Dynamic relocation of chromosomal protein HMG-17 in the nucleus is dependent on transcriptional activity

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Chromosomal proteins HMG-14/-17 are nucleosomal binding proteins, which alter the structure of the chromatin fiber and enhance transcription, but only from chromatin templates. Here we show that in tissue culture cells, HMG-17 protein colocalizes with sites of active transcription. Incubation of permeabilized cells with a peptide corresponding to the nucleosomal binding domains of HMG-14/-17 specifically arrested polymerase II-dependent transcription. In these cells the peptide displaces HMG-17 from chromatin and reduces the cellular content of the protein. These results suggest that the presence of HMG-14/-17 in chromatin is required for efficient polymerase II transcription. In non-permeabilized, actively transcribing cells, the protein is dispersed in a punctate pattern, throughout the nucleus. Upon transcriptional inhibition by α -amanitin or actinomycin D, the protein gradually redistributes until it localizes fully to interchromatin granule clusters, together with the splicing factor SC35. The results suggest that the association of HMG-17 with chromatin is dynamic rather than static, and that in the absence of transcription, HMG-17 is released from chromatin and accumulates in interchromatin granule clusters. Thus, the intranuclear distribution of chromosomal proteins which act as architectural elements of chromatin structure may be dynamic and functionally related to the transcriptional activity of the cell.

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Introduction

A major question related to chromatin structure and gene activity is the mechanism whereby the various protein complexes that act on nucleosomes, such as histone acetylases (reviewed in Grunstein, 1997; Mizzen and Allis, 1998), DNA repair complexes (Smerdon, 1991; Tornaletti and Pfeifer, 1996; Wellinger and Thoma, 1997), or nucleosome-disrupting factors (reviewed in Cairns, 1998; Kadonaga, 1998; Varga-Weisz and Becker, 1998), gain access to their target in condensed chromatin. Likewise, it is not clear whether these protein complexes are permanently associated with the chromatin fiber or whether they access their target in response to a stimulus. The first level of chromatin organization is the linear array of

nucleosomes known as the 10 nm chromatin fiber (Kornberg and Lorch, 1995; Wolffe, 1995). Nucleosomes inhibit the rate at which DNA or RNA polymerase can move along the DNA and often hamper access of regulatory factors to their target (Owen-Hughes and Workman, 1994; Paranjape *et al.*, 1994; Kornberg and Lorch, 1995). The 10 nm chromatin fiber is further folded and compacted into various higher order structures, starting with the 30 nm chromatin fiber and ending with the most compact structure, the metaphase chromosome. It is well documented that chromatin compaction is associated with transcriptional repression and that transcription activation is associated with decompaction of the higher order chromatin structure and nucleosome disruption. These changes in the chromatin structure are mediated by a reduction in the content of histone H1 (Thoma and Koller, 1977), the acetylation of the N-termini of the core histones (Garcia-Ramirez *et al.*, 1992; Hansen and Ausio, 1992; Struhl, 1998) and the activity of energy-requiring protein complexes such as SWI/SNF (Winston and Carlson, 1992), NURF (Tsukiyama and Wu, 1995), ACF (Ito *et al.*, 1996), CHRAC (Varga-Weisz *et al.*, 1995) and RSC (Cairns *et al.*, 1996). In addition, recent studies suggest that the non-histone chromosomal proteins HMG-14 and HMG-17 can also function as architectural elements that unfold the chromatin fiber, facilitating access to the nucleosome and enhancing DNA-dependent activities, but only in the context of chromatin (reviewed in Bustin and Reeves, 1996).

Chromosomal proteins HMG-14 and HMG-17 are the only nuclear proteins known to specifically bind to the 146 bp nucleosome core (reviewed in Bustin and Reeves, 1996). These two structurally similar proteins bind to nucleosomes cooperatively to form complexes containing homodimers of either HMG-14 or HMG-17 and not mixtures containing one molecule of each of these proteins (Postnikov *et al.*, 1995). The binding of HMG-14/-17 proteins to nucleosomes unfolds the higher order chromatin structure thereby enhancing various DNA-dependent activities such as transcription (Crippa *et al.*, 1993; Ding *et al.*, 1994, 1997; Paranjape *et al.*, 1995; Trieschmann *et al.*, 1995a,b; Tremethick and Hyman, 1996) and replication (Vestner *et al.*, 1998). Chromatin unfolding is facilitated by specific interactions between the C-terminal of these HMGs and the N-terminal tail of histone H3 (Trieschmann *et al.*, 1998). In the chromatin fiber, HMG-17-containing nucleosomes are clustered into distinct domains (Postnikov *et al.*, 1997). We suggested that in the HMG-17-containing domains the chromatin is unfolded, and that the presence of this protein reduces the repressive effects of chromatin. Using confocal immunofluorescence we demonstrated that in the nucleus these HMG-14/-17 proteins are organized into multiple distinct foci containing either HMG-14 or HMG-17 (Postnikov *et al.*, 1997). The intranuclear

distribution of the foci is similar to that of transcription sites in cells highly active in transcription (Grande *et al.*, 1997; Zeng *et al.*, 1997). It is therefore possible that the distribution of HMG-14/-17 is relevant to the spatial organization of gene expression within eukaryotic nuclei (Misteli and Spector, 1998).

The intranuclear distribution of various factors involved in gene expression is not uniform and is in some cases dynamic (reviewed in Misteli and Spector, 1998). For example, the transcription of ribosomal genes by polymerase (pol) I is restricted to the nucleolus (Scheer and Weisenberger, 1994), and the transcription of certain genes requiring PSE-binding transcription factors (PTF) is temporarily concentrated into distinct substructures named Oct1/PTF/transcription (OPT) domain (Pombo *et al.*, 1998). In contrast, general pol II transcription occurs at multiple foci throughout the nucleus (reviewed in Singer and Green, 1997). The number and size of these transcription foci vary among cells and change according to transcription intensity (Zeng *et al.*, 1997). Likewise, some of the splicing factors that process pre-mRNA are non-homogeneously distributed in the nucleus and upon staining with specific antibodies elicit a pattern of 'speckles' (reviewed in Spector, 1996). These speckles may serve as storage and supply sites for splicing factors (Huang and Spector, 1996). Their appearance is dynamic: when transcription is inhibited the speckles round up, appear larger, and in some cells types decrease in number (Misteli *et al.*, 1997). At the electron microscopic level the speckles correspond to interchromatin granule clusters and perichromatin fibers (reviewed in Spector, 1996). The number and prominence of the perichromatin fibers are high near actively transcribed regions and decrease when pol II transcription is inhibited (reviewed in Spector, 1996). Thus, in the interphase nucleus the organization of transcription units and splicing factors is dynamic, and correlates with the transcriptional activity of the cell.

The functional organization of non-histone structural proteins, such as HMG-14/-17, has not yet been examined in detail. It is not known whether these proteins are statically associated with chromatin, or whether their intranuclear location is dynamic and dependent on metabolic events, such as the transcriptional activity of the cell. Here we analyze the intracellular distribution of chromosomal proteins HMG-17 in transcriptionally active and inactive cells. We find that the protein colocalizes with nascent transcripts and forms a broad meshwork occupying the non-nucleolar portion of the nucleus with numerous 'dots', or foci, of preferred localization. Unexpectedly, in cells treated with transcription inhibitors, the protein relocates and accumulates in fused interchromatin granule clusters. These results suggest that the intranuclear location of the protein is dynamic and dependent on the cellular level of transcription. Furthermore, incubation of permeabilized cells with specific peptides known to displace HMG-17 (and HMG-14) from chromatin, inhibits pol II transcription. Thus, the presence of HMG-17 in chromatin is required for efficient pol II transcription *in vivo*.

The finding that interchromatin granules serve as storage sites for HMG-17 protein suggests that these sites contain not only factors necessary for pre-mRNA processing (reviewed in Spector, 1996) but also serve as storage

sites for proteins required for efficient transcription from chromatin templates. Thus, this nucleosomal binding protein is not always associated with chromatin. These studies are relevant to understanding processes involving intranuclear trafficking of chromosomal proteins which may lead to changes in chromatin structure during gene activation.

Results

Chromosomal protein HMG-17 colocalizes with many transcription sites

To test whether the spatial distribution of chromosomal protein HMG-17 coincides with that of transcription sites, we permeabilized Hep-2 cells, labeled nascent transcripts with BrUTP, and used double immunofluorescence to visualize the location of HMG-17 and BrU (Figure 1). The intranuclear distribution of nascent transcripts in these cells was typical of that found in actively transcribing cells (Wansink *et al.*, 1993; Zeng *et al.*, 1997). The sites incorporating BrUTP were not uniformly diffuse but were distributed throughout the non-nucleolar portion of the nucleoplasm in a 'granular structure'. The intranuclear distribution of HMG-17 proteins was very similar to that of the nascent transcripts. A merge of the two confocal images indicates that many, but not all of the transcription sites overlap (Figure 1a").

We obtained similar results when we compared the nuclear distribution of HMG-17 with that of RNA pol II, as detected using either monoclonal antibody (mAb) H5 or H14 (not shown). As demonstrated previously by others using the same antibodies to RNA pol II (Bregman *et al.*, 1995; Grande *et al.*, 1997; Zeng *et al.*, 1997), we also found that the sites containing pol II are distributed throughout the entire non-nucleolar nucleoplasm in a pattern very similar to that of nascent transcripts. We find that part of the sites containing HMG-17 overlapped with those containing pol II (not shown). However, as already pointed out by others, with immunofluorescence patterns as complex as those generated by HMG-17, BrUTP and pol II, the actual extent of colocalization can only be approximated (Fay *et al.*, 1997; Zeng *et al.*, 1997).

To obtain more precise information on the extent of colocalization of nascent transcripts and HMG-17, we examined their spatial relation at a higher resolution, using electron microscopy. Secondary antibodies tagged with either 6 or 12 nm gold particles were used to visualize the location of either BrU or HMG-17, respectively. At the ultrastructural level the spatial distribution of the gold particles was similar to that observed by immunofluorescence (Figure 1b). We detected numerous domains containing interspersed clusters of both HMG-17 and nascent transcripts. Frequently the domains were surrounded by chromatin regions that were totally devoid of either BrU or HMG-17.

Suppression of RNA pol II transcription by peptides corresponding to the HMG nucleosomal binding domain

The immunolocalization experiments described above suggest a possible functional relation between pol II transcription and HMG-17 localization. We therefore examined whether efficient pol II transcription requires the presence

Fig. 1. Nascent transcripts colocalize with HMG-17. Nascent transcripts were labeled in permeabilized Hep-2 cells by incorporation of BrUTP for 5 min into RNA. Localization of BrU (a') relative to HMG-17 (a) by confocal laser scanning immunofluorescence. The overlay of both localization patterns is shown in (a''). Note that most of the HMG-17 foci colocalize with the distribution of BrU [yellow in (a'')]. (b) Electron microscopy. BrU-labeled RNA was detected with secondary antibody coupled to 6 nm gold particles (closed arrowheads). HMG-17 was localized with secondary antibody coupled to 12 nm gold particles (open arrowheads). The large arrow points to nuclear domains containing both nascent RNA and HMG-17 surrounded by domains that do not contain these antigens. Bars represent: 15 μ m in (a) and 150 nm in (b).

of chromatin-bound HMG-17. In permeabilized cells, nascent transcripts are present throughout the nucleoplasm excluding the nucleolus, indicating that BrUTP is incorporated into pol II transcripts more efficiently than into pol I (Wansink *et al.*, 1993). We find the same distribution of transcripts in permeabilized Hep-2 cells (Figures 1a and 2a). In the presence of 20 μ g/ml α -amanitin, transcripts are visible only in the nucleolus, indicating that the BrUTP is now preferentially incorporated into pol I transcripts (Figure 2b and b'). Significantly, the immunofluorescent pattern of HMG-17 mirrors the pattern of BrUTP incorporation into pol II transcripts: in the absence of α -amanitin HMG-17 is distributed throughout the nucleus and colocalizes with nascent transcripts, whereas in the presence of α -amanitin the immunofluorescence signal is reduced significantly (Figure 2a' and b'). The protein could be redistributed, dispersed throughout the nucleoplasm, and perhaps even diffuse out of the permeabilized cells (see below). These results suggest that the intranuclear distribution of HMG-17 is linked to pol II transcription.

To correlate better pol II transcription with the spatial distribution of HMG-17, we examined the pattern of BrUTP incorporation in permeabilized cells which were first incubated in media containing excess of a peptide corresponding either to the nucleosomal binding domain of HMG-17 (peptide 2), or a control peptide which has the same amino acid composition but a different sequence to peptide 2 (Bustin *et al.*, 1990a). The nucleosomal

binding domain of HMG-17 is similar to that of HMG-14 (Bustin *et al.*, 1990b). We have demonstrated previously that the 30-amino-acid-long peptide 2 binds to nucleosome cores in a fashion similar to the intact proteins (Crippa *et al.*, 1992). *In vitro*, peptide 2 but not the control peptide, specifically inhibits the HMG-14/-17-mediated transcriptional stimulation of chromatin templates, most probably by competing with the wild-type proteins for binding to nucleosomes (Trieschmann *et al.*, 1995a,b). As demonstrated in Figure 2d and d', the effect of peptide 2 on BrUTP incorporation and HMG-17 localization was indistinguishable from that of α -amanitin. In the presence of this peptide, BrUTP is incorporated only into nucleolar transcripts and the HMG-17 staining is lost. Bound peptide 2 can not be detected because antibodies against HMG17 recognize the nucleosome-binding domain (i.e. peptide 2) only in the free state. When bound to nucleosomes, the epitopes of the nucleosomal binding domain are sterically hindered (Bustin *et al.*, 1990a). The control peptide did not affect the pattern of BrUTP incorporation or the intranuclear location of HMG-17 protein (Figure 2c and c').

As an additional test for the involvement of HMG-17 in pol II transcription we labeled nascent transcripts with [32 P]UTP in permeabilized cells incubated in media containing peptide 2, the control peptide or α -amanitin. As indicated by the specific activity of the various RNA preparations (expressed as c.p.m./EtBr), the RNA extracted from cells grown in the presence of peptide 2 incorporated significantly lower amounts of radioactivity than the RNA

Fig. 2. A peptide corresponding to the nucleosomal binding domain of HMG-14/-17 (peptide 2) suppresses pol II transcription in permeabilized cells and affects the localization of HMG-17. As controls, BrUTP incorporation was performed in the absence (a) or presence (b) of α -amanitin. Nascent transcripts were labeled with BrUTP in the presence of excess of either control peptide (c) or peptide 2 (d). The cells were double stained with specific antibodies and analyzed by confocal microscopy. BrU staining (green) is shown in (a)–(d) and corresponding HMG-17 staining (red) is shown in (a')–(d'), respectively. Overlays are seen in (a'')–(d''). In cells where transcription was blocked by α -amanitin, BrUTP incorporation is either absent or restricted to nucleoli [arrowhead in (b)]. Note that in these cells the HMG-17 staining is weaker, with only few bright foci visible (b'). The same pattern is obtained by incubating the cells with peptide 2 [(d)–(d')]. In contrast, in the presence of a control peptide neither BrUTP (c), nor HMG-17 staining (c'), is affected. Bars represent 15 μ m.

extracted from cells grown in the absence of peptide 2, or incubated with the control peptide (Figure 3). Taken together, the experiments presented in Figures 2 and 3 suggest that displacement of HMG-17 (and probably also HMG-14) from chromatin significantly reduces pol II transcription.

The experiments presented in Figure 2 also suggest that upon displacement from chromatin part of the HMG-17 is lost from the permeabilized cells (compare Figure 2d' with c'). To test this assumption, we measured the amount of HMG-17 protein in cells incubated with either peptide 2 or with the control peptide. After incubation, the proteins present in either the cells or in the buffer (supernatant) were examined by SDS-PAGE. Western analysis with an antibody to peptide 2, that recognized both HMG-17 and peptide 2 but not the control peptide (Bustin *et al.*, 1990a), indicates that addition of peptide 2, but not control peptide, reduces the amount of HMG-17 in the cells (Figure 4A).

Western analysis of the proteins in the supernatant verified that HMG-17 was indeed released from the cells by peptide 2 but not by the control peptide (Figure 4B). The blots were reprobed with antibodies to histone H1 to indicate that peptide 2 does not change the intracellular level of this linker histone (Figure 4A) and that H1 is not released into the supernatant (Figure 4B).

The intranuclear distribution of HMG-17 is related to transcription

To further study the correlation between transcription-related processes and HMG-17 localization, we compared the intranuclear distribution of the splicing factor SC35 with that of HMG-17, in living, non-permeabilized cells. These splicing factors are distributed throughout the nucleoplasm in a speckled pattern; the size and number of the speckles vary between cells and are related to the transcriptional activity in the cell (reviewed in Spector,

Fig. 3. RNA-synthesis in permeabilized cells is specifically suppressed by a peptide corresponding to the nucleosomal binding domain of HMG-14/-17. Permeabilized cells were incubated with NTPs and [32 P]UTP in the absence (lane 1) or presence (lane 2) of α -amanitin, in the presence of a control peptide (lane 3), or in presence of peptide 2 (lane 4). RNA was isolated, separated by gel electrophoresis and stained with EtBr. Incorporation of [32 P]UTP into nascent RNA was visualized by autoradiography after transfer of the RNA to a nylon membrane. The specific activity of each sample was estimated by quantifying the EtBr and the autoradiographic signal with a Molecular Dynamics densitometer. The values presented are the average of five experiments. Note that in the presence of α -amanitin or peptide 2 the incorporation of [32 P]UTP into RNA is reduced significantly.

1996). We found that HMG-17 colocalizes with SC35 in a variable pattern. The strongly fluorescent, large foci of HMG-17 colocalize with the SC35 speckles (Figure 5a and b, solid arrowheads). In cells lacking very bright HMG-17 foci, the colocalization is poor (e.g. the cell indicated by arrows in Figure 5b,b'). However, in these cells, some of the brightest HMG-17 spots localize near the SC35 speckles (open arrowheads in Figure 5b and b').

Since it has been suggested that the largest and most intensely staining SC35 speckles localize to interchromatin granule clusters (Spector, 1996), we also compared the spatial distribution of HMG-17 with that of interchromatin granules. Colocalization of HMG-17 with the interchromatin-granule-specific antibody p105 (Clevenger and Epstein, 1984) reveals that most interchromatin granule clusters contain high local concentrations of HMG-17 (Figure 5c and c'). At the ultrastructural level, we detect interchromatin granules containing high concentrations of both SC35 and HMG-17 (Figure 5d). The presence of HMG-17 in interchromatin granules suggests that not all of the protein is always associated with chromatin.

The experiments described raise the possibility that at low levels of transcription, HMG-17 dissociates from chromatin and accumulates in interchromatin granule clusters. To test this possibility we examined the intranuclear distribution of HMG-17 in cells treated with actinomycin D. Actinomycin D affects various DNA-related activities including transcription, and is known to release HMG-17 from chromatin

(Schroter *et al.*, 1985). After 3 h of exposure to the antibiotic, the punctate immunostaining of HMG-17 became less prominent and the protein was distributed in a highly granular pattern throughout the nucleus (Figure 6a'). The largest and most intensely staining HMG-17 foci colocalize with interchromatin granule clusters (Figure 6a and a'). Indeed, all of the interchromatin granule clusters colocalized with high local concentrations of HMG-17. The effect was significantly more obvious after 5 h of actinomycin D treatment. The intranuclear distribution of HMG-17 was altered totally. The punctate pattern typical of growing cells was extremely faint and practically all of the HMG-17 protein relocated and was concentrated in interchromatin granule clusters (Figure 6b and b'). Similar results were obtained in cells treated with α -amanitin (not shown). In contrast, the intranuclear distribution of the linker histone H1 was not affected significantly by the actinomycin D treatment. This chromosomal protein did not relocate; H1 remained distributed throughout the entire nucleus and was not concentrated in interchromatin granule clusters (Figure 6c and c').

Discussion

Our major finding is that the intranuclear organization of HMG-17 is dynamic rather than static, and is related to the level of transcriptional activity. Furthermore, we show that in tissue culture cells, efficient transcription by RNA pol II requires the presence of HMG-17. Finally, we provide evidence that upon release from chromatin, HMG-17 accumulates in interchromatin granule clusters. Chromosomal protein HMG-17 is a structural element that binds to nucleosomes and stimulates various DNA-dependent activities, but only in the context of chromatin. The results presented here suggest that part of HMG-17 is not associated with chromatin, and that the spatial distribution of HMG-17 in the nucleus is functionally related to the transcriptional state of the chromatin fiber.

The intranuclear organization of HMG-17 is dynamic

We find that HMG-17 is distributed throughout the entire non-nucleolar portion of the nucleus in a broad meshwork with numerous sites, or foci, of preferred localization. The pattern overlaps broadly with the pattern generated with antibodies to BrU that detect sites of active pol II transcription (Bregman *et al.*, 1995; Grande *et al.*, 1997; Zeng *et al.*, 1997). The colocalization of HMG-17 and nascent transcripts was not complete perhaps because some sites contain only HMG-14, or perhaps because some of the sites do not contain these HMG proteins. It is similar to that obtained when the distribution of the nascent transcripts was compared with various antibodies to pol II (not shown). Incomplete overlap between the spatial distribution of nascent transcripts and transcription factors (van Steensel *et al.*, 1995; Grande *et al.*, 1997), or RNA pol II (Bregman *et al.*, 1995), led to the suggestion that not all of the domains which contain transcription factors are actively transcribing (Grande *et al.*, 1997). This might also be the case for the HMG-17 domains. However, our electron microscope localization studies clearly showed that nascent transcripts colocalize with HMG-17.

In most of the tissue culture cells the HMG-17 foci

Fig. 4. HMG-17 is specifically displaced from nuclei by a peptide corresponding to the nucleosomal binding domain of HMG-14/-17 (peptide 2). Permeabilized cells were incubated with equal concentrations of either a control peptide (lanes 1) or peptide 2 (lanes 2). The proteins present in the cell (A) and in the growth medium (solubilized proteins) (B) were separated by SDS-PAGE and analyzed by Western blot with antibodies specific for peptide 2, which also recognized HMG-17. Note that after treatment with peptide 2 the amount of HMG-17 in the cells is lower, and in the medium higher than after treatment with a control peptide. The amount of histone H1 in the cells was not affected by peptide 2.

Fig. 5. The most intense HMG-17 foci colocalize with the splicing factor SC35 and with a marker for interchromatin granule clusters. Hep-2 cells were double labeled with antibodies to HMG-17 [(a'), (b') and (c')] and with antibody to either non-snRNP splicing factor SC35 [(a) and (b)] or to p105 [interchromatin granule, (c)]. Double-stained cells were investigated by confocal laser scanning microscopy. Overlays are shown in (a'')–(c''). Examples of sites where HMG-17 colocalizes with SC35 [(b)–(b'')] or interchromatin granules [(c)–(c'')], are indicated with solid arrowheads. Speckle regions next to bright HMG-17 foci are indicated with open arrowheads [(b)–(b'')]. (d) EM-localization. The arrowhead points to an interchromatin granule cluster containing both SC35 and HMG-17 and surrounded by areas devoid of these antigens. Bars represent 15 μ m in (a)–(c) and 200 nm in (d).

Fig. 6. Redistribution of HMG-17 into interchromatin granules upon transcriptional arrest. Hep-2 cells were cultured in the presence of α -amanitin for either 3 h [(a)–(a'')] or 5 h [(b)–(b'')] and (c)–(c''). After fixation, the cells then were double stained with antibodies to interchromatin granule clusters (p105, green) and with either antibody to HMG-17 [red; (a'), (b')] or an antibody specific to histone H1 [red; (c')]. Corresponding overlays are shown in (a'')–(c''). Upon transcriptional arrest HMG-17, but not H1, accumulates in interchromatin granules. The arrowheads in (a) and (b) point to interchromatin granule clusters. Bar corresponds to 15 μ m.

appeared small, uniform in size and evenly dispersed throughout the non-nucleolar nucleoplasm (i.e. arrow in Figure 5b'; see also Postnikov *et al.*, 1997). However, we also detected numerous cells in which the size of the HMG-17 foci was variable. Some of the foci were small, whereas others appeared large and brightly stained, suggesting that they represent high local concentrations of HMG-17 (Figure 5a'). The large, intensely staining HMG-17 foci colocalized with sites containing high local concentrations of the splicing factor SC35 (Figure 5a), whereas the small dispersed pattern of HMG-17 did not colocalize with SC35. Several investigators demonstrated that the intranuclear distribution of SC35 and pol II, as well as other components associated with the generation and processing of mRNA, are related to transcriptional activity (Bregman *et al.*, 1995; Dirks *et al.*, 1997; Grande *et al.*, 1997; Misteli *et al.*, 1997; Zeng *et al.*, 1997). In transcriptionally active cells, these components are dispersed throughout the nucleus whereas in transcriptionally quiescent cells they relocate and concentrate into a few 'speckles'. At the ultrastructural level the speckles correspond to interchromatin granule clusters and perichromatin fibers (reviewed in Spector, 1996). The interchromatin granule clusters are dynamic structures which represent high local concentrations of SC35, and perhaps other components associated with transcription and RNA processing (Misteli *et al.*, 1997). We find that the dynamics of the spatial organization of HMG-17 is similar to that reported for pol II, and other transcription-associated factors. In transcriptionally active cells HMG-17 proteins are dispersed in multiple small foci, whereas in transcriptionally quiescent cells the proteins relocate and concen-

trate into speckles. This is most obvious in cells treated with transcription inhibitors such as α -amanitin or actinomycin-D. In permeabilized cells the HMG-17 immunofluorescent signal is lost (Figure 2b') most probably because the protein diffused out of the nucleus (Figure 4). In non-permeabilized cells, the protein gradually accumulates into distinct, well-defined sites (Figure 6) and colocalizes fully with interchromatin granule clusters (Figures 5 and 6).

Thus, the intranuclear organization of HMG-17 is dynamic and not, as implied previously, static. It is spatially and functionally linked to factors associated with the transcription and processing of pre-mRNA. It is absent from the nucleolus, dispersed throughout the nucleus in actively transcribing cells and highly localized to interchromatin granule clusters in non-transcribing cells. The intranuclear trafficking of this architectural protein may be functionally related to the structure of the transcriptionally active chromatin fiber.

The organization of HMG-17 in chromatin

In chromatin, HMG-14/-17 cooperatively binds to nucleosomes, forming complexes containing two molecules of HMG-14 or two molecules of HMG-17. Mixed complexes containing one molecule of each HMG-14 and HMG-17 are not detected (Postnikov *et al.*, 1995). Nucleosomes containing either HMG-14 or HMG-17 are clustered into domains, which on average consist of six contiguous HMG-containing nucleosomes (Postnikov *et al.*, 1997). At the resolution of the light microscope, we find that HMG-14 and HMG-17 segregate into distinct nuclear domains, most domains containing either HMG-14 or

HMG-17 (Postnikov *et al.*, 1997). Our present findings that the intranuclear organization of HMG-17 (and presumably HMG-14) is dynamic, and that the proteins can concentrate into speckle-like domains, suggest a mechanism for generating clusters of nucleosomes containing only one type of HMG protein in chromatin. Conceivably, the proteins are recruited, or migrate, from areas of high local concentration of 'free' HMG, to nearby chromatin and bind to nucleosomes. Thus, the HMG content of the nucleosomes in chromatin is dependent on the type of HMG concentrated in the nearby speckle. Accordingly, it is important to determine the mechanism whereby HMG-14 and HMG-17 segregate into separate nucleoplasmic domains.

We wish to emphasize that our studies do not provide any information about the timing or the mechanisms whereby the proteins bind to nucleosomes. It is still possible that these proteins assemble into nascent chromatin together with histones (Bustin *et al.*, 1995), and are subsequently released during periods of low transcriptional activity. Alternatively, only a small fraction of HMG-14/-17 proteins may assemble into nascent chromatin to tag and unfold the local structure of a short region in chromatin. The bulk of HMGs may associate with chromatin in response to a specific cellular signal. This scenario is in agreement with the observations that HMG-14 negates the transcriptional repression of H1 on pre-assembled SV40 minichromosomes (Ding *et al.*, 1997). We speculate that the HMGs relocate from sites of high concentration either to initiate or to maintain chromatin unfolding during transcription or replication.

The effect of HMG-17 on chromatin function

Eukaryotic cells contain various activities designed to overcome the repressive effects of histones and chromatin on DNA-related activities such as transcription and replication. Histone acetylation (reviewed in Turner and O'Neill, 1995; Grunstein, 1997; Mizzen and Allis, 1998), and several specific protein complexes such as SWI/SNF (Winston and Carlson, 1992), NURF (Tsukiyama and Wu, 1995), ACF (Ito *et al.*, 1996), CHRAC (Varga-Weisz *et al.*, 1995) and RSC (Cairns *et al.*, 1996) have been identified as cellular activities associated with chromatin disruption and transcriptional activation. In addition, studies in several experimental systems suggest that the interaction of chromosomal proteins HMG-14/-17 with nucleosomes enhances the transcription and replication potential of chromatin templates. The effect of HMG-14/-17 on DNA-related activities in chromatin has been detected with chromatin templates assembled in *Drosophila* embryonic extracts (Paranjape *et al.*, 1995), in *Xenopus* egg extracts (Crippa *et al.*, 1993; Trieschmann *et al.*, 1995a; Tremethick and Hyman, 1996) and in SV40 minichromosomes isolated from CV-1 cells (Ding *et al.*, 1994, 1997).

Here we demonstrate that a relation between HMG-17 and transcription can also be demonstrated in living cells. First, we show that nascent transcripts colocalize with HMG-17. Secondly, whether the cells are permeabilized or not, we find that dependent upon transcriptional inactivation, HMG-17 protein either diffuses out of the nucleus or accumulates in interchromatin granules. More significantly, we find that incubation of permeabilized cells with a peptide (peptide 2) that corresponds to the

nucleosomal domain of HMG-17, specifically inhibits BrUTP incorporation into the non-nucleolar region of the nucleus. This result is fully consistent with previous *in-vitro* experiments demonstrating that peptide 2 specifically inhibits the HMG-14/-17-dependent enhancement of the transcriptional potential of chromatin templates assembled in *Xenopus* egg extracts (Trieschmann *et al.*, 1995a,b). Furthermore, we also find that truncated versions of this peptide, which do not compete with the intact proteins in the *Xenopus* system (Trieschmann *et al.*, 1995a,b), also fail to show an effect in the Hep-2 cells (not shown). Peptide 2 binds specifically to nucleosome cores with an affinity constant similar to that of the intact proteins (Crippa *et al.*, 1992; Postnikov *et al.*, 1995). We therefore suggest that peptide 2 inhibits transcription by displacing HMG-14/-17 proteins from nucleosomes.

In the run-on transcription experiments, BrUTP preferentially labels abundant transcripts generated from the most actively transcribed genes. We therefore suggest that efficient transcription of these genes is dependent of the presence of chromatin-bound HMG-17 protein. This finding is fully consistent with earlier experiments demonstrating that injection of antibodies to HMG-17 into cells specifically inhibits transcription (Einck and Bustin, 1983).

Material and methods

Cell culture and drug treatments

Hep-2 cells were grown on coverslips in DMEM (Gibco-BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS; Gibco-BRL) at 37°C in a 5% CO₂ incubator. Transcription of cells in the log-phase was blocked with either actinomycin D (Sigma), or α -amanitin (Sigma) added to the culture medium to a final concentration of 10 or 20 µg/ml, respectively. The cells were cultured in the drug-containing media for 4–6 h.

BrUTP incorporation and peptide treatment

BrUTP incorporation into permeabilized cells was performed as recommended elsewhere (Wansink *et al.*, 1993). In brief, cells grown on coverslips were rinsed in phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 7 mM Na₂HPO₄), permeabilized with 0.1% Triton in PBS for 1 min, equilibrated in transcription buffer (TB; 100 mM KCl, 5 mM MgCl₂, 0.5 mM EGTA, 1 mM PMSF, 5 U/ml RNasin, 25% glycerol and 50 mM Tris-Cl, pH 7.4) and incubated for 5 min at room temperature in TBN [TBN: TB containing 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP (Promega) and 0.4 mM BrUTP (Sigma)]. The following peptides were added to TBN to test their effect on RNA synthesis: peptide 2 (KDEPQRRSARLSAKPAPPKPEPKPKKAPAK), corresponding to the nucleosomal binding region of HMG-17 (Bustin *et al.*, 1990a); control peptide (RAKPAKLPKAAPSPKADKERSRPKPQPKPEP), which has the same amino acid composition but a different amino acid sequence to peptide 2 (Trieschmann *et al.*, 1995a). Additional peptides used in experiments whose results are not shown, are described elsewhere (Trieschmann *et al.*, 1995b). All the peptides were HPLC purified and added to a final concentration of 0.4 µg/µl. After 10 min incorporation of BrUTP in the presence of the peptides the coverslips were blotted, rinsed in PBS, immediately fixed in 2% formaldehyde in PBS for 10 min at room temperature, and permeabilized by incubation with 0.1% Triton X-100 in PBS for 10 min. The cells were then washed twice for 3 min. Incorporated BrUTP was detected using anti-BrdUTP (Boehringer Mannheim; diluted 1:25).

Antibodies

The following antibodies were used: affinity pure rabbit antibodies to either HMG-17 (2 µg/ml), peptide 2 or histone H1 (1.5 µg/ml), mouse to either histone H1 (Calbiochem), SC35 (1:1000, Sigma) or mAbs p105 (anti-PANA, ICN). The rabbit antibodies have been characterized extensively and used by many investigators (Bustin, 1989; Bustin *et al.*, 1990a). In addition, the antibodies to HMG-17 and histone H1 were retested by Western analysis for specificity in the nuclear protein

preparations prepared from Hep-2 cells. The bands detected by the antibodies had the appropriate molecular weight.

Immunofluorescence microscopy

Cells grown on coverslips were rinsed in PBS and fixed with 2% formaldehyde in PBS for 10 min at room temperature. After fixation, the coverslips were processed as follows: washed twice for 3 min in PBS, incubated for 10 min in PBS containing 0.1% Triton X-100, rinsed in PBS and washed twice for 5 min in PBS. The primary and secondary antibodies were added to the coverslips for 45 min and then removed by blotting and washing the coverslips twice for 5 min between the incubation steps. Bound rabbit antibodies were detected with Texas-Red-coupled goat anti-rabbit (Dianova). Bound mouse antibodies were detected with DTAF-coupled goat anti-mouse (Dianova). The preparations were counterstained with 1 µg/ml Hoechst 33258 for 5 min. Finally, the coverslips were blotted, rinsed and washed twice for 5 min in PBS and mounted in Mowiol (Hoechst, Frankfurt). For comparison with the BrUTP incorporation protocol, the cells were permeabilized before fixation. Cell permeabilization did not affect the fluorescence pattern.

Confocal laser scanning microscopy and image processing

Optical sections of double-stained cells were recorded with a Leica confocal laser scanning microscope (TCS-NT) equipped with a 63×/1.30 Neofluar oil-immersion objective. Red and green fluorescence were scanned with a dual wavelength channel to excite DTAF and TRITC/Texas Red at 488 and 568 nm, respectively, with the help of the TCS-NT software. Settings were identical for all scanning procedures. Single optical sections were taken with an oil-immersion objective (×63, NA = 1.4, with an axial resolution of 500 nm and a lateral resolution of 300 nm) with zoom 2 or 4, and a 4× accumulation. Sampling distance was 60 nm lateral and 266 nm axial. No crosstalk could be observed. Fluorescent signals of both fluorochromes were recorded simultaneously at one scan. The degree of colocalization was also controlled by linescans (TCS-NT software). The red and green voxel values were plotted along single lines drawn arbitrary through optical sections of multiple image pairs. For the BrU/HMG-17 double localization, more than 80% of the peaks obtained from the red signal coincided with the peaks of the green signal. In control preparations of HMG-14/HMG-17 (Postnikov et al., 1997) and Topo II/HMG-17 (not shown) <5% of the green and red peaks coincided.

Electron microscopy

For electron microscopy, cells grown on coverslips were fixed and incubated with the first antibody as described above. Secondary gold-coupled antibodies (Dianova, diluted 1:10) were incubated for either 4 h at room temperature (6 nm, goat anti-mouse) or overnight at 4°C (12 nm goat anti-rabbit). The coverslips were then rinsed and washed in PBS twice for 10 min, rinsed in 0.05 M sodium cacodylate buffer (SCB, pH 7.2), fixed with 2.5% glutaraldehyde in SCB for 15 min, and post-fixed with 2% OsO₄ (in SCB) for 20 min. The cells then were dehydrated and flat-embedded in Epon 812. Ultrathin sections were stained according to standard protocols and examined in an electron microscope (EM10, Zeiss) at 80 kV.

RNA extraction and analysis

Cells grown on culture plates (3.5 cm in diameter, 1×10⁶ cells/plate) under the same conditions as used for coverslips, were washed twice with PBS containing 10 U/ml RNasin and were permeabilized with 0.1% Triton in PBS for 1 min at room temperature. Cells were then washed twice with PBS/RNasin and equilibrated in TB. TB was removed and cells were incubated in 500 µl TB containing ATP, CTP, GTP (0.5 mM each) and 10 µCi [³²P]αUTP (ICN), in either the presence or absence of the indicated peptides (0.4 µg/µl) or 20 µg/ml α-amanitin (Sigma). After incubation, the TBN was removed, the cells were washed twice with PBS, and lysed directly with 1 ml of Trizol (Gibco-BRL). RNA was isolated with Trizol according to the manufacturer's procedure. The RNA pellet was washed with 1 ml of 75% EtOH, resuspended in RNA sample buffer (20 mM MOPS, 5 mM NaAc, 1mM EDTA, 50% formamide, 6% formaldehyde, pH 7.2) and solubilized at 70°C for 5 min. The RNA was electrophoresed in 1% agarose-formaldehyde gel at 100 V. After electrophoresis the gel was stained with EtBr and photographed, and the RNA was transferred onto a nylon membrane by Northern transfer. The membrane was finally exposed to X-ray film.

Western blot analysis of peptide-treated cells

Cells grown on culture dishes were treated as described above for RNA analysis and incubated with TBN containing either 0.4 µg/µl control

peptide or peptide 2 (nucleosomal binding domain) for 10 min at room temperature. The TBN media were collected and the proteins precipitated with 6.5 volumes of ice-cold acetone. Cells were then washed twice with PBS and harvested in 1 ml of PBS. The cell pellet was washed again in PBS and resuspended in Roti-Load (Roth). The proteins were resolved in a 13% acrylamide gel containing SDS. For immunoblot, the proteins were transferred to PVDF-membrane (Immobilon-P, Millipore), the membrane was blocked with 5% non-fat dried milk in TBS (140 mM NaCl, 10 mM Tris-Cl pH 7.4), and incubated with affinity pure antibodies directed to peptide 2 for 2 h at room temperature. The concentration of the antibody was 0.1 µg/ml diluted in blocking buffer. After four washes in TBS, secondary antibodies (goat anti-rabbit IgG conjugated with peroxidase; Dianova; diluted 1:10 000) were added and the blots incubated for 1 h. After five washes, the bound antibodies were visualized using the enhanced chemiluminescence detection system (ECL, Amersham, UK). For detection of histone H1 the membrane was stripped and reprobed with affinity pure antibodies to histone H1 (0.1 µg/ml) following the same steps as for anti-peptide 2.

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