The effect of histone H1 and DNA methylation on transcription

Colin A. JOHNSON, John P. GODDARD and Roger L. P. ADAMS*
Institute of Biomedical and Life Sciences, Division of Biochemistry and Molecular Biology, University of Glasgow, Glasgow G12 8QQ, U.K.

We have previously shown that DNA methylation acts as a focus for the formation of inactive chromatin *in vivo*. We have investigated the mechanism further by *in vitro* transcription of a template containing two tRNA genes and an extensive (G+C)-rich sequence characteristic of a CpG island. The extent of transcription from the unmethylated or fully methylated template was assayed in the presence of varied levels of histone H1. The transcriptional activity of both templates was inhibited by increasing amounts of histone H1, although inhibition with the methylated template occurs at a lower H1:DNA ratio. The H1c variant shows the greatest preferential inhibition of the methylated template. We demonstrate that histone H1 complexed to DNA is one of the factors that inhibits transcription by preventing the formation of initiation complexes, particularly on methylated template, rather than the formation of disordered H1·DNA aggregates.

INTRODUCTION

Histone H1 is the internucleosomal histone of higher eukaryotes [1]. It has been implicated in the establishment of stable and tissue-specific gene repression [2–5], as it is presumed to have a function in the formation of the 30 nm chromatin fibre [6,7], although molecular models of chromatin compaction and transcriptional repression by histone H1 are open to debate. It has been shown recently that histone H1 is located in the interior of the 30 nm chromatin fibre [8], which supports the solenoidal model of chromatin compaction. Histone H1 has been shown to be abundant in heterochromatin [9] and to be associated with nucleosomes containing 5-methylcytosine [10]. Conversely, it is absent from CpG island chromatin [11], which is characteristic of active autosomal housekeeping genes, and is depleted in chromatin which contains active genes [6,12]. The effects of histone modification and DNA modification, specifically the methylation of DNA at CpG dinucleotides, on the structure and properties of nucleosomes and chromatin has not been clarified.

DNA methylation [13] correlates with the inactivity of many tissue-specific genes in vertebrates [14–16]. Two models have been proposed to explain the mechanisms underlying this correlation. Methyl groups in a promoter may directly interfere with the binding of some transcription factors, such as the cyclic AMP-responsive element binding protein [17], and the transcription factor AP-2 [18]. Alternatively, methylation may direct the formation of an inactive chromatin structure that is inaccessible to transcription factors [19]. Several studies have shown that methylated DNA in both chromatin and nuclei is inaccessible to certain nucleases, such as DNAase I [20] and *Msp*I [21], and it is presumed to be similarly inaccessible to transcription factors. It has been proposed that the inactivation process requires the participation of proteins that bind preferentially to methylated DNA [22]. Several such proteins have been described, namely methyl-CpG binding proteins (MeCPs) [23,24] and methylated DNA binding proteins (MDBPs) [25,26]. MeCP-2 has been shown to be associated with centromeric heterochromatin [23], and MDBP-2 shares sequence identities with histone H1 [27]. These results imply that both proteins have a function in inactive chromatin. The literature is undecided as to whether histone H1 binds preferentially to methylated DNA [28,29], although it has recently been shown to repress preferentially *in vitro* transcription from methylated template DNA [28].

Our earlier studies [30] have shown that, following the transfection of a methylated construct for a polymerase (pol) II gene into dividing mammalian cells, the reporter gene is inactivated as a result of its incorporation into inactive chromatin. Using a plasmid construct containing two tRNA genes and an adjacent CpG island in an *in vitro* transcription assay, the present study provides evidence that histone H1 is centrally involved in the repression of transcription from methylated DNA. Besser et al. [31] also have shown that DNA methylation inhibits transcription by RNA pol III of a chicken tRNA gene, following its microinjection into *Xenopus* oocytes, and they also concluded that repression involved the formation of inactive chromatin structures.

MATERIALS AND METHODS

Plasmid preparations

Supercoiled plasmid pArg/Leu was used as a template for *in vitro* transcription. It was constructed by ligation of a *Bam*HI–*Eco*RI fragment containing a human $tRNA^{Arg}$ and $tRNA^{Leu}$ gene into the pUC19 vector (Figure 1a). The insert was derived by subcloning the two genes from a cluster of tRNA genes, that has been cloned previously from human placenta by Dr. D. Bourne [32]. Plasmid DNA was cloned in *Escherichia coli* JM109 cells and supercoiled DNA was isolated from cell lysates by CsCl isopycnic centrifugation using standard procedures. Covalently closed circular plasmid was obtained by digestion with DNA topoisomerase I (Promega), under conditions recommended by the manufacturer, and linear pArg/Leu was obtained by digestion with *Not*I.

Abbreviations used: DTT, dithiothreitol; MDBP, methylated DNA binding protein; MeCP, methyl-CpG binding protein; NP-40, Nonidet-P40; PMSF, phenylmethanesulphonyl fluoride; pol, polymerase; Sarkosyl, *N*-laurylsarcosine.

* To whom correspondence should be addressed.

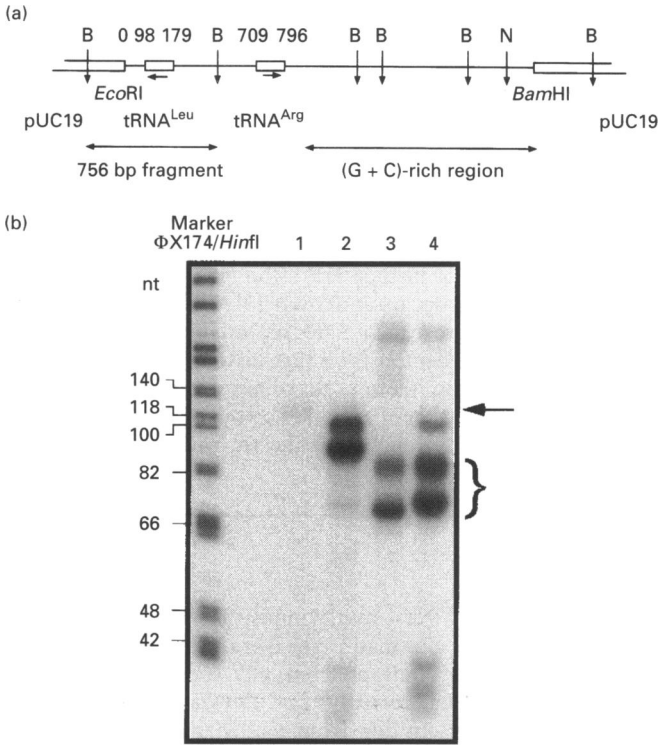

Figure 1 Structure and *in vitro* transcription of pArg/Leu

(**a**) Linear representation of the circular plasmid pArg/Leu, showing a *Bam*HI–*Eco*RI fragment of 2.7 kbp containing a human $tRNA^{Arg}$, a $tRNA^{Leu}$ gene and a (G + C)-rich region. This region resembles a mammalian CpG island, but the exact sequence is unknown. The location of the two genes is indicated in base-pairs from the *Eco*RI site. A *Not*I site (N), *Bgl*I sites (B) and a *Bgl*I–*Bgl*I fragment of size 756 bp, which contains the $tRNA^{Leu}$, are also indicated. (**b**) Transcription assays to determine the transcription products from the pArg/Leu construct. Lane 1: transcription in the absense of added DNA template, showing an unknown endogenous product (indicated by arrow). Lane 2: transcription with a plasmid construct containing only the $tRNA^{Arg}$ gene. Lane 3: transcription of $tRNA^{Leu}$ gene. Lane 4: transcription of pArg/Leu plasmid template. Refer to the main text for the identification of the transcription products. The marker is denatured end-labelled ΦX174/*Hin*fI DNA, with sizes indicated in nucleotides (nt). The transcription products used for subsequent figures and quantitative analyses are indicated by a brace.

In vitro DNA methylation

Template or competitor DNA was methylated with M.*Sss* I methylase (New England Biolabs) under conditions recommended by the manufacturer. Mock-methylated DNA was prepared in parallel by incubation in the absence of M.*Sss* I, and was used as the unmethylated DNA control in all experiments. The extent of methylation was assessed by digestion with *Hpa*II or *Hha*I, and the reaction was terminated by phenol–chloroform extraction. Precise concentrations of plasmid DNA were determined by using a Hoefer TKO 100 DNA minifluorometer. Calf thymus or pUC19 DNA was used as a standard.

Covalently closed circular patch-methylated templates were constructed by methylating the restriction fragments obtained by digestion of pArg/Leu with *Bgl*I, separating and purifying the fragments by standard procedures, and ligating suitable combinations of methylated fragments with unmethylated fragments. A 756 bp fragment which contained the $tRNA^{Leu}$ gene (Figure 1a) was either unmethylated with all the flanking regions methylated, or was itself methylated with all other regions unmethylated. Ligations were carried out at 16 °C for 16 h using T4 DNA ligase (Promega) at a concentration of 3 Weiss units/μg of DNA. Religated covalently closed circular plasmid was separated and purified from unligated fragments by gel electrophoresis in 0.7% (w/v) low melting-point agarose (Gibco BRL).

In vitro transcription

HeLa nuclear extract was prepared as described by Dignam et al. [33]. Histone H1 was depleted from the extract by the selective precipitation of proteins by the addition of 1.25 vol. of 4 M ammonium sulphate [4], buffered with 50 mM Hepes/KOH, pH 7.9. The proteins were pelleted by centrifugation at 100000 *g* for 30 min at 4 °C, and were resuspended in buffer D [20 mM Hepes/KOH, pH 7.9/20% (v/v) glycerol/0.1 M KCl/0.2 mM EDTA/0.5 mM phenylmethanesulphonyl fluoride (PMSF)/0.5 mM dithiothreitol (DTT)] [33].

The conditions for transcription by pol III were optimized by Dr. E. S. Gonos (personal communication). A reaction mixture (final volume 20 μl) contained 5 μl of extract (7.0 mg of protein/ml) and final concentrations of 14 mM Hepes/KOH, pH 7.9, 80 mM KCl, 20 mM NaCl, 3.5 mM $MgCl_2$, 10% (v/v) glycerol, 0.3 mM DTT and 0.3 mM PMSF. The reaction mixture included the desired amount of template DNA (usually 100 ng), with pUC19 DNA as a non-specific carrier, if required, to a total of 150–300 ng of DNA per assay. The mixture was preincubated at 30 °C for 20 min, after which transcription was initiated by the addition of unlabelled ribonucleoside triphosphates at pH 7.0 (600 μM ATP, CTP and GTP; 25 μM UTP), 2.5 μCi of [α-^{32}P]UTP (3000 Ci/mmol, Amersham) and 10 mM creatine phosphate. The transcription reaction was allowed to proceed at 30 °C for 60 min and terminated by the addition of SDS to 0.5% (w/v), 100 μg of proteinase K (Sigma) and 40 μg of *E. coli* crude tRNA as carrier. The mixture was incubated at 37 °C for 20 min and then an equal volume of 1.0 M ammonium acetate was added. The mixture was extracted with phenol–chloroform, and this was followed by a second extraction in the presence of 0.5 M ammonium acetate. The RNA in the aqueous phases was ethanol-precipitated, washed with 70% (v/v) ethanol, dried thoroughly and resuspended in formamide loading buffer (deionized formamide containing 10 mM EDTA, pH 8.0, 0.1% w/v Bromophenol Blue and 0.1% w/v xylene cyanol). Samples were analysed by electrophoresis on a 12% (w/v) polyacrylamide, 4 M urea denaturing slab gel, followed by autoradiography. Transcription was quantified either by scintillation counting or by using a Fuji BAS1000 phosphoimager.

Histone H1 preparation

Total acid-extracted calf thymus histone H1 (Boehringer–Mannhiem Gmbh) was used in initial experiments. Lyophilized protein was dissolved in buffer H [10 mM Tris/HCl, pH 7.5, 1.0 mM DTT, 0.4 mM PMSF, 5 mM EDTA, 0.01% (v/v) Nonidet-P40 (NP-40) and 10% (v/v) glycerol]. NP-40 was included in all buffers containing histone H1 to prevent non-specific adsorption to plastic or glass [4]. The presence of EDTA facilitates histone renaturation [34]. Samples were renatured by step-dialysis [35] to the histone H1 buffer, starting from buffer containing 2 M NaCl and 5 M urea. Preparations were stored frozen at −70 °C. Total core histones (H2A, H2B, H3 and H4) were used as controls for histone H1 as they are highly positively charged but do not repress *in vitro* transcription under these conditions (see below, Figure 9). Core histone octamers were purified from HeLa cell nuclei by hydroxyapatite adsorption chromatography [36] and concentrated to 1–2 mg/ml using low-molecular-mass cut-off Centriprep concentrators (Amicon).

Somatic histone H1 variants H1a–e were partially purified by reversed-phase h.p.l.c. chromatography [37], as modified by Caiafa and coworkers [38]. Protein fractions were lyophylized and renatured as described above. Total histone H1 and H1 variants were analysed and characterized by SDS/12% (w/v) PAGE. Variant preparations were not fully homogenous, because of the incomplete resolution obtained by reversed-phase h.p.l.c. chromatography. Variants were characterized as previously described by Caiafa and coworkers [38], from which it could be concluded that the preparations of H1a and H1d were 95% homogeneous, whereas the H1c preparations were contaminated with H1e. The H1e preparation was also contaminated with H1c, and H1b was not resolved using this method of chromatography. Protein concentrations were determined by Bradford's procedure [39], after correction for the anomalous effect of histones on colorimetric assays.

Formation of histone H1:DNA complexes

Total histone H1 was allowed to bind to 100 ng of supercoiled template DNA under conditions that facilitated the formation of a 'slow' complex, as defined by Clark and Thomas [40]. 'Slow' complexes are presumed to form by the non-cooperative and reversible binding of H1 to DNA. Histone H1 dissolved in buffer H was mixed, in various proportions, with DNA in 20 mM Hepes/KOH, pH 7.0, and 2.0 mM $MgCl_2$ [41] in the presence of 15 mM NaCl. Mixtures were incubated at 27 °C for 60 min, after which the complexes were used in subsequent procedures. The formation of histone H1·DNA complexes, under these conditions, was assayed by agarose (1.0%, w/v) gel electrophoresis in 0.25 × Tris-borate/EDTA electrophoresis buffer and 20% (v/v) glycerol.

RESULTS

In vitro transcription products

Transcription assays with plasmid constructs containing only the $tRNA^{Arg}$ gene or only the $tRNA^{Leu}$ gene enabled the transcription products from pArg/Leu to be determined (Figure 1b). Unlike the $tRNA^{Leu}$gene, the $tRNA^{Arg}$ gene contains an intron [32]. Nascent RNA, which is transcribed from the $tRNA^{Arg}$gene, is processed by the nuclear extract from an original size of 100 nt,

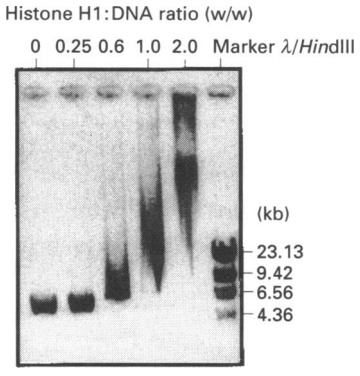

Figure 2 Formation of histone H1·DNA complexes

Histone H1·DNA complex formation is assayed by gel electrophoresis in agarose, at increasing levels of histone H1 (H1:DNA ratio 0–2.0 w/w, with respect to 250 ng of unmethylated plasmid DNA). 'Slow' complexes are retarded during electrophoresis, at H1:DNA ratios <1.0 (w/w), but are not localized to the wells of the gel as would be expected for an aggregate of 'fast' complexes. This is evident at an H1:DNA ratio of 2.0 (w/w).

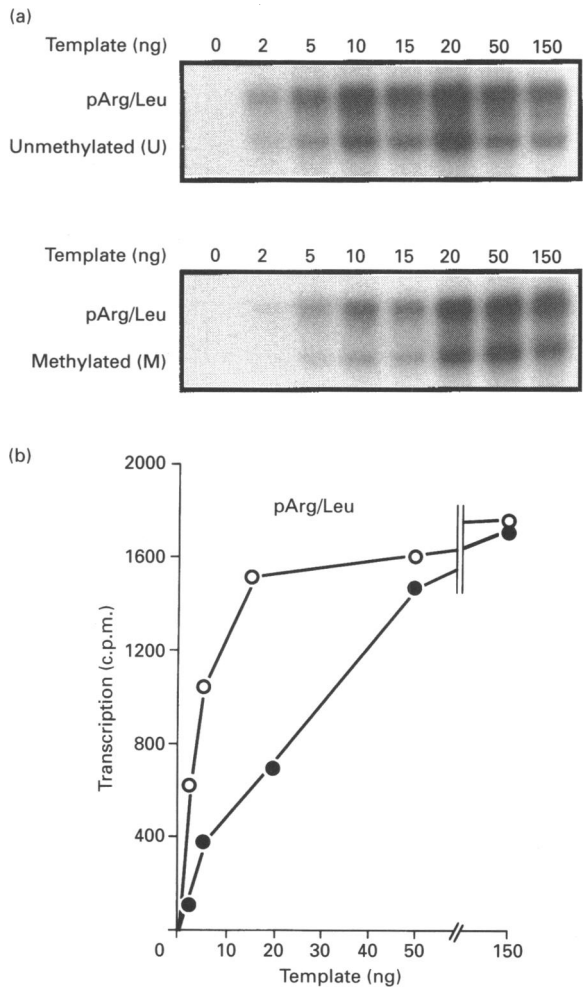

Figure 3 Effect of template methylation on *in vitro* transcription

(**a**) Transcription is inhibited preferentially from the methylated template (M) at low levels of template (2–20 ng of DNA per assay) and a constant concentration of extract. At saturating levels of unmethylated (U) or methylated (M) template (100–150 ng of DNA per assay), transcriptional activity of the methylated template is at least 80% that of the unmethylated template. (**b**) Transcription from both genes was quantified using a phosphoimager. The graph shows the preferential inhibition of methylated template (●) compared with the unmethylated template (○).

to a mature tRNA of size 85 nt. During processing, half-molecule intermediates of size 35–40 nt are also formed. Nascent RNA that is transcribed from the $tRNA^{Leu}$gene is also processed to a mature tRNA of size 75 nt. There is also a small amount of longer (150 nt) transcript, which we ascribe to read-through at the usual termination signal following the gene and subsequent termination at a T-rich sequence 70 nt downstream [32]. In the absence of added DNA template, the nuclear extract also supports transcripition by RNA pol III (as shown by α-amanitin sensitivity) of a small amount of unknown product. To simplify the interpretation of subsequent results, only the major products of transcription (i.e. nascent and processed RNA) were used for quantitative analyses and for subsequent Figures. Preliminary experiments showed that the degree of processing was highly reproducible among samples under the standard conditions for *in vitro* transcription (see the Materials and methods section), and that processing was not affected by the methylation status of the template (results not shown).

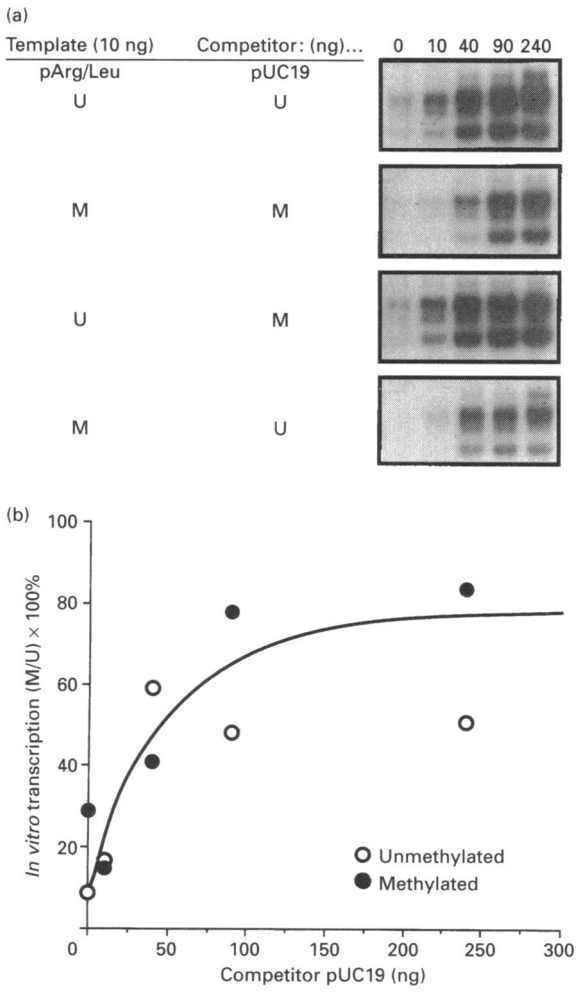

Figure 4 Removal of inhibitors from the nuclear extract with competitor DNA

(**a**) In the presence of 10–240 ng of unmethylated (U) or methylated (M) pUC19 competitor DNA, transcription from 10 ng of template is increased considerably, in comparison with the transcription in the absence of competitor. (**b**) The graph expresses the difference in transcriptional activity for the methylated (M) and unmethylated (U) templates as (M/U × 100%), in the presence of one type of competitor; either unmethylated (○) or methylated (●) competitor.

Histone H1·DNA complexes

Histone H1·DNA complexes were formed as described in the Materials and methods section. The conditions used in these studies favoured the formation of 'slow' complexes, in which the histone H1 was reversibly bound to the DNA and did not form aggregates. 'Slow' complexes retard the movement of DNA during agarose gel electrophoresis (Figure 2) at H1:DNA ratios < 1.0 (w/w). Clark and Thomas [40] also describe the formation of 'fast' complexes, which are presumed to form by the co-operative binding of H1 molecules at high concentrations of salt (>20 mM) or high H1:DNA ratios (>1.0, w/w). They are disordered aggregates of H1 and DNA which are transcriptionally inactive and which are retarded significantly during electrophoresis, in comparison with slow complexes and naked DNA. In Figure 2, both slow and fast complexes appear to form at an H1:DNA ratio of 2.0; the fast complexes (in the form of an aggregate) localize in the well of the gel, whereas the slow

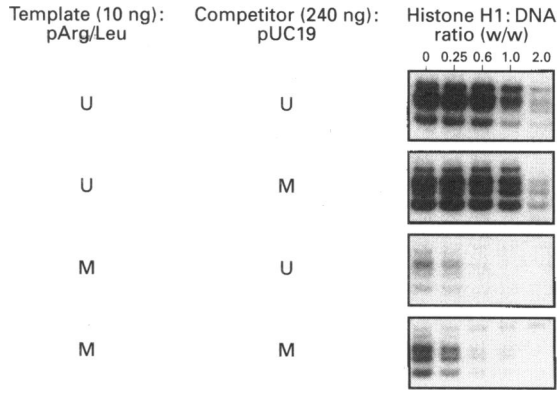

Figure 5 Reversal of stimulated transcription on addition of histone H1

Transcription from 10 ng of unmethylated (U) and methylated (M) template in the presence of 240 ng of unmethylated or methylated pUC19 competitor DNA and increasing levels of histone H1 (0–2.0 w/w ratio, with respect to template DNA).

complexes are retarded but move freely. There are no differences in the extent of slow complex formation for either unmethylated or methylated DNA (results not shown).

Inhibition of transcription by methylation

At low levels of template (2–20 ng of DNA/assay), little transcription is observed from the methylated pArg/Leu in comparison with the mock-methylated control. This difference largely disappears at high levels of template (>50 ng/assay) (Figure 3), though there remains a ~20% reduction in transcription from the methylated template. (The extent of this reduction was rather variable, showing differences between the batches of nuclear extract.) These results indicate the presence of a limiting amount of a selective inhibitor in the nuclear extract. Similar results have been described for transcription of pol II genes by Boyes and Bird [41], who suggested that MeCP-1 was the limiting protein.

In the presence of 10–240 ng of unmethylated or methylated pUC19 competitor DNA, transcription from 10 ng template is increased considerably (Figure 4). The activating effect of competitor was consistently less effective with the methylated template, reaching only 80% of the level obtained from the unmethylated template even at the highest levels of competitor tested. Transcription in the presence of methylated competitor is somewhat better than in the presence of unmethylated competitor but, in both cases, transcription is increased at least 10-fold. It is assumed that the stimulation of transcription is caused by the binding to the competitor DNA of inhibitory proteins present in the nuclear extract. As the inhibitory factors bind to unmethylated and methylated competitor DNA, this observation is not consistent with the limiting inhibitory factor being MeCP-1 (which binds only to methylated DNA). It also appears to rule out the involvement of MeCP-1 in the general lowered template activity of the methylated template, as this is still observed in the presence of excess methylated competitor. It is, however, consistent with a role for histone H1 as the limiting component responsible for *selectively* inhibiting transcription from a methylated template. Histone H1 is known to be present in low amounts in the HeLa nuclear extracts (C. A. Johnson, J. P. Goddard and R. L. P. Adams, unpublished work) [4] and will be removed by binding to competitor DNA.

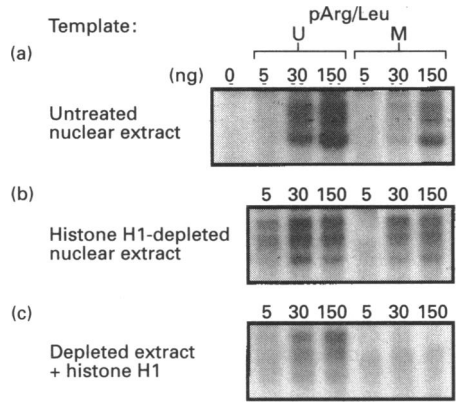

Figure 6 Transcription with H1-depleted nuclear extract

Depletion of the nuclear extract of histone H1, by precipitation of general transcription factors with 2.2 M ammonium sulphate, increases the level of transcription from the methylated template to that from the unmethylated template. (**a**) Control, assaying transcription of increasing amounts of unmethylated (U) or methylated (M) template with untreated extract (results comparable with those of Figure 3). (**b**) Low levels of methylated template (30 ng) are not preferentially inhibited with H1-depleted extract. (**c**) The effect is reversed by the addition of exogenous total histone H1 to treated extract at an H1:DNA ratio of 0.6 (w/w).

Preincubation of the template DNA with histone H1 leads to an inhibition of the stimulated transcription and this inhibition is selective for methylated template (Figure 5). This remains true whether or not the competitor DNA is methylated. In other words, we have evidence for histone H1 alone being essential (and limiting) for the *selective* inhibition of transcription from the methylated template. No evidence has been found that a second factor (e.g., MeCP-1) is required for the *selective* inhibition of transcription exerted by histone H1 on methylated templates.

Transcription with H1-depleted nuclear extract

Another way to remove histone H1 is to subject the nuclear extract to ammonium sulphate fractionation (see the Materials and methods section). In comparison with the normal Dignam nuclear extract that was used in the studies described above, a nuclear extract that is depleted of histone H1 shows increased transcription with low levels of methylated or unmethylated template (Figure 6). The addition of histone H1 to the depleted extract causes inhibition of transcription particularly with methylated templates. We presume that inhibition is caused by the formation, during the preincubation stage of the transcription assay, of H1·DNA complexes that inhibit transcription from methylated and, to a lesser extent, unmethylated constructs.

Histone H1 selectively inhibits transcription from methylated templates

With amounts of template in excess of 50 ng/assay, the difference between transcription from methylated and unmethylated templates is small (Figure 3). However, at high levels of template DNA, transcription is inhibited from both templates onto which slow complexes of total calf thymus histone H1 have been deposited (see the Materials and methods section). Transcriptional inhibition occurs at a lower H1:DNA ratio for methylated templates, in comparison with unmethylated or mock-methylated templates (Figure 7). Transcription from unmethylated templates is inhibited by 95% at an H1:DNA ratio

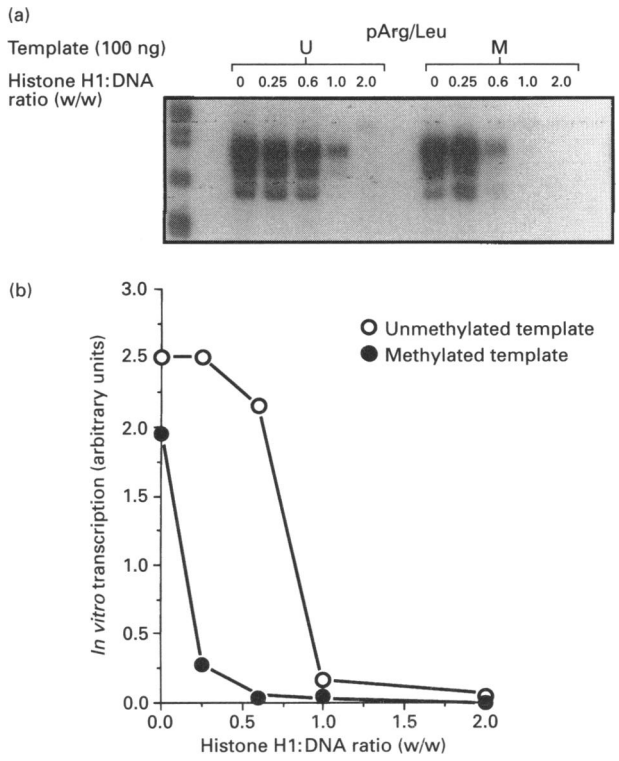

Figure 7 Histone H1 selectively inhibits transcription from methylated template

(**a**) The extent of transcription from 100 ng of unmethylated (U) or methylated (M) template is assayed in the presence of varied levels of total histone H1. Transcriptional inactivation by histone H1 is effective at lower H1:DNA ratios with methylated templates (0.25–0.6, w/w), in comparison to unmethylated templates (>1.0, w/w). Complete inactivation of all types of template is obtained with a further increase in H1 levels (2.0, w/w). (**b**) The graph shows the preferential inactivation of methylated template at a lower H1:DNA ratio than the unmethylated template.

of 1.0 (w/w). Methylated templates are inhibited by 95% at a ratio of 0.25–0.6 (w/w). Complete repression is obtained at high levels of H1 (1.0–2.0 ratio), for both types of template. Control experiments using core histones (see the Materials and methods section) over the same range of protein:DNA ratios showed no inhibition with either template (Figure 9, and see below).

The preferential inhibition of transcription from methylated templates by H1 is also seen for covalently closed circular (form II topoisomer) and linear pArg/Leu template, although there is a decrease in the absolute level of transcription by 2- and 5-fold respectively, in comparison with the supercoiled template (results not shown). These results suggest that the overall supercoiled conformation of the template affects neither the inhibitory activity of histone H1 nor the differential transcription from the two templates. To test whether the number or density of methylated CpGs also has an effect, a 943 bp fragment containing both tRNA genes but lacking the (G+C)-rich region was used as a template. Preferential inhibition of transcription from the methylated template was still observed, despite a reduced methyl CpG density.

Histone H1 prevents the formation of initiation and elongation transcription complexes preferentially on methylated templates

It has previously been shown that histone H1 inhibits RNA pol

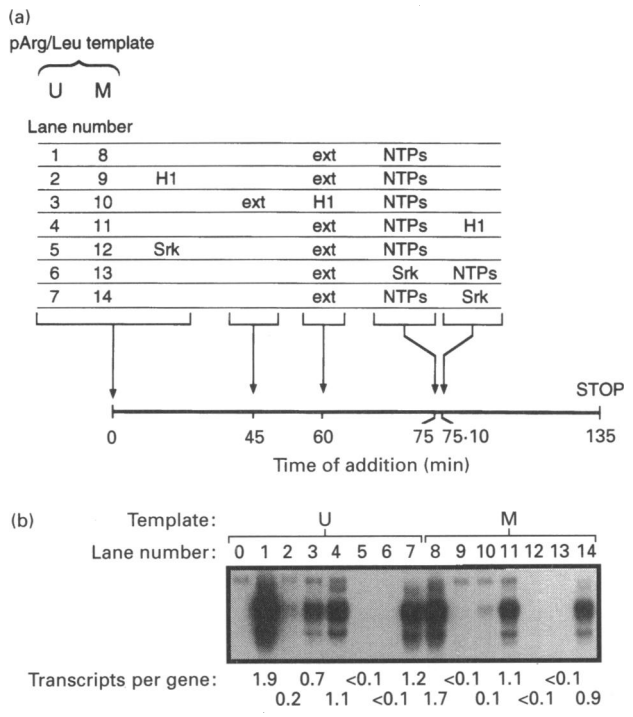

Figure 8 Initiation of transcription on methylated template

(**a**) Incubations were carried out at 30 °C with 100 ng of unmethylated (U) or methylated (M) pArg/Leu, as outlined in the scheme (see text for details). Histone H1 and HeLa nuclear extract (ext) were added at the indicated times to either unmethylated (lanes 1–7) or methylated (lanes 8–14) template as described in the Materials and methods section. Transcription was initiated with ribonucleoside triphosphates (NTPs). Initiation or elongation complex assembly was inhibited by the addition of 0.025% Sarkosyl (Srk) at the indicated times. Sections of gels corresponding to the transcripts for one particular gene were excised and quantified by scintillation counting. Transcriptional efficiency was expressed as transcripts per gene.

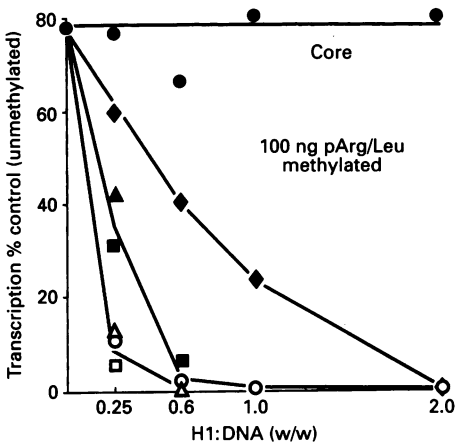

Figure 9 Effect of different histone H1 variants on transcription

Somatic variants of histone H1 were separated by reversed-phase h.p.l.c. chromatography, with core histones as a control: ●, core histones; ◆, histone H1a; ▲, histone H1d; ■, histone H1e > c; ○, histone H1e = c; □, histone H1c; △, total histone H1. Increasing levels of each variant were assayed for transcriptional inactivation of 100 ng of unmethylated and methylated templates, in the same way that total histone H1 is assayed in Figure 7. The amount of transciption of the methylated template is expressed as a percentage of that of the unmethylated temlplate (the control value of 100%), and is shown for each histone H1 variant at H1:DNA ratios of 0–2.0 (w/w).

II transcription by preventing the assembly of initiation complexes on template DNA [4]. We demonstrate that a similar interaction occurs with the general RNA pol III transcriptional machinery.

The results of Figure 8 show that pre-incubation of the template with histone H1 at a H1:DNA ratio of 1.0 (w/w) prevents the assembly of initiation complexes on both unmethylated (lane 2) and methylated (lane 9) template. Histone H1 also inhibits the conversion of pre-assembled initiation complexes into elongation complexes on the addition of ribonucleoside triphosphates, although this inhibition is less effective on unmethylated template (lane 3) than on methylated template (lane 10). However, the presence of H1 does not inhibit elongation from either template (lanes 4 and 11), although transcription is limited to a single round in both cases, as shown by comparison with transcription assays containing 0.025% (w/v) *N*-lauryl-sarcosine (Sarkosyl) (lanes 7 and 14). Sarkosyl prevents the formation of initiation complexes (lanes 5 and 12) and the conversion of pre-assembled initiation complexes into elongation complexes (lanes 6 and 13) but, like H1, does not stop the elongation by the polymerase once ribonucleoside triphosphates have been added [42]. As a consequence, the presence of Sarkosyl prevents re-initiation of transcription, limiting the transcription to one round. This was confirmed by excising gel slices containing the labelled RNA transcripts for one particular gene, counting in a liquid scintillation counter and determining the transcriptional efficiency in terms of transcripts per gene.

We conclude that histone H1, like Sarkosyl, acts by preventing the formation of initiation complexes, particularly on methylated templates, rather than by preventing elongation, which proceeds at similar rates from unmethylated and methylated templates.

Different variants of histone H1 inhibit transcription to unequal extents

The contribution of different variants to the formation of inactive chromatin is not known but it has been suggested that they may act at different sites to regulate the stability of the 30 nm chromatin fibre. The different variants of histone H1 were complexed with methylated or unmethylated (control) DNA templates and used in transcription assays as described above. Preparations containing predominantly H1c show inhibition at H1:DNA ratios comparable with those at which inhibition is shown by total histone H1 (Figure 7). The unmethylated template is inhibited at H1:DNA ratios of 0.60–1.0 (w/w), whereas the methylated template is inhibited at a ratio of 0.25 (w/w). The preparations which contain predominantly H1e or H1d have a reduced, selective inhibitory activity, whereas H1a has little selective inhibitory activity (Figure 9). Core histones do not have any inhibitory activity, as reported above.

Gene methylation is not essential for H1-mediated inhibition of transcription

Plasmids were constructed which were methylated either in all regions of plasmid pArg/Leu except a *BglI*–*BglI* fragment of size 756 bp (Figure 1a) which contained the $tRNA^{Leu}$ gene, or methylated only in this 756 bp fragment. Transcription was compared with that from fully methylated or unmethylated plasmid templates (Figure 10). It is clear that methylation of the flanking region alone is sufficient to preferentially reduce transcription of the unmethylated $tRNA^{Leu}$ gene in the presence of increasing levels of histone H1. The construct which contains an

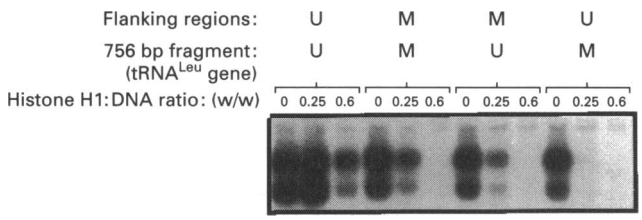

Figure 10 Flanking region methylation inhibits *in vitro* transcription of the distal tRNA^{Leu} gene

Patch-methylated constructs of the pArg/Leu template were constructed as described in the text. The unmethylated construct is inhibited only at higher levels of histone H1 (>0.6, w/w) compared with the fully or partly methylated constructs that are strongly inhibited at an H1 : DNA ratio of 0.25 (w/w).

unmethylated tRNA^{Leu} gene in a methylated vector is inactivated at similar H1:DNA ratios as is the fully methylated construct. The construct which contains the methylated tRNA^{Leu} is inactivated to the greatest extent.

DISCUSSION

H1·DNA complexes as a model for inactive chromatin

We have used complexes of H1 and unmethylated or methylated naked DNA as models for inactive chromatin. This, we believe, is justifiable because the interactions between histone H1 and either naked DNA or DNA in chromatin appear to be similar [4]. For example, H1 requires a similar salt dependence to bind to DNA and chromatin [43]. Co-operative binding of H1 to DNA, leading to the compaction of nucleosomes into the 30 nm fibre, occurs over the same range of salt concentrations as salt-induced folding [44]. H1 appears to interact mostly with linker DNA in chromatin, rather than with core histones [45], although the globular domain of the molecule can be cross-linked to H3, H2A and H2B in the core octamer [46]. H1 increases the nucleosome repeat length of chromatin reconstituted *in vitro* from 180 bp (in the absence of H1) to 220 bp [47]. This increase can be explained if H1 extends the linker DNA.

Repression from unmethylated template requires an H1:DNA ratio of 0.60–1.0 (w/w), which corresponds to one molecule of H1 per 32–55 bp of DNA. It is generally accepted that the nucleosome core particle contains 146 bp of DNA [1], with 40 bp of linker DNA per nucleosome that is accessible to H1 in the chromatin of mammalian cells. The observation that the spacing of histone H1 molecules is similar in both H1·DNA complexes and chromatin suggests that the interactions of H1 with DNA are similar in naked DNA and in chromatin. In addition, the reversible binding of H1 to DNA *in vitro* under certain conditions is comparable with the exchange of H1 within chromatin *in vivo* [48]. The complexes formed between H1 and DNA have been thoroughly studied [40], and there have been two recent studies of H1-mediated inhibition of class II genes on naked DNA templates [4,28]. However, it still remains necessary to extend the studies of H1-mediated inhibition of unmethylated templates that have been reconstituted with nucleosomes [47,49,50] to include methylated templates.

Histone H1 preferentially inhibits transcription from methylated templates

We made the initial observation that *in vitro* transcription from low levels of a methylated template was less efficient than from low levels of an unmethylated template. This suggested the presence of an inhibitor in the nuclear extract that is specific for methylated DNA. There is no evidence that the binding of transcription factors to pol III genes is affected by DNA methylation, but HeLa nuclear extract does contain low levels of histone H1 [4], and would also be expected to contain MeCP-1. Two methods were used to remove histone H1 from nuclear extracts.

Addition of competitor DNA, either methylated or unmethylated, led to enhanced transcription from both templates, implying that an inhibitor was present that bound to both methylated and unmethylated DNA. This inhibitor cannot, therefore, be MeCP-1 (which binds only to methylated DNA) but could be histone H1, which has similar affinities for methylated and unmethylated DNA. The differential inhibition from the methylated template was considerably reduced in the presence of high levels of competitor DNA, which is consistent with the structure of the complex between histone H1 and methylated DNA being different from that formed with unmethylated DNA, as reported by Higurashi and Cole [29]. Addition of histone H1 alone is able to re-impose the strong preferential inhibition of transcription from the methylated template. We presume that the methylated competitor has removed MeCPs as well as histone H1 and so these cannot be involved in the selective inhibition exerted by histone H1 on transcription from the methylated template.

Depletion of the nuclear extract of histone H1 using ammonium sulphate precipitation also abolished the preferential inhibition from the methylated template. This differential effect could be re-imposed by addition of histone H1 alone, again indicating that this protein is able to preferentially inhibit *in vitro* transcription from the methylated template. In this case, it is presumed that MeCP-1 remains in the H1-depleted nuclear extract and it alone is insufficient to inhibit transcription from methylated templates.

Histone H1 functions as a repressor of transcription for both pol-II-transcribed genes [6,12,50] and pol-III-transcribed genes [49] in the context of chromatin or nucleosomes. We show here that this inhibition is more efficient for methylated pol III templates. However, histone H1 does not prevent RNA chain elongation, which suggests that the movement of the transcription complex is not impeded by the H1·DNA complexes, and the results presented in Figure 8 show that histone H1 exerts its differential effect on transcription of methylated DNA during the formation of the initiation complex. Figure 10, however, shows that methylation of flanking regions alone is able to bring about H1-mediated inhibition of transcription of an unmethylated gene. We suggest that binding of histone H1 to methylated DNA may be a co-operative event that leads to a change in nucleoprotein conformation, thereby rendering the promoters inaccessible to transcription factors.

Besser et al. [31] have shown that methylation inhibits tRNA gene transcription in *Xenopus* oocytes, and their results are consistent with the model proposed here.

The possible roles of histone H1 variants

Different histone H1 variants occur in different species, cell types and developmental stages. In some vertebrates, the presence of certain histone H1 variants has been correlated with distinct developmental stages, that involve changes in chromatin structure before changes in gene transcription [2,51]. For example, the special linker histone H5 of chicken erythrocytes compacts and inactivates the entire erythrocyte nucleus by the formation of heterochromatin. In the sea-urchin there are well characterized

changes of histone H1 variants during embryo development [52]. We have shown that different somatic variants of calf thymus histone H1 inhibit transcription to different extents (Figure 9). Recently, a mixture of two variants, H1c and H1e, has been shown to specifically inhibit *in vitro* enzymic DNA methylation [38], and it has been proposed that H1c/H1e acts at CpG islands to inhibit their methylation by DNA methyltransferase, thereby maintaining the islands in an unmethylated state. This follows from the finding that H1e is the only variant that can bind to DNA rich in CpG dinucleotides (6 CpG dinucleotides/44 bp). Here we demonstrate that H1c is particularly effective at inhibiting *in vitro* transcription from methylated templates where the overall CpG concentration is ~400/5600 bp (i.e. ~3 methylCpG dinucleotides/44 bp). Island DNA undergoing maintenance methylation during DNA replication could thus be packaged into inactive chromatin by H1c, whereas unmethylated genes would remain active.

This work was supported by the Medical Research Council and the Wellcome Trust. We thank Stefan Kass and Sriharsa Pradhan for friendly advice and criticism. We also thank Professor Paola Caiafa for helpful discussions and the supply of the fractionated H1, and Professor Charles A. Fewson and the University of Glasgow for the provision of facilities.

REFERENCES

1 Wolffe, A. P. (1992) Chromatin: Structure and Function, Academic Press, London
2 Schlissel, M. S. and Brown, D. D. (1984) Cell **37**, 903–913
3 Wolffe, A. P. (1989) EMBO J. **8**, 527–537
4 Croston, G. E., Kerrigan, L. A., Lira, L. M., Marshak, D. R. and Kadonaga, J. T. (1991) Science **251**, 643–649
5 Kamakaka, R. T. and Thomas, J. O. (1990) EMBO J. **9**, 3997–4006
6 Garrand, W. T. (1991) BioEssays **13**, 87–88
7 Felsenfeld, G. and McGhee, J. D. (1986) Cell **44**, 375–377
8 Graziano, V., Gerchman, S. E., Schneider, D. K. and Ramakrishnan, V. (1994) Nature (London) **368**, 351–354
9 Weintraub, H. (1984) Cell **38**, 17–27
10 Ball, D. J., Gross, D. S. and Garrard, W. T. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 5490–5494
11 Tazi, J. and Bird, A. (1990) Cell **60**, 909–920
12 Bresnick, E. H., Bustin, M., Marsaud, V., Richard-Foy, H. and Hager, G. L. (1991) Nucleic Acids Res. **20**, 273–278
13 Adams, R. L. P. (1990) Biochem. J. **265**, 309–320
14 Bird, A. P. (1992) Cell **70**, 5–8
15 Razin, A. and Cedar, H. (1991) Microbiol. Rev. **55**, 451–458
16 Hergersberg, M. (1991) Experientia **47**, 1171–1185
17 Iguchi-Ariga, S. M. M. and Schaffner, W. (1989) Genes Dev. **3**, 612–619
18 Comb, M. and Goodman, H. M. (1990) Nucleic Acids Res. **18**, 3975–3982
19 Graessmann, M. and Graessmann, A. (1993) in DNA Methylation: Molecular Biology and Biological Significance (Jost, J. P. and Saluz, H. P., ed.), pp. 404–424, Birkhäusen Verlag, Basel
20 Keshet, I., Lieman-Hurwitz, J. and Cedar, H. (1986) Cell **44**, 535–543
21 Antequera, F., Macleod, D. and Bird, A. P. (1989) Cell **58**, 509–517
22 Ehrlich, M. and Ehrlich, K. C. (1993) in DNA Methylation: Molecular Biology and Biological Significance (Jost, J. P. and Saluz, H. P., ed.), pp. 145–168, Birkhäusen Verlag, Basel
23 Meehan, R. R., Lewis, J. D., McKay, S., Kleiner, E. L. and Bird, A. P. (1989) Cell **58**, 499–507
24 Lewis, J. D., Meehan, R. R., Henzel, W. J., Maurer-Fogy, I., Jeppesen, P., Klein, F. and Bird, A. (1992) Cell **69**, 905–914
25 Zhang, X.-Y., Asiedu, C. K., Supakar, P. C., Khan, R., Ehrlich, K. C. and Ehrlich, M. (1990) Nucleic Acids Res. **18**, 6253–6260
26 Jost, J.-P., Saluz, H.-P. and Pawlak, A. (1991) Nucleic Acids Res. **19**, 5771–5775
27 Jost, J.-P. and Hofsteenge, J. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 9499–9503
28 Levine, A., Yeivin, A., Ben-Asher, E., Aloni, Y. and Razin, A. (1993) J. Biol. Chem. **268**, 21754–21759
29 Higurashi, M. and Cole, R. D. (1991) J. Biol. Chem. **266**, 8619–8625
30 Kass, S. U., Goddard, J. P. and Adams, R. L. P. (1993) Mol. Cell. Biol. **13**, 7372–7379
31 Besser, D., Götz, F., Schulze-Förster, K., Wagner, H. and Simon, H. (1990) FEBS Lett. **269**, 358–362
32 Bourn, D., Carr, T., Livingstone, D., McLaren, A. and Goddard, J. P. (1994) DNA Sequence, in the press
33 Dignam, J. D., Lebovitz, R. M. and Roeder, R. G. (1983) Nucleic Acids Res. **11**, 1475–1489
34 Caiafa, P., Reale, A., Allegra, P., Rispoli, M., D'Erme, M. and Strom, R. (1991) Biochim. Biophys. Acta **1090**, 38–42
35 Hentzen, P. C. and Bekhor, I. (1985) in Progress in Nonhistone Protein Research (Bekhor, I., ed.), vol. I, pp. 75–101, CRC Press, Boca Raton, FL
36 Workman, J. L., Taylor, I. C. A. and Kingston, R. E. (1991) in Methods in Cell Biology; Functional Organisation of the Nucleus: A Laboratory Guide (Hamlako, B. A. and Elgin, S. C. R., eds.), vol. 35, pp. 419–445, Academic Press, San Diego, CA
37 Quesada, P., Farina, B. and Jones, R. (1989) Biochim. Biophys. Acta **1007**, 167–175
38 Santoro, R., D'Erme, M., Mastrantonio, S., Reale, A., Marenzi, S., Saluz, H.-P., Strom, R. and Caiafa, P. (1995) Biochem. J. **305**, 739–744
39 Bradford, M. (1976) Anal. Biochem. **72**, 248–254
40 Clark, D. J. and Thomas, J. O. (1986) J. Mol. Biol. **187**, 569–580
41 Boyes, J. and Bird, A. (1991) Cell **64**, 1123–1134
42 Hawley, D. K. and Roeder, R. G. (1987) J. Biol. Chem. **262**, 3452–3461
43 Clarke, D. J. and Thomas, J. O. (1988) Eur. J. Biochem. **178**, 225–233
44 Clarke, D. J. and Kimura, T. (1990) J. Mol. Biol. **211**, 883–896
45 Thoma, F., Koller, T. and Klug, A. (1979) J. Cell Biol. **83**, 403–427
46 Thomas, J. O. and Khabaza, A. J. A. (1980) Eur. J. Biochem. **112**, 501–511
47 Rodriguez-Campos, A., Shimamura, A. and Worcel, A. (1989) J. Mol. Biol. **209**, 135–150
48 Louters, L. and Chalkley, R. (1985) Biochemistry **24**, 3080–3085
49 Shimamura, A., Sapp, M., Rodriguez-Campos, A. and Worcel, A (1989) Mol. Cell. Biol. **9**, 5573–5584
50 Laybourne, P. J. and Kadonaga, J. T. (1991) Science **254**, 238–245
51 Dimitrov, S., Almouzni, G., Dasso, M. and Wolffe, A. P. (1993) Dev. Biol. **160**, 214–227
52 Lai, Z. C. and Childs, G. (1988) Mol. Cell. Biol. **8**, 1842–1844

Received 20 June 1994/9 September 1994; accepted 15 September 1994