepression (compare lanes 5 and 7 for HMG-Y and lanes 6 and 8 for HMG-Y $\Delta$ E) and in the concomitant repression of the non-SAR template. Thus, HMG-I/Y does not solely work as an anti-repressor which needs to be added to the template prior to histone H1 as has been observed with several transcription activators (Croston *et al.*, 1991). In this *in vitro* system, HMG-I/Y behaves as a true derepressor of H1-mediated inhibition of transcription.

#### **HMG-I/Y mediates the redistribution of histone H1 from SAR to non-SAR DNA fragments**

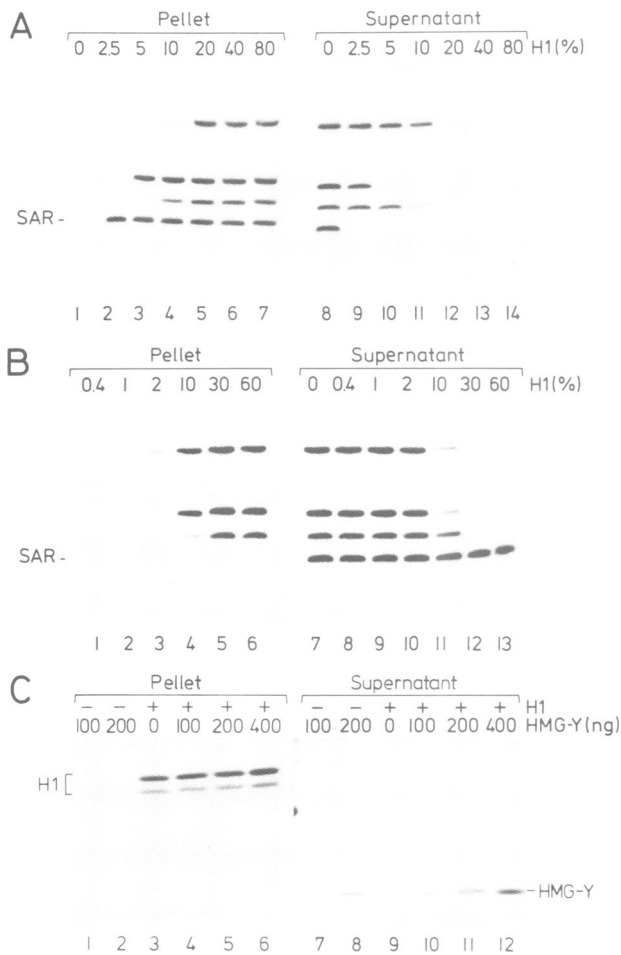
In Figures 1B and 2, derepression of transcription from the SAR template by HMG-I/Y led in turn to a linked repression of the non-SAR transcript. This reversal is likely to be due



**Fig. 3.** HMG-I/Y mediates the redistribution of histone H1 from SAR to non-SAR fragments. 20  $\mu$ l samples containing 240 ng of competitor DNA and radioactively labeled SAR and non-SAR DNA fragments were titrated at different histone H1:DNA weight ratios as indicated above the gel. Samples also received 0 or 20 ng of HMG-I as shown by minus or plus signs. The samples were then digested with DNase I and the purified DNA samples were analyzed by agarose gel electrophoresis. The DNA probe used was derived from plasmid K38 digested with *Xba*I and *Eco*RI which generates a 3.0 kb vector non-SAR fragment, a 1.0 kb fragment containing the 657 bp histone SAR and a 0.25 kb non-SAR fragment. Nuclease digestion products migrate as a smear ahead of the 0.25 kb DNA fragment.

to an HMG-induced redistribution of histone H1. We used the following approaches to demonstrate this directly. DNA–H1 complexes are known to be DNase I-resistant along the entire length of the DNA fragment due to the highly cooperative polymerization of H1 (Izaurralde *et al.*, 1989). If the derepression mediated by HMG-I/Y is due to the redistribution of H1 from the SAR to the non-SAR fragment, then the DNase I-resistance should shift accordingly. In the following experiment we titrated a mixture of appropriately end-labeled SAR and non-SAR DNA fragments with an increasing amount of histone H1 and subsequently challenged the samples with DNase I. At the particular dose of nuclease used, complete degradation of test fragments is observed in the absence of added H1 or following the addition of HMG-I near the optimal derepression level (Figure 3, lanes 1 and 2). Upon titration of the samples with H1 in the absence of HMG-I, preferential protection of the SAR fragment can be noted as expected (lanes 3, 5 and 7). This is in contrast to the non-SAR fragments which are nuclease-sensitive; their intensity is strongly reduced to a level of  $\sim 10\%$  of the input or less. This situation is completely reversed upon addition of HMG-I/Y. In this case we note a strong enhancement of nuclease resistance of the non-SAR fragment, while the SAR fragment is completely degraded (lanes 6, 8 and 10). This is most likely due to the redistribution of histone H1 from the SAR to the non-SAR fragment. At high H1:DNA ratios, all fragments are H1-bound and nuclease-resistant (lane 11).

A different assay can also be used to follow the selective interaction of H1 with SARs: histone H1–DNA complexes form aggregates that can be collected by centrifugation (Izaurralde *et al.*, 1989). This simple assay is useful as it is possible to follow the partitioning of several DNA fragments as well as that of the input proteins into pellet and supernatant fractions. We appropriately digested and end-labeled a plasmid clone containing the entire *Drosophila* histone gene repeat. Titration of this probe with H1 led to the quantitative and selective aggregation of the SAR (Figure 4A, compare lanes 2 and 9). Addition of more H1 resulted in the progressive aggregation of the non-SAR



**Fig. 4.** HMG-I/Y mediates the selective dissociation of histone H1-SAR complexes. Plasmid clone DM506, which contains the 5.0 kb *Drosophila* histone gene repeat, was digested with *EcoRI*, *XhoI* and *HindIII* and end-labeled to yield a mixture of SAR and non-SAR DNA fragments (Mirkovitch *et al.*, 1984). The probe was mixed with 250 ng of competitor DNA and incubated with histone H1 at the different H1:DNA weight ratios shown. Samples were fractionated by centrifugation and pellet and supernatant DNA fractions were analyzed by gel electrophoresis (A). Samples in panel B were incubated for an additional 15 min in the presence of 20 ng of purified HMG-I prior to centrifugation. Panel C shows the distribution of HMG-Y and histone H1 into the supernatant and pellet fractions from a scaled-up experiment. DNA samples containing 5  $\mu$ g salmon sperm DNA and 1  $\mu$ g histone H1 were incubated with 0, 100, 200 or 400 ng of HMG-I prior to centrifugation. The purified proteins were analyzed by SDS-PAGE.

fragments as well (lanes 3–7 and 10–14). In contrast, H1 titration of these fragments in the presence of HMG-I led to a complete reversal: the SAR fragment became in this case the most soluble fragment and was quantitatively retained in the supernatant even at the highest concentrations of H1 tested (Figure 4B, compare lanes 1–6 and 7–13).

Do histone H1 and HMG-I/Y form mixed complexes? To answer this question, we examined by SDS-PAGE the proteins recovered in the pellet and supernatant fractions of a scaled up experiment. A fixed amount of H1 (20% H1:DNA weight ratio) was used to aggregate SARs selectively. We observed that all of the added H1 partitioned as a DNA-protein complex into the pellet, irrespective of added HMG-Y (Figure 4C, lanes 9–12). HMG-Y, however, remained in the supernatant fraction when added at HMG-I/Y:DNA weight ratios of 1:50 or 1:25 (lanes 4

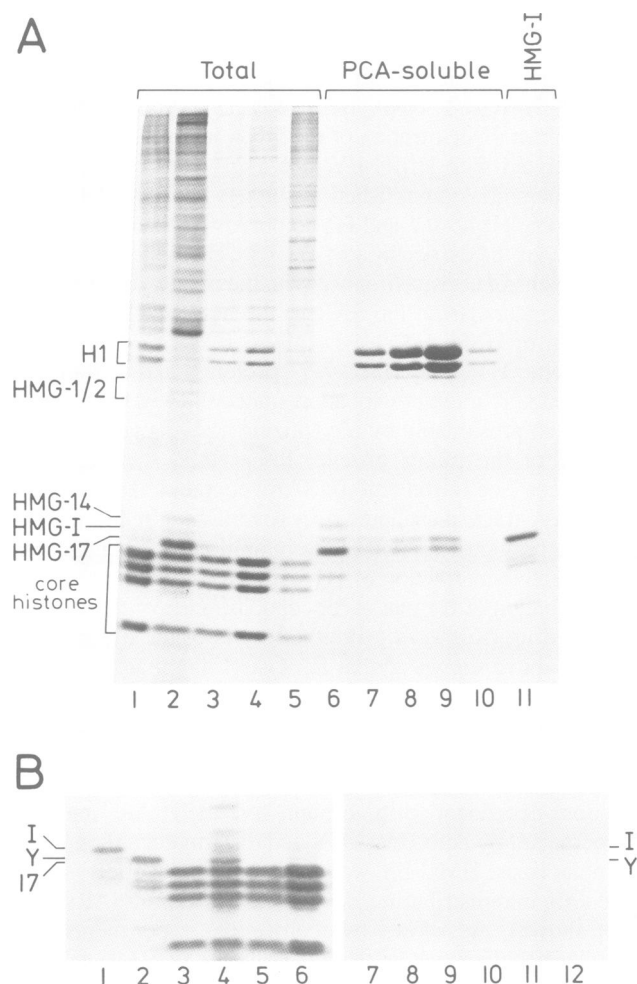
and 5) which mediate selective displacement of H1 from SAR-containing DNA (see Figure 2B, lanes 4–6). The pelleting of a minor fraction of the input HMG-Y at the highest HMG-I/Y:DNA ratio of 1:12.5 tested (lane 12) results from over-titration of the DNA in the system; DNA over-titrated with HMG-I/Y also aggregates (K.Zhao and U.K.Laemmli, unpublished results). Thus, under these conditions, HMG-I/Y and H1 do not form mixed complexes and, given the association of H1 with the pellet DNA, H1 must exchange, in accordance with the results of the DNase I experiments, from the SAR to the non-SAR DNA upon addition of HMG-I/Y.

Histone H1, the HMG-I/Y proteins and distamycin preferentially interact with short tracts of dA·dT base pairs (A-tracts) presumably by recognizing a particular structural feature of the minor groove, rather than specific DNA sequences (see Introduction). SARs contain a large number of such A-tracts distributed over several hundred base pairs and we previously reported the inhibition by distamycin of the selective interaction of H1 with SARs and the resulting redistribution of H1 that occurs upon titration of the A-tracts by the drug (Käs *et al.*, 1989). Results of the experiments shown above suggested that HMG-I/Y might act similarly. We performed centrifugation assays similar to those shown in Figure 4 to compare HMG-I/Y and distamycin directly and found that both behaved similarly in displacing histone H1 from a SAR-containing DNA fragment. We observed a dose-dependent displacement of the SAR into the supernatant by both HMG-I/Y and distamycin although, on a molar basis, HMG-I/Y was ~50 times more effective than the drug in redistributing H1 from SARs to non-SARs (data not shown). As shown by footprinting experiments, this similar behavior results from the binding of HMG-I/Y and distamycin to identical sequences of the histone SAR (E.Käs, unpublished results).

#### **HMG-I/Y is strongly enriched in a histone H1-depleted subfraction of active chromatin**

Nuclei are known to contain a subfraction of chromatin that is soluble in magnesium-containing buffers. This chromatin fraction, called S1, is depleted of histone H1 and is enriched in active sequences. The remaining chromatin can be further fractionated in EDTA-containing low ionic strength buffers into soluble (S2) and insoluble (P) fractions. The S2 fraction contains the bulk of the input chromatin (Rose and Garrard, 1984; Huang and Garrard, 1989). If HMG-I/Y is involved in the displacement of H1 in chromatin, it would therefore be expected to be enriched in H1-depleted, active chromatin.

We used this biochemical fractionation procedure to examine the partitioning of HMG-I/Y in chromatin from HeLa nuclei. We prepared S1, S2 and P fractions from HeLa chromatin, and the total proteins recovered in each fraction were examined by SDS-PAGE (Figure 5A, lanes 1–5). These protein fractions were also extracted with perchloric acid (PCA), which selectively solubilizes histone H1 and the HMG proteins (Johns, 1982). PCA-soluble proteins in each fraction were then similarly analyzed by SDS-PAGE (lanes 6–10). As shown in Figure 5A, S1 chromatin is strikingly depleted of histone H1 (compare lanes 1 and 2), this is particularly evident in PCA-extracted samples (compare lane 6 with lanes 7–10). This striking depletion occurs in parallel with a very strong general enrichment for the following HMG proteins: HMG-I, Y, 14, 17, 1 and 2. The identity of these HMG proteins was established on the basis



**Fig. 5.** A histone H1-depleted subfraction of active chromatin is enriched for HMG-I/Y. (A) Different amounts of samples from intact HeLa nuclei or from S1, S2 and P chromatin fractions (see text for details) were loaded in each lane to yield comparable amounts of core histones (lanes 1–5) or of HMG proteins (lanes 6–10). Lanes 1–5 show unfractionated protein samples while lanes 6–10 show PCA-soluble proteins from each fraction. Note that ~50% of the protein in the HMG-17 band (lane 2) is micrococcal nuclease. This contaminant is lost following acid extraction (lane 6). The amounts loaded are expressed in  $A_{260}$  unit-equivalents of starting material. Lane 1: intact nuclei, 0.1  $A_{260}$  unit; lane 2: S1, 4 unit-equivalents; lanes 3 and 4: S2, 0.1 and 0.2 unit-equivalents; lane 5: P, 0.4 unit-equivalent; lane 6: S1, 4 unit-equivalents; lanes 7, 8 and 9: S2, 0.5, 1 and 2 unit-equivalents; lane 10: P, 2 unit-equivalents. Lane 11 contained 1  $\mu$ g of purified HMG-I protein. Samples were analyzed by SDS-PAGE on a 15% acrylamide gel and stained with Coomassie brilliant blue. The positions of H1, HMG-1, HMG-2, HMG-14/17 and HMG-I are indicated at the left of the gel. (B) Protein samples were electrophoresed on a 15% acrylamide-SDS gel and stained with Coomassie brilliant blue (lanes 1–6) or blotted on to nitrocellulose and probed with an antibody directed against HMG-I (lanes 7–12). The samples contained 0.5  $\mu$ g of HMG-I (lanes 1 and 7), 0.5  $\mu$ g of HMG-Y (lanes 2 and 8), 0.1  $A_{260}$  unit of intact nuclei (lanes 3 and 9), 4 unit-equivalents of the S1 fraction (lanes 4 and 10), 0.1 unit-equivalent of the P fraction (lanes 5 and 11) and 0.5 unit-equivalent of P (lanes 6 and 12). The protein samples were loaded on the basis of equal core-histone contents (lanes 3–5 and 9–11), or overloaded by 5-fold (lanes 6 and 12). Note that in this experiment, the pellet fraction represents bulk chromatin recovered after a single centrifugation and is therefore a mixture of the S2 and P fractions shown in panel A. The low HMG-Y signal is due to a weaker cross-reactivity with the HMG-I antibody.

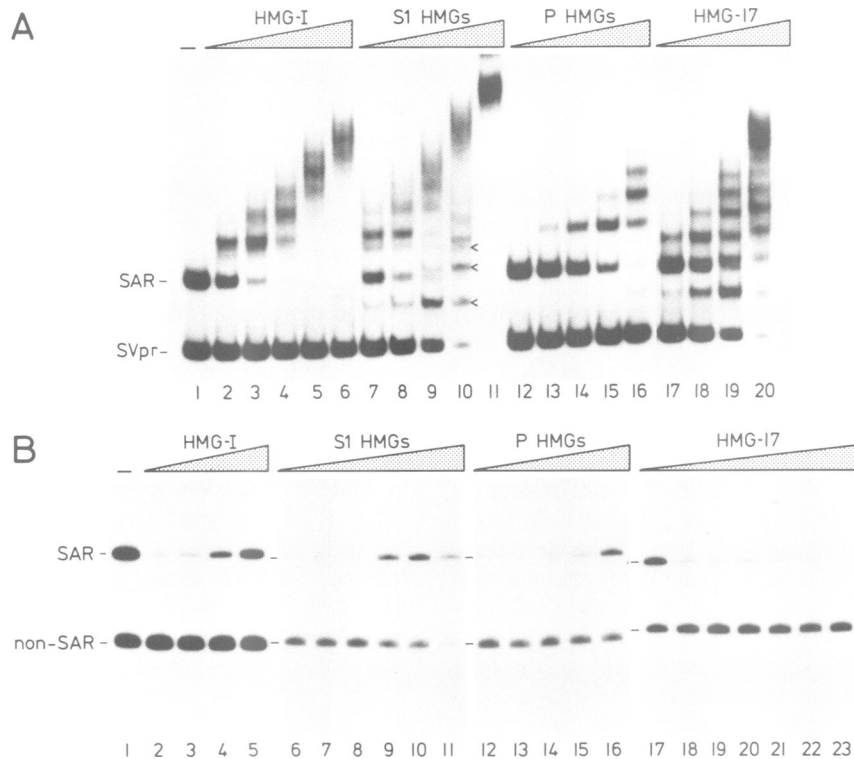
of their acid-solubility and their co-migration with purified proteins on 15% acrylamide-SDS or acid-urea gels (data not shown). HMG-I/Y was further identified by immunoblotting (Figure 5B).

Interestingly, HMG-I is strongly enriched in S1 chromatin. In order to match the intensity of the HMG-I band in S1 with that in S2, it is necessary to overload the S2 fraction by 5- to 10-fold on the basis of core-histone content (compare lane 6 and lanes 7–8 in Figure 5A). This is more clearly seen in Figure 5B which shows a Western blot of S1 and bulk (S2 plus P) fractions probed with an antibody raised against HMG-I (compare lane 10 with lanes 11 and 12). HMG-17 is the most abundant HMG protein found in S1 chromatin. A comparably intense HMG-17 band is detected in S2 only when overloaded by ~20-fold on a core-histone basis (compare lanes 6 and 9 in Figure 5A). A less abundant, high molecular weight, acid-soluble protein is also enriched in S1 and was identified as HMG-1/HMG-2 by migration on acid-urea gels (lanes 2 and 6 and data not shown).

The biochemical enrichment of HMG-I in the S1 fraction can also be revealed by using crude HMG fractions derived from S1 and bulk chromatin in a band-shift assay. The crude HMG fraction from S1 generates a SAR-specific ladder identical to and quantitatively matched by that obtained with purified HMG-I (Figure 6A, compare lanes 2–6 and 7–11). In agreement with the biochemical analysis shown above, on a nucleosome basis, a 5- to 10-fold lower SAR-binding activity is detected in the crude HMG fraction derived from bulk chromatin (lanes 12–16). Careful inspection of the band-shift products derived from the crude S1 HMG fraction reveals a second distinct ladder of shifted products (indicated by arrowheads in Figure 6A). This intermediate binding activity was identified as being due to HMG-17 using purified protein (compare with lanes 17–20) and is correspondingly weaker in the crude HMG fraction from bulk chromatin. Binding of HMG-17 to DNA occurs at much higher amounts of added protein and is not SAR-specific. The crude HMG fractions were also tested in the T7 derepression assay. As shown in Figure 6B, a derepression activity similar to that of HMG-I was detected in S1 extracts, and in much lower amounts in bulk HMG extracts (compare lanes 7–11 and 12–16 with lanes 3–5). This activity could be attributed to HMG-I but not to HMG-17, which, in agreement with its failure to bind SAR-DNA specifically, failed to mediate derepression (lanes 19–23).

Table I summarizes the results of a time-course experiment which demonstrates that the recovery of HMG-I in the S1 fraction is dependent on digestion by micrococcal nuclease and roughly parallels the release of nucleosomes into that fraction. We estimate that, on a core-histone basis, HMG-I is enriched by ~7-fold in histone H1-depleted S1 chromatin. Maximally, ~5% of the total chromatin partitions into S1 together with ~35% of the total HMG-I/Y content of the nucleus. In contrast, most of the HMG-17 recovered in the S1 fraction is released within 1 min of digestion by micrococcal nuclease (see Discussion). Although we observe a nuclease-dependent release of HMG-I/Y into the S1 fraction, centrifugation studies indicate that the protein is no longer chromatin-bound (data not shown). The S1 fraction is primarily composed of mono-nucleosomes (data not shown and see Huang and Garrard, 1989) and it is reasonable to





**Fig. 6.** A SAR-specific binding activity indistinguishable from HMG-I/Y is present in H1-depleted active chromatin and relieves H1-mediated inhibition of T7 transcription. Purified HMG proteins and HMG-containing fractions from S1 and bulk chromatin (see Materials and methods) were tested for SAR-specific binding and derepression of T7 transcription. Panel A shows SAR-specific binding detected by the gel shift assay described in Figure 2A. DNA samples were incubated with 0, 1, 2, 4, 8 and 16 ng of purified HMG-I (lanes 1–6) or with increasing amounts of HMG fraction from S1 chromatin (0.035–0.7  $A_{260}$  unit-equivalents, lanes 7–11), or from P chromatin (0.00085–0.017 unit-equivalents, lanes 12–16), or with 10, 20, 40 and 80 ng of purified HMG-17 (lanes 17–20). The amounts of S1 and P HMG fractions used correspond to equal amounts of core histones. HMG-17–DNA complexes are indicated by arrowheads (see text). Panel B shows the results of a derepression experiment similar to that shown in Figure 1B. SAR and non-SAR DNA templates were transcribed with T7 RNA polymerase without protein addition (lanes 1 and 17) or in the presence of histone H1 (40% weight ratio, lanes 2, 6 and 18). Other samples were incubated with histone H1 and HMG proteins as follows: 2, 5, 10 ng of purified HMG-I (lanes 3–5), increasing amounts of the HMG fraction from S1 chromatin (0.14–1.4  $A_{260}$  unit-equivalents, lanes 7–11) or from bulk chromatin (0.0068–0.068 unit-equivalents, lanes 12–16), or with 10, 20, 40, 80 or 160 ng of purified HMG-17 (lanes 19–23). The amounts of S1 and P HMG-protein fractions added correspond again to equal core-histone contents.

**Table I.** Enrichment of HMG-I in the histone H1-depleted subfraction of active chromatin

Length of digestion by MNase (in min)	0	1	2	4	8	30
Fraction of chromatin released in S1 (% total)	0	ND	0.3	1.25	2.5	5
HMG-I released in S1 per $A_{260}$ unit of nuclei (ng)	5	5.2	11	30	48	110
Nucleosomes per HMG-I molecule in S1	–	–	1.6	1.6	1.9	1.57
Enrichment of HMG-I in S1 (relative to bulk)	–	–	6.9 ×	6.9 ×	5.8 ×	7.0 ×
HMG-17 released in S1 per $A_{260}$ unit of nuclei (ng)	50	500	500	500	500	500

The release of HMG-I and of HMG-17 in the soluble (S1) chromatin fraction described in the text was measured as a function of micrococcal nuclease (MNase) digestion. Amounts of protein are shown per  $A_{260}$  unit-equivalent of starting nuclei and were estimated from DNA binding activity measurements relative to known amounts of purified recombinant proteins (Figure 6A). Note that small amounts of HMG-I and HMG-17 (~5 and 50 ng per  $A_{260}$  unit of nuclei, respectively) are extracted in the absence of MNase digestion (time-point 0). The enrichment of HMG-I in the S1 fraction is relative to whole nuclei, in which we measured an average of 11 nucleosomes per HMG-I molecule (~300 ng per  $A_{260}$  unit, of which up to 110 ng are recovered in the S1 fraction). Nuclei contain ~600 ng of HMG-17 per  $A_{260}$  unit of which up to 500 ng are recovered in the S1 fraction. The amounts of HMG-I and HMG-17 in nuclei were estimated by comparison with known amounts of purified recombinant protein. For our calculations we assumed 37  $\mu$ g DNA per  $A_{260}$  unit of nuclei and 170 bp of DNA per nucleosome.

suggest that the dissociation of HMG-I/Y from chromatin might be brought about by digestion of the nucleosomal linker DNA.

## Discussion

Chromatin domains that contain transcriptionally active genes are structurally altered at several different levels and are more open or accessible than bulk chromatin (reviewed by Felsenfeld, 1992). Major biochemical modifications of active chromatin include a sub-stoichiometric content of histone H1 (Ericsson *et al.*, 1990; Kamakaka and Thomas, 1990; Tazi and Bird, 1990) and the presence of gaps in the nucleosomal array that are associated with the regulatory regions of genes (reviewed by Grunstein, 1990). Recent studies have dealt with the mechanism whereby such nucleosomal gaps are created (Schmid *et al.*, 1992; Workman and Kingston, 1992). In this report, we have focused on the initial unfolding of the transcriptionally silent chromatin fiber that results from the displacement of histone H1 and which is thought to represent an early, obligatory step leading to transcriptional activation.

A simple mechanism whereby histone H1 might be displaced from chromatin is through mobilization by interference with its preferential interaction with A-tracts in DNA (reviewed by Laemmli *et al.*, 1992). Such a mobilization would be expected to result in a new equilibrium of H1–DNA interactions and thus lead to H1 redistribution. Strong support for this hypothesis is based on our previous studies with the oligopeptide distamycin, which selectively binds to A-tracts, and which we used as a tool to study SAR function both *in vivo* and *in vitro*. As tested experimentally, addition of distamycin to cells or nuclei leads to a reduced occupancy of nucleosomal linkers presumably due to the displacement of histone H1. This conclusion is based on the significant distamycin-dependent enhancement of cleavage by topoisomerase II or by restriction enzymes observed *in vivo* and *in vitro* at the nucleosomal linkers of SARs as well as by a number of biochemical extraction and assembly experiments (Käs *et al.*, 1993). We formulated a model based on these observations and proposed that open, H1-depleted chromatin regions may be generated by titration of the A-tracts of SARs by putative distamycin analogs ('D-proteins'). The resulting displacement of the tightly bound H1 molecules would lead to a local opening of chromatin originating at SARs which may spread to adjacent regions depending on the extent of cooperative H1–H1 interactions in chromatin.

As assayed by sensitive T7 RNA polymerase transcription repression/derepression experiments, our studies demonstrate that HMG-I/Y functions as a D-protein *in vitro*. Following the highly selective repression of a SAR-containing template by histone H1, addition of HMG-I/Y leads to equally selective derepression (Figures 1 and 2). The components of this assay system are in equilibrium since the order of addition of HMG-I/Y and of histone H1 is of no importance (Figure 2C). Thus, HMG-I/Y behaves as a genuine derepressor and does not solely work as an anti-repressor which needs to be added to the template prior to histone H1 as has been observed with transcription factors such as GAL4, VP16 and Sp1 (Croston *et al.*, 1991).

HMG-I/Y is known to bind preferentially to A-tracts containing four to seven or more dA·dT base pairs (Solomon *et al.*, 1986; Reeves *et al.*, 1987). The 657 bp histone SAR

used in these experiments contains 11–13 tight HMG-I/Y binding sites as determined by band-shift and footprinting experiments. The selective titration of about half of these sites by HMG-I/Y is sufficient to achieve complete derepression of the SAR-containing template which is observed at HMG-I/Y to histone H1 weight ratios ranging from 1: 25 to 1:10 (Figure 2A and B). The 1200 bp SAR template used in our transcription experiments can bind about 30 cooperatively interacting histone H1 molecules (one H1 per 40 base pairs, Izaurralde *et al.*, 1989) which can be displaced by the specific binding of about 6 to 8 HMG-I/Y molecules to the histone SAR (Figure 2). A greater number of H1 molecules would of course be displaced if longer transcription templates were used.

The binding of HMG-I/Y to the SAR template leads to the mobilization of histone H1 and to its redistribution on to non-SAR DNA fragments (Figure 1B); no soluble (free) H1 is observed experimentally (Figure 4C). This redistribution is demonstrated by the shift of transcriptional repression, of DNase I-resistance (Figure 3) and of the aggregation behavior from the SAR to the non-SAR templates (Figure 4). In the presence of HMG-I/Y, the cooperative association of H1 with the non-SAR fragments is thus energetically more favorable. This shift in equilibrium is brought about by the titration by HMG-I/Y of the A-tracts of the SAR which also constitute the preferential H1 binding sites, and results in the inactivation (or functional deletion) of the SAR as nucleation sites of H1 assembly. This is most likely due to the steric impediment, imposed by SAR-specific HMG-I/Y binding, to the propagation of an uninterrupted H1 polymer stabilized by cooperative interactions. In addition, the titration of the clustered A-tracts of the SAR by HMG-I/Y may also, by exerting a *Circe* effect (Mirkovitch *et al.*, 1984; Adachi *et al.*, 1989), facilitate and favor further interactions with the sparser A-tracts of flanking DNA regions rather than with identical sites present in the non-SAR fragment. In essence, SAR-containing fragments, once titrated by HMG-I/Y, acquire the behavior of GC-rich DNA which binds H1 poorly (Käs *et al.*, 1989). The acidic tail of HMG-I/Y, common to many transcriptional activators and anti-repressors (reviewed by Ptashne, 1988; Sigler, 1988) and which, arguably, might be required to displace histone H1, is dispensable in our test system: the HMG-YΔE mutant is as efficient as the wild-type protein in inducing H1 redistribution (Figure 2C). We cannot, however, rule out the possibility that such a domain might play a role in the context of a complete eukaryotic RNA polymerase transcription system—and *a fortiori* in chromatin—where it might be required for interaction with other factors.

Our experiments do not at present establish a causal relationship between SAR-specific HMG-I/Y binding and the displacement of histone H1 in chromatin, but an extension of our conclusions to chromatin seems quite reasonable. Major arguments in support of this hypothesis include the strong enrichment of HMG-I/Y in the H1-depleted S1 chromatin fraction (Figures 5 and 6), the results of our previous experiments with distamycin (Käs *et al.*, 1993) and the fact that H1 appears to bind similarly to DNA and to chromatin, a conclusion which, although not directly proven, is not in contradiction with the available experimental evidence. Cross-linking studies indeed demonstrate a similar H1 oligomerization as a function of binding to DNA or to chromatin (Clark and Thomas, 1986; De Bernardin *et al.*, 1986). Furthermore, the subfraction of H1 in chromatin that



is resistant to extraction by poly-glutamic acid and is specifically displaced by distamycin behaves identically to H1 bound to SAR-DNA *in vitro* (Käs *et al.*, 1993).

The enrichment factor for HMG-I/Y in S1 over bulk chromatin is ~7-fold, corresponding to an average of 1.7 nucleosomes per molecule. The histone H1-depleted S1 fraction, which is known to be enriched in actively transcribed genes (Rose and Garrard, 1984), also contains a general abundance of HMG proteins, the predominant species being HMG-17 (Figure 5; see also Jackson *et al.*, 1979; Chambers and Rill, 1984). The release of HMG proteins into the S1 supernatant fraction is nuclease-dependent, and the solubilization of HMG-I/Y, in contrast to HMG-17, parallels that of nucleosomes. Interestingly, most of the HMG-17 is released from nuclei within 1 min of digestion by micrococcal nuclease (Table I). HMG-17 might interact preferentially with torsionally stressed chromatin domains and might be displaced by their relaxation as a result of DNA nicking induced by brief digestion. The disruption of higher-order nucleosome folding by nuclease digestion might also cause this rapid release (Mathew *et al.*, 1979). The HMG proteins are thought to be implicated in the formation of active chromatin (Goodwin *et al.*, 1979; Jackson *et al.*, 1979; Albanese and Weintraub, 1980; Sandeen *et al.*, 1980), but only one of these—HMG-I/Y—is active in the displacement of histone H1 (Figure 6). As discussed above, low HMG-I/Y:histone H1 weight ratios ranging from 1:25 to 1:12.5 are sufficient to effect derepression of a SAR template *in vitro*. Such ratios are in fact remarkably similar to the HMG-I:H1 ratio of ~1:12 that we observe in HeLa nuclei (Figure 5).

The chromosomal pattern of repressed and open chromatin domains appears to be stably propagated during cell growth (Weintraub, 1985). During cellular differentiation, SARs and HMG-I/Y might be involved in the establishment of the overall pattern of open and closed chromatin domains rather than in the fine tuning of genomic activity. HMG-I/Y may be considered to be a structural component of chromatin, albeit one with a variable stoichiometry. HMG-I/Y levels are high in rapidly dividing cells while low or undetectable levels are observed in differentiated, non-proliferating cells (Johnson *et al.*, 1988, 1990). This observation provides general support for the argument that HMG-I/Y may be involved in the formation of active chromatin, but how is the regional specificity of chromatin activation achieved? Biological specificity, such as the firing of an engaged RNA polymerase, results from interactions involving a multi-component system and the regional activation of chromatin is similarly expected to result from consecutive and collaborative events. In this scheme, HMG-I/Y would mobilize histone H1 at or near SARs; the subsequent H1 redistribution would then spread to adjacent regions and facilitate in turn the binding of additional factors, such as those involved in the displacement of nucleosomes. HMG-I/Y activity might itself be regulated by reversible phosphorylation (Palvimo and Linnala-Kankkunen, 1989; Nissen *et al.*, 1991; Reeves *et al.*, 1991). Additional events, such as histone acetylation, might then occur to 'freeze' a particular chromatin conformation that is accessible to transcription factors. These and other mechanisms that might impart specificity to chromatin opening, as well as the possible interaction between SARs and other specialized DNA elements thought to play a role in the control of long-

range chromatin organization, have recently been reviewed (Laemmli *et al.*, 1992).

SARs are generally observed in close association with the regulatory elements of tissue-specific genes of *Drosophila* (see Gasser and Laemmli, 1987 for review) and of several mammalian genes (for example, Cockerill and Garrard, 1986; Cockerill *et al.*, 1987; Bode and Maass, 1988; Jarman and Higgs, 1988; Phi-Van and Strätling, 1988). Notable exceptions are the SARs associated with the *Drosophila* histone gene cluster and with the heat-shock genes (Mirkovitch *et al.*, 1984) which are ubiquitously expressed. Common to these genes, however, is their high level of transcriptional activity during a limited stage of the cell cycle or in a restricted number of cells during development. We suggest that SARs may serve to facilitate the rapid temporal activation of these genes as initiated by HMG-I/Y and effected by available tissue-specific factors. We note that HMG-I/Y has previously been reported to play a role in DNA replication and transcription (reviewed by Reeves, 1992). More recent evidence conclusively demonstrates the involvement of HMG-I/Y in the viral induction of the human IFN- $\beta$  gene where it facilitates binding of the NF- $\kappa$ B transcription factor (Thanos and Maniatis, 1992). The role for HMG-I/Y discussed here does not by any means exclude a more extended involvement of this protein as an auxiliary factor.

If SARs work according to the chromatin switch model, one would expect that SARs flanking a test gene would have either a repressive or stimulatory effect on the level of its transcription, depending at first approximation on the ratio of the putative D-proteins and histone H1 (reviewed in Laemmli *et al.*, 1992). A number of gene expression studies have demonstrated that SARs flanking homologous and heterologous reporter gene constructs stimulate transcription by a factor of 10- to 20-fold (Stief *et al.*, 1989; Phi-Van *et al.*, 1990; Klehr *et al.*, 1991), while deletion of the intronic SAR of the mouse immunoglobulin kappa gene leads to a 3- to 4-fold reduction in gene expression (Blasquez *et al.*, 1989; Xu *et al.*, 1989). Common to these studies is the observation that SARs, in contrast to transcriptional enhancer elements, exert their stimulatory effect only if the reporter gene is stably integrated into the genome but have no effect in transient transfection assays. These observations are consistent with the chromatin switch model. Transiently transfected genes, in contrast to stably integrated ones, are quite efficiently transcribed and they appear to adopt an open chromatin structure (discussed by Weintraub, 1985); an element facilitating chromatin opening would be expected to be unnecessary and therefore without effect in this case.

The 5'-regulatory regions of housekeeping genes as well as those of several tissue-specific genes are associated with regions of unmethylated CpG-rich DNA (Bird, 1987; Gardiner-Garden and Frommer, 1987). The chromatin of these GC-rich islands is known to be largely—and conveniently—free of histone H1 (Tazi and Bird, 1990), perhaps as a result of the low affinity of H1 for GC-rich DNA (Käs *et al.*, 1989). In a cell with a sub-stoichiometric histone H1 content, the distribution of this histone would be skewed toward AT-rich sequences. As a corollary, given the 'default' open chromatin conformation of CpG islands, we suggest that cells need a special mechanism—methylation-specific interactions with the methyl-CpG-binding proteins (MeCPs) described by Bird and collaborators—to repress

certain genes. The prediction would be that MeCPs are specialized histone H1-like proteins. Indeed, two such proteins, MeCP2 and MDBP-2, have been shown to contain putative DNA-binding motifs strikingly similar to those found in histone H1 and/or in HMG-I/Y (Lewis *et al.*, 1992; Jost and Hofsteenge, 1992).

Mapping studies within the 90 kb human  $\beta$ -globin cluster have identified at least eight SARs in close association with the regulatory elements of the  $\beta$ -globin genes (Jarman and Higgs, 1988). In contrast, no SARs were detected within a 140 kb region of the human  $\alpha$ -globin cluster whose genes are all associated with CpG islands (Vyas *et al.*, 1992). Interestingly, the  $\alpha$ -globin cluster is contained within an open (DNase I-sensitive) chromatin domain in both erythroid and non-erythroid cells. In contrast, the chromatin of the  $\beta$ -globin cluster is opened in a tissue-specific manner only in erythroid cells (Vyas *et al.*, 1992). We suggest that, given the constitutively open chromatin configuration of the  $\alpha$ -globin gene cluster, gene-associated SARs are dispensable at this locus. This contrasts with the  $\beta$ -globin locus which may acquire an open chromatin conformation with the help of HMG-I/Y-like proteins acting via SARs, tissue-specific factors and other components of active chromatin. These considerations suggest that eukaryotic cells may have adopted two strategies for chromatin opening, SARs and CpG islands. Future experiments will further define the mechanisms whereby these sequences and the proteins they interact with modulate long-range chromatin organization.

## Materials and methods

### Plasmids and DNA methods

Plasmids K38 and K40 are diagrammed in Figure 1C and were constructed as follows. K38 contains a 1.5 kb *EcoRI*–*HindIII* insert cloned in pUC18, consisting of the 657 bp *HinfI*–*EcoRI* histone SAR (Mirkovitch *et al.*, 1984). The histone SAR was linked to an SV40 promoter-containing fragment (the *PvuII*–*HindIII* fragment from pSV2cat; Gorman *et al.*, 1982) and to an oligonucleotide containing a T7 RNA polymerase promoter. We inserted a fragment of the CAT gene (the *HindIII*–*NcoI* fragment from pSV2cat) 3' of the T7 promoter. The 1.5 kb *EcoRI*–*HindIII* insert of plasmid K40 was also cloned in pUC18 and contains the 774 bp *Sall*–*AvaI* fragment of pBR322 inserted 5' of a 170 bp *BamHI* fragment spanning the promoter region of the rat albumin gene (Mueller *et al.*, 1990), followed by the T7 promoter and CAT gene fragments similar to those in K38. Appropriate adapters were inserted during plasmid construction to produce the restriction sites shown in Figure 1C.

A bacterial expression vector containing human HMG-Y coding sequences was cloned in our laboratory by PCR amplification of a cDNA clone provided by Max Birnstiel (Eckner and Birnstiel, 1989). The 5' and 3' primers used (5'-CATATGAGTGTGAGCTCGAGCTCGAAGTCC and 5'-GGATCCTCACTGCTCCTCTCCGAGGACTC, respectively) generated an *NdeI*–*BamHI* amplification product that was cloned in expression vector pET3b and expressed in *E. coli* strain BL21(DE3)pLysE (Studier *et al.*, 1990). The mutant HMG-Y clone lacking the C-terminal acidic tail (HMG-Y $\Delta$ E) was generated by PCR amplification using the same 5' primer and 5'-GGA-TCCTCACTTCTCCAGTTTTTGGGTCTGCC as the 3' primer, yielding a truncated gene product lacking the acidic C-terminal domain that was cloned and expressed as above.

### Protein purification

Histone H1 from rat liver nuclei was purified as described by Izaurralde *et al.* (1989). Purified recombinant HMG-I was kindly provided by Raymond Reeves and purified HMG-17 was generously given by Michael Bustin. Recombinant HMG proteins were isolated from PCA-extracted bacterial lysates and purified by HPLC (Elton and Reeves, 1986). Briefly, 100–300  $\mu$ g of lyophilized crude HMG protein resolubilized in 100  $\mu$ l of 0.1% trifluoroacetic acid (TFA), 0.1 M DTT and injected in a Vydac-C<sub>4</sub> silica column were eluted as follows at a flow rate of 1 ml/min: 10 min with 0.1% TFA followed by 0–80% buffer B (0.08% TFA, 75% acetonitrile)

over the next 50 min and 100% buffer B for 10 min. 1 ml fractions were collected and analyzed by SDS–PAGE (Laemmli, 1970). Peak fractions were lyophilized, resuspended in water and stored at –70°C.

### T7 transcription assays

DNA mixtures containing competitor DNA (200 ng of *BglI*-digested pUC18 or sonicated salmon sperm DNA) and the various templates described in the figure legends were incubated with purified histone H1 in MB buffer (20 mM Tris–HCl, pH 7.4, 20 mM KCl, 70 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.05 mM spermine, 0.125 mM spermidine, 0.1 mM PMSF, 0.1% digitonin, 0.1% Trasylol) for 15 min at room temperature before addition of HMG-I/Y and the incubation was then continued for 15 min. In some experiments, HMG-I/Y was added first (Figure 2C). Reaction volumes ranged from 5 to 6  $\mu$ l. 5  $\mu$ l of a T7 transcription mix were then added (1.2 mM each of ATP, CTP, GTP, 0.012 mM UTP, 24 mM DTT, 0.24 mg/ml BSA, 3 units RNasin, 12 units T7 RNA polymerase and 3  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]UTP, 800 Ci/mmol). After 15 min at 37°C, reactions were stopped by addition of 100  $\mu$ l TE containing 100 mM NaCl and 1% SDS, the RNA products were then purified and analyzed by electrophoresis on 7.5% acrylamide/7 M urea/1  $\times$  TBE denaturing gels.

### DNA binding assays

For band-shift experiments, binding was performed in 10 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 5% glycerol or in MB buffer. 20  $\mu$ l reactions contained 250 ng of competitor DNA (*BglI*-digested pUC18 or sonicated salmon sperm DNA), radioactively labeled probe and protein as indicated in the figure legends and were incubated for 15 min at room temperature. After addition of 2  $\mu$ l of loading mix (0.1% bromophenol blue, 20% Ficoll 400), samples were electrophoresed in 4% acrylamide/0.25  $\times$  TBE gels for 4 h at 4°C (10 V/cm). The running buffer (0.25  $\times$  TBE) was recirculated between the lower and upper chambers. Gels were dried and autoradiographed. For the experiment shown in Figure 3, DNA samples (probe plus 240 ng of linearized pUC18 DNA) were incubated with histone H1 for 15 min at room temperature. HMG-I was added as indicated in the figure legend and samples were incubated for an additional 15 min before digestion with 200 ng DNase I (DPFF grade, Worthington) for 1 min at room temperature. The DNA samples were then purified and analyzed by agarose gel electrophoresis. Similarly, for the experiments shown in Figure 4, DNA samples were incubated with histone H1 and H1–DNA complexes were collected by centrifugation (Izaurralde *et al.*, 1989) after an additional incubation in the absence or presence of HMG-I. Soluble and insoluble fractions were analyzed by gel electrophoresis. The distribution of H1 and HMG-I proteins in the supernatant and pellet fractions from a scaled-up experiment was analyzed by SDS–PAGE (Figure 4C).

### Chromatin fractionation and HMG protein purification

Nuclei were prepared from exponentially growing HeLa cells and stored at –20°C as described by Mirkovitch *et al.* (1984). For chromatin fractionation, 20 A<sub>260</sub> units of nuclei were washed twice in MB buffer and resuspended in 500  $\mu$ l MB buffer containing 2 mM CaCl<sub>2</sub>. Resuspended nuclei were incubated 5 min at 37°C before addition of 1 unit of micrococcal nuclease ( $\mu$ M units, Sigma) and digested for 2 min at 37°C before addition of EGTA to 3 mM. In some experiments, digestion with 0.2 units of micrococcal nuclease was allowed to proceed for 1–30 min (see Table I). After 10 min on ice, samples were centrifuged for 10 min in the cold at 12 000 g and the H1-depleted active chromatin supernatant (S1) was collected. The nuclear pellet was lysed by resuspension in 500  $\mu$ l of ice-cold 1 mM EDTA, pH 7.4 and resuspended as above. The supernatant (S2) was collected and the pellet (P) was resuspended in 1 mM EDTA, pH 7.4. In some experiments, the bulk chromatin pellet obtained after the first centrifugation was used without further fractionation into S2 and P. Acid-soluble proteins were purified by extraction of the chromatin fractions with 5% perchloric acid followed by acetone precipitation. Samples were analyzed by SDS–PAGE as described in the figure legends. For the experiments shown in Figure 6, histone H1 was removed from acid-soluble proteins of bulk chromatin by chromatography on a heparin column (0.2 ml bed volume) equilibrated in column buffer (20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.1 mM PMSF) containing 0.1 M NaCl. HMG protein fractions were eluted with 0.2 ml of column buffer/0.2 M NaCl followed by 0.6 ml of column buffer/0.5 M NaCl. 0.1 ml fractions were collected, checked by SDS–PAGE and pooled HMG samples were then tested in the band-shift and T7 transcription assays as described above. Acid-soluble proteins from the H1-depleted S1 fraction could be used directly but were similarly fractionated.

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