High mobility group chromosomal protein 1 binds to the adeno-associated virus replication protein (Rep) and promotes Rep-mediated site-specific cleavage of DNA, ATPase activity and transcriptional repression

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High mobility group protein 1 (HMG1) is an abundant non-histone chromosomal protein which plays a role in several nuclear events involving DNA. Here we demonstrate that HMG1 physically interacts with the human adeno-associated virus (AAV) Rep protein. HMG1 promotes the formation of Rep–DNA complexes and stimulates the activity of Rep in site- and strand-specific cleavage of DNA and the hydrolysis of ATP, functions required for viral gene regulation, replication and site-specific integration of viral DNA into human chromosome 19. We show that HMG1 enhances Rep-mediated repression of the AAV p5 promoter in transfected cells, suggesting that HMG1 and Rep also interact *in vivo*. HMG1, Rep and DNA can be immunoprecipitated as a ternary complex. Kinetic studies indicate that complexes of Rep with DNA have similar stabilities in the presence and absence of HMG1. These results suggest that the effect of HMG1 on Rep binding is exerted at the step of complex formation and thereby may reflect an activity of HMG1 in promoting the assembly of complex cellular nucleoprotein structures.

Keywords: AAV/ATPase/DNA endonuclease/HMG1/transcription

Introduction

High mobility group 1 (HMG1) is a member of the abundant high mobility group of non-histone chromosomal proteins found ubiquitously in eukaryotic cells. It has a tripartite structure, consisting of two internal repeats (HMG domains A and B) and an acidic carboxy-terminal tail (Bustin *et al.*, 1990). HMG domains mediate DNA binding and have been identified both in widely expressed proteins which bind DNA with little or no apparent sequence specificity and in some sequence-specific DNA-binding proteins found in only certain cell types (Grosschedl *et al.*, 1994). HMG1 belongs to the first group, binding single-stranded and double-stranded DNA independently of the sequence. Notably, however, it binds

to cruciform or kinked DNA with a significantly higher affinity than to linear B-form DNA (Bianchi *et al.*, 1989; Pil and Lippard, 1992).

The function of HMG1 has not been defined precisely although it has been implicated in several eukaryotic cellular processes including transcription (Ge and Roeder, 1994; Ogawa *et al.*, 1995), DNA replication (Alexandrova and Beltchev, 1988) and recombination (Bianchi *et al.*, 1989; Paull *et al.*, 1993). Central to all these processes may be the ability of HMG to increase DNA flexibility and promote the formation of nucleoprotein complexes. HMG1 bends DNA without sequence specificity. It has been shown to enable the ligation to circular forms of short DNA fragments which are otherwise too inflexible for self-ligation (Pil *et al.*, 1993; Paull *et al.*, 1993). Onate *et al.* (1994) observed enhanced binding of the progesterone receptor to its DNA recognition element in the presence of HMG1 and, having found that HMG1 is an effective DNA-flexing protein, proposed a structural alteration of the progesterone receptor DNA target site as a possible mechanism for the enhanced binding. However, increasing DNA flexibility is not the sole means by which HMG may influence cellular processes. By physically interacting with TATA-binding protein, HMG1 indirectly inhibited class II gene transcription (Ge and Roeder, 1994). Stimulation of transcription by HMG1 has also been reported (Ogawa *et al.*, 1995). Recently, Zappavigna *et al.* (1996) showed that HMG1 interacts with the HOXD9 protein and enhances its sequence-specific DNA-binding activity *in vitro*. HMG1 stimulated transcriptional activation by HOXD9 *in vivo*. The closely related HMG2 was isolated in a screening for proteins which interact with Oct2 (Zwilling *et al.*, 1995). HMG2 increased specific DNA binding by Oct2 and stimulated Oct2-dependent transcription.

Adeno-associated virus type 2 (AAV2) is a small DNA-containing virus which infects humans (reviewed by Berns and Linden, 1995). For efficient replication, AAV requires co-infection with a helper virus, for example adenovirus or herpes virus; however, a low level of helper-independent AAV replication can occur in cells exposed to genotoxic stress (Yakobson *et al.*, 1987, 1989; Yalkinoglu *et al.*, 1988). An outstanding feature of the life cycle of AAV in cell culture is the establishment of latent infection by integration of the viral DNA into the cellular genome. This occurs when conditions for productive infection are not met. AAV is unique among eukaryotic viruses in its ability to integrate its DNA site-specifically (Kotin *et al.*, 1990; Samulski *et al.*, 1991) and is receiving much attention as a potential vector for the transfer and integration of foreign DNA into eukaryotic cells. AAV DNA is a linear single-stranded molecule of ~4.7 kb flanked by palindromic inverted terminal repeats (ITRs) which con-

tain the replication origins (reviewed by Berns and Linden, 1995). For replication, AAV requires, in addition to functions provided by the host cell, non-structural proteins encoded by its own *rep* gene. Four overlapping Rep proteins are made (Rep 78, Rep 68, Rep 52 and Rep 40). The two larger proteins (Rep 78 and Rep 68) regulate many aspects of the viral life cycle, including DNA replication, transcription and site-specific chromosomal insertions. They are multifunctional proteins with properties including DNA binding and site- and strand-specific endonuclease, helicase and ATPase activities. The ITR DNA contains a binding site for Rep as well as a site (called TRS) at which Rep nicks the DNA in a sequence- and strand-specific manner, a step which is critical for DNA replication. The site in human chromosome 19 into which AAV DNA integrates (the pre-integration site) has been characterized (Kotin *et al.*, 1992; Giraud *et al.*, 1994, 1995) and contains a Rep DNA recognition sequence (Weitzman *et al.*, 1994) and a putative TRS site for Rep-mediated cleavage. These two sequences have been identified as the minimum recognition signals for site-specific AAV recombination (Linden *et al.*, 1996). Kotin (1994) and Linden *et al.* (1996) have proposed a model for site-specific integration in which the initial event is the Rep-mediated binding of AAV DNA to the Rep-binding motif on the pre-integration DNA. Nicking by Rep of the putative DNA cleavage site in the pre-integration locus would then serve as a step to initiate recombination.

The central role played by the Rep proteins in the latent and the productive cycles of AAV makes it likely that they interact with host cellular proteins. In the work reported here, we looked for such proteins and, in doing so, identified a novel type of function for the cellular high mobility group protein, HMG1.

Results

HMG1 enhances the specific binding of Rep to DNA

Analysis of the binding of Rep to DNA by electrophoretic mobility shift assay (EMSA) shows a characteristic series of specific complexes (Owens *et al.*, 1993; McCarty *et al.*, 1994). We observed such a pattern following incubation of an oligonucleotide containing the Rep DNA-binding site, Δ ITR (see Figure 1E and Materials and methods; Chiorini *et al.*, 1994), with a whole cell protein extract of HeLa cells which had been co-infected with AAV2 and helper adenovirus type 2 (Figure 1A, lane 3). These complexes were not observed when extracts from uninfected cells or cells infected with adenovirus only were used (data not shown). The series of complexes is believed to represent multimers of Rep molecules and bound oligonucleotide (McCarty *et al.*, 1994). In initial experiments, we observed that supplementing the binding reaction mixtures with nuclear extracts from a number of different cell types led to increased Rep binding. This suggested that a commonly occurring accessory factor may facilitate the Rep–DNA interaction. The known ability of HMG1 to enhance the binding of the progesterone receptor to DNA, its high affinity for cruciform or bent DNA (the ITR can form a T-shaped hairpin structure) and

its possible role in promoting recombination made HMG1 a potential candidate.

The high mobility group proteins are soluble in 2–5% perchloric acid, a property used in their extraction. When a perchloric acid extract of HeLa cell nuclei was added to a mobility shift reaction mix containing Rep, an alteration in the pattern of Rep–DNA binding was observed (Figure 1A, lanes 3 and 4). An overall enhancement was seen along with an increase in the amount of slowly migrating complexes and a corresponding decrease in the amount of faster migrating complexes. The perchloric acid extract by itself did not react with the Δ ITR probe (Figure 1A, lane 2). The proteins in this type of extract include three distinct families of high mobility group proteins, HMG1/2, HMG14/17 and HMG1. To confirm a possible interaction between Rep and HMG1, we used purified proteins. Rep 78 was synthesized in *Escherichia coli* as a fusion protein with glutathione *S*-transferase (GST) and purified using glutathione affinity chromatography. The addition of limiting amounts of purified GST–Rep 78 to a binding reaction gave rise to weak specific complex formation with the Δ ITR (Figure 1B, lane 2). However, when purified HMG1 was added, a strong enhancement of shifted complexes was seen (Figure 1B, lane 3). As before, an increase in the amount of slowly migrating complexes and a corresponding decrease in the amount of faster migrating complexes was observed. The control, GST alone, did not bind to the Δ ITR oligonucleotide, (Figure 1B, lane 4), and addition of HMG1 did not stimulate any GST binding (Figure 1B, lane 5). HMG1 by itself also gave no detectable binding to the Δ ITR (Figure 1B, lane 6). Thus, addition of purified HMG1 to a Rep–DNA binding reaction is alone sufficient to give the observed enhanced binding. We examined whether HMG1 would provoke binding of Rep to an oligonucleotide which does not contain a Rep-binding element. No binding of Rep to such an oligonucleotide (HPV16 E2 recognition sequence) was observed either in the absence or in the presence of HMG1 (data not shown), indicating that HMG1 does not lead to non-specific DNA binding by Rep. Addition of HU, the prokaryotic DNA-bending protein (a kind gift of J. Wagner and D. Pettijohn), to an electrophoretic mobility shift binding reaction did not enhance Rep binding to DNA (Figure 1C, lane 3).

We examined whether the enhanced binding observed with the Δ ITR would also be detected with the complete cruciform-like ITR (see Figure 1D and Materials and methods). As seen in Figure 1D, using an amount of GST–Rep78 which gave barely detectable complex formation with the ITR, a strong increase in binding was observed upon supplementing the reaction with the HMG-containing perchloric acid extract of HeLa cells. Complexes of this nature were not observed when this extract by itself was added to the reaction (Figure 1D, lane 4). A similar enhancement in DNA binding was obtained with Rep 68 purified (Chiorini *et al.*, 1994) as a bacterial maltose-binding protein (MBP) fusion (data not shown).

Complexes of Rep with DNA have similar stabilities in the presence and absence of HMG protein

The observed enhancement in Rep DNA binding could be the result of an HMG-induced increase in the stability

Fig. 1. HMG1 enhances the binding of Rep to DNA. (A) Mobility shift assay containing 0.02 pmol of the labeled Δ ITR oligonucleotide incubated with 1 μ g of a perchloric acid extract of HeLa nuclei alone (lane 2) or 0.5 μ g of a whole cell extract of HeLa cells co-infected with AAV and Ad2 in the absence (lane 3) or in the presence (lane 4) of 1 μ g of a perchloric acid extract of HeLa nuclei. Lane 1 contains the Δ ITR probe alone. (B) Mobility shift assay containing 0.02 pmol of labeled Δ ITR oligonucleotide incubated with 25 ng of purified GST-Rep 78 or 25 ng of GST in the absence (lanes 2 and 4 respectively) or in the presence of 100 ng of purified HMG1 (lanes 3 and 5 respectively). Lane 6 contains 100 ng of HMG1 alone with the oligonucleotide probe. (C) Mobility shift assay containing 0.02 pmol of labeled Δ ITR oligonucleotide incubated with 25 ng of purified GST-Rep 78 in the absence (lane 2) or in the presence of 25 ng of purified HU (lane 3) or 25 ng of purified HMG1 (lane 4). Lane 1 contains the Δ ITR probe alone. (D) Mobility shift assay containing 0.1 ng of the labeled entire inverted terminal repeat (ITR, see Materials and methods) incubated with 100 ng of purified GST-Rep 78 in the absence (lane 2) or in the presence (lane 3) of 1 μ g of a perchloric acid extract of HeLa nuclei. Perchloric acid extract (1 μ g) alone was incubated with the ITR (lane 4). (E) A schematic representation of the ITR and the oligonucleotide (Δ ITR) with the Rep-binding site (Rep) and cleavage site (TRS) indicated. F in (A–C) denotes unbound probe.

of Rep–DNA complexes. To examine if the complexes were indeed more stable in the presence of HMG, we allowed complexes to form with or without HMG protein

in the reaction mixture. Pre-formed complexes were then challenged by the addition of an excess of unlabeled DNA substrate. The autoradiogram is shown in Figure 2A

Fig. 2. Dissociation of Rep–DNA complexes. Labeled Δ ITR (0.02 pmol) was incubated with 0.5 μ g of a whole cell extract of HeLa cells infected with AAV and Ad2 in the absence (lanes 2–5) and in the presence (lanes 6–9) of 1 μ g of HMG proteins from a perchloric acid extract of HeLa nuclei. After 20 min, complexes were challenged with a 260-fold excess of unlabeled Δ ITR for 10, 20 or 30 min, as indicated. Complexes (denoted a, b, c and d) were resolved on a 6% TBE polyacrylamide gel. **(B)** The amount of complexes in **(A)** was quantified with a PhosphorImager (Molecular Dynamics) and plotted as a function of time after addition of competitor.

and the results quantified in Figure 2B. Examination of dissociation of Rep from DNA is complicated by the fact that there are several specific complexes formed. Addition of competitor initially caused a decay of the slowest migrating complexes (d, c and b) and an increase in the amount of complex a. Subsequently, complex a also decayed. In the presence of HMG, there was, at equilibrium, more of complexes d and c and less of complexes a and b. However, the higher order complexes d and c rapidly decayed upon addition of competitor accompanied

by an increase in the amount of complexes b and a. Complex b subsequently decayed. The presence of HMG protein in the reaction mixtures was clearly not reflected by greater stability of the slowest migrating complexes. The fact that, both in the presence and absence of HMG, complex a increases after addition of competitor strongly suggests that more of this complex is formed through decay of higher order complexes. This, together with the higher amount of complexes initially formed in the presence of HMG, would explain the less rapid disappearance of complex a. We conclude that higher order Rep–DNA complexes (d, c and b) formed in the presence of HMG do not have a greater stability than complexes formed in its absence. However, we cannot totally exclude stabilization of complex a by HMG.

It is important to note that HMG1 enhanced Rep binding to DNA without altering the mobility of the complexes. This is most clearly seen in Figure 2A. In the absence of HMG and competitor DNA (lane 2), four complexes (a, b, c and d) are seen. Complexes a, b and c are clearly visible, while complex d is weak. Under the same conditions but in the presence of HMG (lane 6), the higher order complexes are favored, with a and b becoming weaker, c and d, stronger. However, no new complexes were observed in the presence of HMG (lanes 6–9). This is also true for the experiments shown in Figure 1 where a low concentration of Rep was used and thus only lower order complexes were observed. Addition of HMG1 caused the appearance of Rep-shifted bands of lower mobility; however, this can occasionally be observed with greater concentrations of Rep alone (data not shown). Nevertheless, as discussed later, the presence of HMG in the complexes cannot be excluded.

Immunoprecipitation of a ternary complex containing Rep, HMG1 and DNA

GST–Rep 78, purified HMG1 or both were incubated with labeled Δ ITR. DNA complexes were then immunoprecipitated with either a Rep-specific or an HMG1/2-specific antiserum using protein A–Sepharose as an immuno-adsorbent. After extensive washing of the beads, the amount of bound labeled DNA was measured (see Materials and methods). The results are presented in Figure 3. Specific co-immunoprecipitation of the Rep–DNA complex by Rep antibody was increased ~7-fold in the presence of HMG1, reflecting our observations in mobility shift assays. Rep–DNA complexes were not precipitated non-specifically by an HMG1/2 antiserum; neither did this serum co-precipitate HMG1 and DNA. By contrast, the HMG1/2 antiserum did co-immunoprecipitate labeled DNA probe in the presence of both Rep and HMG1. These results show that Rep and HMG1 are able to bind in a ternary complex with DNA.

HMG protein enhances the site-specific cleavage of DNA by Rep

To determine the functional significance of the enhancement in Rep's DNA-binding activity, we examined the effect of HMG on the ability of Rep site-specifically to nick the AAV terminal repeat DNA. Endonuclease action at the Rep cleavage site of the 5'-labeled ITR-containing DNA used in this assay (see Materials and methods) should generate a short labeled molecule of 69 bases.

Fig. 3. Co-immunoprecipitation of labeled DNA probe in the presence of Rep and HMG. Purified GST-Rep 78 (30 ng) and pure HMG1 (150 ng) were incubated with 0.1 ng of 32 P-labeled Δ ITR and immunoprecipitated, using protein A-Sepharose beads as an immunoadsorbent, with antisera against Rep or HMG1/2 as indicated. After several washes, the amount of labeled DNA bound was determined by scintillation counting.

Figure 4 shows the effect of adding to the endonuclease assay increasing concentrations of GST-Rep 78, in the absence (lanes 2, 4, 6 and 8) and in the presence of a fixed concentration of HMG-containing perchloric acid extract (lanes 3, 5, 7 and 9). In the absence of HMG, endonuclease activity was detected weakly and only at the highest concentration of GST-Rep 78 used. By contrast, when HMG was present, Rep endonuclease activity was detected even at the lowest concentration of GST-Rep 78 and this activity increased with increasing GST-Rep 78. Neither HMG by itself nor GST nicked the ITR in this assay. This enhancement in Rep endonuclease activity was seen when HMG was added in a crude form (perchloric acid extract), or as a purified mixture of HMG1 and HMG2 or highly purified HMG1 alone.

To quantify the enhancement in endonuclease activity, we measured the level of nicking achieved by increasing concentrations of GST-Rep 78 in the absence or presence of a constant amount of HMG. Figure 5 shows that low concentrations of GST-Rep 78 alone were ineffective at nicking the template ITR; at the lowest amounts used (18 and 37 ng of protein), there was no detectable endonuclease activity. The first amount of GST-Rep 78 used which gave rise to nicked product was 75 ng. Under these conditions, 1.25% of the substrate was converted to nicked product. It would appear that a critical threshold concentration of Rep is required before it achieves a detectable level of activity. By contrast, in the presence of HMG, site-specific endonuclease activity was detected with the lowest amount of GST-Rep 78. At 75 ng per reaction, GST-Rep 78 displayed 28 times more endonuclease activity in the presence of HMG than in its absence. The degree of enhancement became less marked as the concentration of GST-Rep 78 was increased and the amount of substrate became limiting. At this point, neither a further increase in Rep protein nor addition of HMG led to increased nicking. It thus appears that HMG1 facilitates the interaction between Rep and DNA necessary for Rep-mediated cleavage. The effect of HMG is largest at low concentrations of Rep.

Fig. 4. The site-specific endonuclease activity of Rep is enhanced by HMG1. Under conditions of Rep-induced cleavage of DNA (see Materials and methods), 0.1 ng of 32 P-end-labeled ITR was incubated without proteins (lane 1), or with increasing concentrations of GST-Rep 78 (50 ng, lanes 2 and 3; 100 ng, lanes 4 and 5; 150 ng, lanes 6 and 7; 200 ng, lanes 8, 9 and 10) in the absence (-) or presence (+) of 250 ng of a HMG-containing perchloric acid extract of HeLa nuclei. Lane 10 shows the result with 250 ng of ovalbumin instead of the HMG-containing extract, and lane 11 the result with 250 ng of the extract alone. Lanes 12-14 show the result of a similar experiment performed using 100 ng of pure HMG1 and GST-Rep 78 (50 ng, lanes 12 and 13; and 100 ng, lanes 14 and 15). Following the reaction, the DNA was extracted with a mixture of phenol and chloroform, precipitated with ethanol and dissolved in loading buffer containing 90% formamide. Samples were denatured and electrophoresed on a 6% polyacrylamide denaturing gel containing 7 M urea. Size markers (in nucleotides) are shown in lane M. The arrow denotes the position of the cleavage product. Under these gel conditions, the ITR itself gives multiple bands possibly due to lack of complete denaturation of this palindromic DNA.

HMG1 stimulates the ATPase activity of Rep

To elucidate further the mechanism by which HMG1 acts on Rep, we decided to investigate its influence on an enzymatic function of Rep which is independent of site-specific DNA binding, namely ATPase activity. Wonderling *et al.* (1995) showed that a Rep 68-MBP fusion protein has the capacity to hydrolyze ATP to ADP, and this activity is stimulated by a factor of 2 upon the addition of single-stranded DNA. We examined the effect of single-stranded DNA, HMG1 and both on the ATPase activity of GST-Rep 78 and GST-Rep 52. As a control for possible co-purifying ATPases we used a Rep 78 mutant which contains a lysine to histidine change in the NTP-binding site at position 340 and has been reported to lack any significant ATPase activity (Wonderling *et al.*, 1995).

Figure 6 shows the ATP hydrolysis we observed using similar concentrations of GST-Rep 78, GST-Rep K340H and GST-Rep 52 in the presence of single-stranded DNA, HMG1 and both. The mutant Rep protein did not show any significant hydrolysis of ATP, implying that the

Fig. 5. Quantitation of the effect of HMG1 on Rep endonuclease activity. A Rep endonuclease assay was carried out as above with increasing amounts of GST–Rep 78 in the absence (●) or in the presence (■) of 250 ng of an HMG-containing perchloric acid extract of HeLa nuclei. The amount of substrate ITR nicked in each reaction was quantified with a PhosphorImager (Molecular Dynamics) and expressed as a percentage of total ITR in that reaction. The percentage of nicked substrate was plotted as a function of the amount of GST–Rep 78.

purified Rep proteins are free of unrelated ATPases. It was not influenced by addition of HMG1, single-stranded DNA or a combination of both, showing that neither the single-stranded DNA nor HMG1 contain contaminating ATPase activity. As expected, GST–Rep 78 hydrolyzed ATP. Addition of single-stranded DNA led to an increase in ATPase activity by a factor of 2.4 (consistent with the findings of Wonderling *et al.*, 1995). In the presence of HMG1, hydrolysis of ATP rose by a factor of 2.2. The addition of both single-stranded DNA and HMG1 had a slightly more than additive effect, increasing hydrolysis of ATP to ADP by a factor of 6.5. Rep 52 overlaps with the Rep 78 sequence in the carboxy-terminus and therefore contains the NTP-binding site, but lacks the N-terminal DNA-binding domain. The ATPase activity of Rep 52 has not been reported previously. We show here that GST–Rep 52 can hydrolyze ATP and does so as effectively as GST–Rep 78 (Figure 6B). In contrast to the effect observed with GST–Rep 78, single-stranded DNA did not affect this activity. This may be due to the lack of the DNA-binding domain. Interestingly, addition of HMG1 led to an increase by a factor of 2, similar to that observed with Rep 78. This stimulation was not increased further by the addition of a combination of both single-stranded DNA and HMG1. Similar results were observed in several experiments, and varying the amount of the three different Rep proteins from 5 to 15 ng and HMG1 from 5 to 50 ng did not significantly alter the fold stimulation due to single-stranded DNA, HMG1 or both. The fact that Rep 52 exhibits ATPase activity which is not stimulated by addition of single-stranded DNA, yet can be enhanced by HMG1, suggests that the stimulation occurs through direct protein–protein interaction.

Rep and HMG1 physically interact

HMG1 may exert the effect we observed on Rep activity by altering the DNA which is the target for Rep or by physically interacting with Rep, or both. We examined the possibility of a direct interaction between the two proteins by incubating GST–Rep 78 immobilized on glutathione–Sepharose or, as control, GST immobilized on glutathione–Sepharose with a perchloric acid extract from HeLa cells. Following incubation and extensive

Fig. 6. Effect of single-stranded DNA and/or HMG1 on the ATPase activity of GST–Rep proteins. Standard ATPase assays (Materials and methods) were performed with 0.25 μ Ci of [γ - 32 P]ATP, and the percentage of ATP hydrolyzed was quantified. (A) A typical experiment showing the mean hydrolysis of ATP by 10 ng of GST–Rep 78 (black bars) and 10 ng of GST–Rep K340H (white bars) in the absence or presence of single-stranded DNA (0.2 μ g of M13mp18) and/or HMG1 (10 ng) as indicated. (B) As for (A) except that hydrolysis of ATP by 6 ng of GST–Rep 52 was measured. ‘Input’ refers to the amount of ATP hydrolyzed in the absence of Rep. Error bars represent the standard deviation of two independent values.

washing of the Sepharose beads, proteins retained on them were fractionated by SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose filters and probed using antibodies to HMG proteins. Figure 7A (lane 1) shows that HMG1 did indeed associate with Rep 78. The control beads with GST alone, in contrast, did not bind HMG1 (Figure 7A, lane 2). Thus, the GST part of the fusion protein was not responsible for the observed interaction.

To determine if there is a particular region of Rep important for its interaction with HMG1, we synthesized GST–Rep fusion proteins containing different parts of the Rep molecule. These are summarized in Figure 7B. In Figure 7A (lanes 3–7), we show that both the N-terminal and central portion of Rep 78 bound to HMG1, while the C-terminal part did not bind. Similar results were obtained using both an antiserum against HMG1 and HMG2 and a monoclonal antibody that recognized HMG1. Thus there are at least two regions of Rep 78 that can interact with HMG1, one of which is located in the N-terminal portion and the other in the central portion which is shared by all four Rep proteins. Binding was stable in physiological salt concentrations but was sensitive to mild detergents.

HMG1 enhances Rep-mediated transcriptional repression

To determine whether HMG1 and Rep interact functionally *in vivo*, we carried out transfection assays with cultured cells. AAV uses three promoters, one of which, p5, directs the synthesis of Rep 78 and Rep 68. This promoter contains a Rep recognition sequence and, in the absence of helper virus, Rep binding to this sequence represses transcription from p5 (Kyöstiö *et al.*, 1995). We have observed, by EMSA, that Rep binding to the p5 Rep-binding element is stimulated by HMG1 (data not shown). Given this and our other observations on HMG1–Rep

Fig. 7. HMG1 directly interacts with Rep. (A) Glutathione beads containing 20 μ g of GST, GST-Rep 78 or mutant forms of GST-Rep fusion proteins, as indicated, were incubated with 40 μ g of a perchloric acid extract of HeLa cells, washed extensively and boiled in SDS gel loading buffer. Samples were fractionated by SDS-PAGE, transferred to nitrocellulose and probed with a polyclonal antibody to HMG1 and HMG2. Size markers are shown in kDa. A background band of ~80 kDa appears in all samples regardless of whether Rep is present or not. (B) Schematic representation of the GST-Rep fusion proteins tested for binding to HMG. Amino acid numbers shown refer to Rep 78 except where indicated otherwise.

interactions, we tested whether the down-regulation of the p5 promoter is enhanced by overexpression of HMG1. We constructed a plasmid (p5luc) containing p5 fused to the luciferase reporter gene. This plasmid was transfected alone or in combination with plasmids expressing HMG1 (pCHMG1), Rep 78/68 (pYNRep) or both into the human kidney embryonic cell line 293T which has a reproducibly high transfection efficiency. The p5 promoter activity in these cells is increased due to the constitutive expression of the adenovirus E1a gene (Chang *et al.*, 1989). As Rep has been shown to down-regulate a variety of heterologous promoters (Hermonat, 1994; Horner *et al.*, 1995), the luciferase activity was normalized by the protein concentration of the lysate. The p5 activity in the absence of any treatment was set arbitrarily to 100%. As seen in Figure 8, this activity increased slightly when the cells were transfected with p5luc and increasing amounts of pCHMG1 (white bars), indicating that the HMG1 expression plasmid by itself does not inhibit this promoter. As expected, co-transfection of p5Luc and pYNRep caused a down-regulation of p5 transcription, as reflected in a decrease in luciferase activity. p5 promoter activity was decreased by a factor of 2.6 in the presence of Rep. Co-transfection of pYNRep and pCHMG1 caused a further inhibition of this promoter (black bars). The inhibition of p5 promoter activity increased to 4.9- and 7.4-fold follow-

Fig. 8. Rep-mediated down-regulation of p5 is stimulated by HMG1. 293T cells were transfected with 0.5 μ g of the p5luc luciferase reporter construct in the presence of increasing amounts of pCHMG1 (0, 1.5 and 3 μ g), either without (white bars) or with (black bars) 1.5 μ g of the Rep 78/68 expression plasmid pYNRep. p5 activity in the absence of treatment was set arbitrarily to 100%, and all other values were expressed as a function of this. The luciferase activity was normalized to the protein concentration in the lysate. Bars represent the standard deviation from at least three independent experiments, each carried out in triplicate.

ing transfection of 1.5 and 3.0 μ g of pCHMG1 respectively. These results indicate that HMG1 co-operates with Rep in its function as a transcriptional repressor *in vivo*.

Discussion

We have shown that the ubiquitous chromosomal protein HMG1 interacts with AAV Rep proteins. This interaction is accompanied by the stimulation of several functions of Rep: binding to AAV DNA, sequence-specific cleavage of AAV DNA termini and ATPase activity. HMG1 can form a ternary complex with Rep and DNA. We also provide evidence that HMG1 and Rep functionally interact *in vivo*. In transfection assays, HMG1 enhanced Rep-mediated repression of transcription from an AAV promoter.

How might HMG1 affect the different functions of Rep? Rep can exist as multimers, and these are postulated to be the active form of the protein (Kyöstiö and Owens, 1996; Hermonat and Batchu, 1997). HMG1 could promote the multimerization of Rep. Alternatively, HMG1 could, by binding to Rep, induce an allosteric change rendering the protein more active. Finally Rep-DNA complex formation could be facilitated by HMG1 as a result of a structural change, such as bending, of the DNA or through contacts with both reaction partners.

The enhanced binding of Rep to DNA in the presence of HMG1 could be due to an effect either on complex formation or on complex stability. We measured the stability of complexes formed in the presence and absence of HMG. After addition of unlabeled competitor DNA, higher order Rep-DNA complexes rapidly dissociated regardless of whether HMG was present or not. Mobility shift analysis of Rep complexed with DNA reveals a series of bands, thought to result from different Rep multimers. In the presence of HMG1, there was increased formation of the complexes, especially the more slowly migrating ones. However, no new shifted bands were observed in the presence of HMG1, that is there was no additional shift which could be attributed to the presence of HMG1 in the complexes. These results are consistent with others showing no change, due to the presence of HMG1, in the mobility of electrophoretically shifted complexes of DNA and the HOXD9 protein (Zappavigna *et al.*, 1996) or DNA and the progesterone receptor (Onate *et al.*, 1994). Likewise, the presence of HMG2 did not alter the electrophoretic mobility of Oct2-DNA complexes (Zwilling *et al.*, 1995). The failure to detect HMG1 in the complexes in the mobility shift assays may have several reasons: (i) it is possible that HMG1 does not participate in the final Rep-DNA complex; (ii) HMG may not cause an alteration in mobility because it is small in size compared with Rep; (iii) HMG1 may form a ternary complex with Rep and DNA, but under conditions of electrophoresis the HMG1 component dissociates. We could show, in co-immunoprecipitation experiments, that a ternary complex containing HMG1, Rep and DNA can form. In these experiments, an HMG1/2-specific antibody precipitated labeled Δ ITR only when both HMG1 and Rep were present in the binding reaction.

Several lines of evidence indicate a physical and functional interaction between HMG1 and Rep. We showed that HMG1 specifically binds to the Rep proteins. In

addition, from the results of our ATPase assays, we conclude that HMG1 has the ability to influence Rep function independently of specific DNA binding. Both GST-Rep 78 and GST-Rep 52, which lacks the N-terminal DNA-binding domain, hydrolyze ATP to ADP to the same extent. The ATPase activity of GST-Rep 78 was stimulated by both single-stranded DNA and HMG1. In contrast, GST-Rep 52 was stimulated by HMG1 but not by the addition of single-stranded DNA. This suggests that the effect of HMG1 on GST-Rep 52 results from a direct interaction of the two proteins as HMG1-induced DNA alterations can be ruled out in this case. We found that HMG1 bound to GST-Rep 78. A truncated GST fusion protein containing the first 243 amino acids of Rep and a protein containing the central portion of Rep also bound HMG1. However, binding was not observed with a fusion protein containing the C-terminal 138 amino acids. These observations suggest that HMG1 may bind at least two regions in Rep. The nature of the binding domains is not yet clear. HMG1 has been shown to interact with the HOX homeodomain (Zappavigna *et al.*, 1996) and with TBP (Ge and Roeder, 1994), and HMG2 with Oct2 through its POU domain (Zwilling *et al.*, 1995). AAV Rep, however, does not have sequence similarity with any of these proteins. The fact that the ternary complex (HMG, Rep and DNA) exists and that DNA with bound HMG1 can be precipitated by anti-HMG1/2 antibodies only when Rep is also present is a further hint for the interaction of the two proteins.

While these data support a functional role for the physical interaction between HMG1 and the Rep proteins, they do not preclude a contribution of HMG1-induced DNA alterations. HMG1 binds to DNA in general and to cruciform and bent DNA structures with a higher affinity. It is possible that in the DNA binding and nicking experiments, HMG1 behaves like a chaperone, bringing the specific DNA-binding protein, Rep, and its DNA recognition sequence together or facilitating multimerization of Rep. The termini of AAV DNA, the ITRs, can adopt a cruciform-like structure (see Figure 1E) containing DNA loops and single-stranded regions. Rep-binding sites are contained within ITRs, and HMG proteins would be expected to interact with ITR DNA (although under the conditions used in our experiments, no binding was detected). It should be noted, however, that we saw HMG-mediated enhancement of Rep binding to its recognition site in both ITR DNA and the linear oligonucleotide. Since the prokaryotic DNA-bending protein HU did not enhance Rep binding to DNA, it appears that DNA bending alone is not sufficient to stimulate Rep-DNA interaction.

HMG1 was found to stimulate Rep endonuclease and ATPase activities. Whereas the increase in nicking could be due to enhanced binding to target DNA, the results obtained with the ATPase activity of GST-Rep 52 clearly show a direct stimulation of an enzymatic activity by HMG1. This is a novel function for HMG1.

We suggest that the observed interaction of AAV Rep with HMG1 is evidence for a general cellular activity of HMG1 in promoting the assembly of nucleoprotein structures (Paull *et al.*, 1993; Grosschedl *et al.*, 1994). For AAV, this interaction could be of critical significance in all aspects of the viral life cycle, including productive

and latent infections. Regulation of AAV gene expression is complex, with Rep acting alternately as a repressor and an activator of transcription. AAV uses three promoters, p5, p19 and p40. In the absence of helper virus, all four Rep proteins repress p5 and p19 transcription (Beaton *et al.*, 1989; Kyöstiö *et al.*, 1994; Horer *et al.*, 1995). In the presence of helper virus, Rep acts as both a repressor and an activator of transcription (Pereira *et al.*, 1997). We examined one aspect of the role of Rep 78/68 in transcription—their ability to down-regulate the p5 promoter in the absence of helper virus—and found that the transient expression of HMG1 in transfected cells significantly enhanced Rep-mediated repression. Repression of p5 by Rep is dependent on site-specific DNA binding and the consensus ATP-binding motif (Kyöstiö *et al.*, 1995). We observed enhanced binding of Rep to the p5 Rep-binding element in the presence of HMG1 *in vitro*. It is likely, therefore, that the increased repression seen *in vivo* is at least partially due to increased binding of Rep to this site.

The replication of the linear single-stranded AAV genome with its flanking hairpins (ITRs) occurs by a strand displacement mechanism (reviewed by Berns and Linden, 1995). The 3' hairpin serves as a DNA primer which is extended by cellular polymerase giving rise to a duplex intermediate structure. Since the hairpin is the primer, it is not replicated. The introduction, by Rep, of a site-specific nick in the parental strand, exposing a 3' OH group for extension, solves this problem. The hairpin is thus transferred to the previously newly synthesized strand, and the parental strand gains a copy of it. This end can now reform a hairpin shape and prime a new round of synthesis. Enhancing the activity of Rep to bind and nick the ITR could therefore lead to increased viral DNA replication. The site-specific endonuclease activity of Rep is ATP dependent. It is possible that part of the observed stimulation of this activity is due to enhanced Rep ATPase activity in the presence of HMG1.

In latent infection by AAV, viral DNA integrates into the host genome principally, but not exclusively, at a specific locus on human chromosome 19. Current theories on the mechanism of site-specific integration propose that Rep plays a central role (Kotin, 1994; Linden *et al.*, 1996). Weizmann *et al.* (1994) identified a sequence within the AAV integration locus of chromosome 19 which specifically binds Rep 68 and Rep 78. Using an *in vitro* assay, they showed that Rep could mediate the simultaneous binding of the AAV hairpin and DNA of human chromosome 19 and suggested that this may have a function in the integration event. Linden *et al.* (1996) propose that the AAV genome is brought into register with chromosome 19 DNA by the formation of a complex generated by Rep binding both to the viral genome and to the chromosome 19 target sequence. The nicking activity of Rep at the adjacent chromosome 19 TRS initiates a series of reactions which lead to integration. Clearly, HMG1 enhancement of both Rep binding and nicking activities could be crucial to the proposed mechanism. The amount of Rep present in non-productively infected cells destined to become latently infected is extremely low. Significantly, it was at low concentrations of Rep that we observed HMG1 to have the greatest effect on Rep function. Recently, Aiyar *et al.* (1996) showed, using

an *in vitro* assay, that HMG1 increased the efficiency of integration of linear retroviral DNA by the avian sarcoma virus integrase.

In addition to the sequence elements which direct targeting, viral integration may also depend upon alterations in the flexibility of chromosomal DNA. There is evidence for the formation of complex nucleoprotein structures in site-specific recombination in prokaryotes. In *Salmonella*, gene inversion is mediated by the formation of a nucleoprotein structure called the Hin invertosome. Formation of this structure requires extensive DNA bending, and HU stimulates Hin-mediated site-specific recombination (Haykinson and Johnson, 1993). HMG1 and HMG2 have been shown to be able to promote the assembly of the Hin invertosome and enhance Hin-mediated DNA cleavage (Paull *et al.*, 1993). In an analogous way, in eukaryotic cells, HMG1 might facilitate the correct formation of the nucleoprotein complexes required for integration of the AAV genome and for recombination in general.

Materials and methods

Whole cell protein extracts

HeLa cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS). For infection, the medium was removed from cultures (in 9 cm diameter plates) that were 50% confluent and replaced by 2 ml of medium to which was added 2×10^9 p.f.u. of adenovirus 2, 2×10^9 infectious units of AAV or both. No virus was added to control plates. After 90 min at 37°C, 10 ml of medium containing 5% FCS was added. At 23 h post-infection, whole cell protein extracts were prepared as described (Pagano *et al.*, 1992). Briefly, cells were harvested from two Petri dishes by scraping with a rubber policeman, collected by centrifuging at 800 g for 5 min and washed twice with a solution containing 10 mM Tris pH 8, 150 mM NaCl. After the second wash, the cell pellet was resuspended by gentle pipetting in 200 µl of the following solution: 20 mM Tris pH 8, 1 mM EDTA, 20% glycerol, 1 M NaCl, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 1.6 mM dithiothreitol (DTT), 1.6 µg/ml pepstatin, 2.1 µg/ml aprotinin and 1.3 µg/ml leupeptin. The lysate was then centrifuged for 1 h at 100 000 g. Aliquots of the supernatant (which usually contained between 2 and 5 µg/ml of protein) were stored at -70°C. All manipulations were carried out between 0 and 4°C.

Isolation of HMG proteins from HeLa cells

HeLa cells were grown to 80% confluence on 15 cm diameter dishes, washed twice with phosphate-buffered saline (PBS) and collected by scraping. Cells were pelleted (800 g, 5 min) and resuspended in five packed cell volumes of hypotonic buffer containing 10 mM HEPES-KOH pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 10 mM β-mercaptoethanol, 1% NP-40, 2 µg/ml pepstatin, 2.0 µg/ml leupeptin and 1 mM PMSF. Following a 20 min incubation on ice, cells were disrupted with a Dounce homogenizer and nuclei recovered by centrifuging the lysate at 1000 g for 10 min. Perchloric acid extraction was performed as described previously (Stros *et al.*, 1994). Nuclei from 20 dishes were resuspended in 5 ml of 5% perchloric acid, incubated with rotation for 30 min at 4°C and centrifuged at 10 000 g for 20 min. The supernatant was adjusted with HCl to a final concentration of 0.35 M and precipitated with 6 volumes of acetone. Precipitated proteins were washed with 90% acetone, air dried and resuspended in the following solution: 10 mM HEPES pH 7.6, 5 mM potassium glutamate, 5% glycerol, 5 mM DTT, 0.1% Tween-20. For some purposes, whole cells were extracted as follows: after initial washing and pelleting of cells, they were resuspended immediately in hypertonic buffer (hypotonic buffer + 500 mM KCl). Following a 20 min incubation on ice, the solution was centrifuged (10 000 g for 5 min). Perchloric acid was added to the supernatant to a final concentration of 5% and the extraction continued as described above. Purified HMG1 (which shows a single silver-stained band at 28 000 Da and was confirmed immunologically using both polyclonal and monoclonal HMG1 antibodies, Onate *et al.*, 1994) was a kind gift of P. Prendergast and J. Wagner.

Construction of recombinant AAV2 Rep–GST fusion proteins and expression plasmids

The AAV2 Rep 78 gene (nucleotides 321–2252) and the Rep 52 gene (nucleotides 993–2252) were amplified by PCR from pAV2 (Laughlin *et al.*, 1983) using the following primers containing *Bgl*III sites 5' GAAGATCTATGCCGGGTTTACGAG (nt 321–338), 5' GAAGATCTATGGAGCTGGTCGGGTGG (nt 993–1010) and 3' GAAGATCTTCAGAGAGAG TGTCTCGAGCCAAT (nt 2252–2232). The generated PCR fragments were digested with *Bgl*II and cloned into the *Bgl*II site of pSP72 (Promega). The plasmids were named pspRep 78 and pspRep 52. Using PCR-mediated mutagenesis and pspRep 78 as a template, amino acid 340 of Rep was mutated from lysine to histidine. The mutant fragment was cloned into the *Bgl*II site of pSP72 and named pspRep K340H. The sequences were confirmed. The *Bgl*III fragment and the *Bgl*III–*Bam*HI fragment of pspRep 78, the *Bgl*III fragment of pspRep 52 and the *Bgl*III fragment of pspRep K340H were subcloned into the *Bam*HI site of pGex-2T (Pharmacia) in-frame with the gene encoding GST, generating pGexRep 78, pGexRep 78 1–243, pGexRep 52 and pGexRep K340H. The C-terminal truncation of Rep 52 (pGexRep52 1–260 or pGexRep 78 225–484) was generated by *Eco*RI digestion of pGexRep 52, releasing nt 1767–2252, gel purification of the plasmid and religation. In order to obtain the C-terminal region of Rep 78, the *Hind*III–*Bgl*II fragment of pspRep 78 was filled in by the Klenow polymerase and subcloned into the filled-in *Eco*RI site of pGex-2T, leading to pGexRep 78 522–621. Constructs were introduced into *E. coli* (TB1 strain) for protein expression. The eukaryotic Rep expression vector pYNRep was constructed by subcloning the *Bgl*III fragment of pspRep 78 into the *Bam*HI site of pYN ATC under the control of the Moloney murine leukemia virus long terminal repeat (MoMuLV LTR). The luciferase reporter construct p5luc was constructed by subcloning the *Bgl*III–*Nco*I fragment (AAV2 nt 1–625) from pAV2 (Laughlin *et al.*, 1983) into the *Bgl*II and *Nco*I sites of pGL3 basic (Promega). In this construct, the first 100 amino acids of Rep78 are fused in-frame with the luciferase gene. The sequence was confirmed. The HMG1 eukaryotic expression clone pCHMG1, which contains the rat HMG1 gene inserted next to the cytomegalovirus (CMV) promoter in pCDNA1Amp (Invitrogen) was a kind gift of M. Binchi and D. Edwards.

Expression of GST–Rep fusion proteins

Individual colonies transformed with Rep fusion protein expression vectors were used to inoculate 10 ml of 2× YT medium. Following growth overnight at 37°C with vigorous shaking, cultures were inoculated into 1 l of 2× YT. Bacteria were grown at 25°C until an absorbance at 600 nm of 0.3–0.5 was obtained. Expression of the different Rep fusion proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM, and incubation was continued for another 4–6 h at 25°C. Cells were harvested by centrifugation at 2500 g for 15 min. Bacteria were resuspended in 50 ml of lysis buffer (50 mM Tris–HCl pH 8.0, 50 mM NaCl, 1 mM EDTA, 10% glycerol, 10 mM β-mercaptoethanol, 1 mM azide) and stored at –20°C until required.

When required, bacterial suspensions were thawed and processed immediately. The cells were lysed by sonication. Insoluble material was removed by centrifugation for 20 min at 15 000 r.p.m. at 4°C. Bacterial lysates were incubated at 4°C for 1 h with glutathione resin (Pharmacia), previously equilibrated with lysis buffer. Following incubation, the resin was washed extensively with lysis buffer. The washed beads were either stored in lysis buffer at 4°C or the fusion protein was eluted in a small volume of elution buffer (25 mM HEPES–KOH pH 7.6, 1 mM DTT, 5 mM potassium glutamate and 5 mM glutathione). Eluted proteins were stored in storage buffer (10 mM HEPES–KOH pH 7.6, 5 mM potassium glutamate, 5% glycerol, 5 mM DTT, 0.1% Tween-20) at –70°C.

Preparation of radiolabeled probes

The probes are shown schematically in Figure 1E. Plasmid psub201 containing the cloned AAV genome (Samulski *et al.*, 1987) was digested with the restriction enzymes *Xba*I and *Pvu*II, releasing a 177 bp fragment containing the AAV ITR sequence. This fragment was gel purified and alkaline phosphatase treated. Approximately 10 ng was 5' end labeled using T4 polynucleotide kinase and [γ -³²P]ATP and subsequently boiled and quick-cooled to allow denaturation of the double strand and formation of the hairpin ITR conformation. The product consists of a modified ITR plus ~41 nucleotides on the viral side. This DNA was then used in mobility shift as well as in endonuclease assays. ΔITR (Chiorini *et al.*, 1994) is a 52 bp oligonucleotide comprising only the arm of the ITR (kindly provided by R. Kotin). The ΔITR probes were made by 5'

end labeling either oligomer and annealing with an excess of the complementary oligonucleotide and were used in mobility shift assays.

Mobility shift assay

Mobility shift assays were carried out as described previously (Im and Muzycka, 1989) with modifications. In brief, reaction mixtures contained (in a final volume of 20 μl) 25 mM HEPES–KOH (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 2% glycerol, 0.25 μg bovine serum albumin (BSA), 50 mM KCl, 0.01% NP-40, 0.5 μg of HeLa whole cell protein extract or the indicated amount of purified GST–Rep (between 10 and 100 ng). Then 500 ng of poly(dI–dC) was added to reaction mixtures containing HeLa extracts; 50 ng was added to those containing purified GST–Rep. Where indicated, purified HMG or a perchloric acid extract of HeLa nuclei was added to this reaction mixture. The reaction mixture was incubated on ice for 15 min. Approximately 0.02 pmol of ³²P-labeled ΔITR oligonucleotide or 0.1 ng of ³²P-labeled ITR was then added and the reaction continued for a further 20 min on ice. (In competition experiments, a 260-fold excess of unlabeled ΔITR was added at this point and reactions incubated further for specified durations of time.) Ficoll 400 was added to a final concentration of 5% and the reaction mixtures were loaded onto 6% polyacrylamide gels (39:1; acrylamide: bisacrylamide w/w) containing 0.5× TBE (89 mM Tris, 89 mM boric acid, 0.2 mM EDTA) (pH 7.6). Gels were electrophoresed in the same (0.5× TBE) buffer at 4°C for 2.5 h at 200 V. They were then transferred to Whatmann 3MM filter paper, dried and autoradiographed.

Co-immunoprecipitation experiments

Co-immunoprecipitations were performed as described by Onate *et al.* (1994) with modifications. The DNA-binding reaction mixture was identical to that used for mobility shift assay. Either purified HMG1 (150 ng), purified GST–Rep 78 (30 ng) or both were added as indicated. The mixture was incubated at 4°C for 15 min. Then 1 ng (200 000 c.p.m./ng) of ³²P-labeled ΔITR was added and the reaction continued for a further 45 min. One μl of rabbit antiserum (anti-Rep or anti-HMG1/2) was added and the incubation continued as before for 1 h with frequent mixing. Protein A–Sepharose beads (30 μl) in 50 μl of binding buffer were added to the samples and incubated for 1.5 h at 4°C with frequent mixing to maintain the resin in suspension. The protein A–Sepharose was then washed five times with 1 ml of binding buffer. Labeled DNA probe which remained bound to the beads was quantified with a liquid scintillation counter.

Rep endonuclease assay

Rep endonuclease assays were carried out as described previously (Im and Muzycka, 1990) with slight modifications. In a final volume of 20 μl, the indicated amounts of purified GST–Rep were incubated with ~0.5 ng of ³²P-end-labeled ITR (40 000 c.p.m.) in a solution containing the following: 25 mM HEPES–KOH (pH 7.5), 5 mM MgCl₂, 1 mM DTT, 0.4 mM ATP, 1 μg of ovalbumin and 100 ng of poly(dI–dC). Where indicated, purified HMG or a perchloric acid extract of HeLa nuclei was added. The reaction began by the addition of MgCl₂ and was incubated for 1 h at 37°C, followed by the addition of 50 μl of phenol–chloroform and 30 μl of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). Following extraction with phenol–chloroform, the reaction mixture was precipitated with ethanol. The precipitates were dissolved in 90% formamide containing 0.1% bromophenol blue and 0.1% xylene cyanol and were electrophoresed on denaturing gels containing 6% acrylamide and 7 M urea. Gels were dried and autoradiographed. The distance between the TRS site and the 5'-labeled end of the *Xba*I site in the ITR as prepared above is 69 nucleotides.

ATPase assay

ATPase assays were carried out as described previously (Wonderling *et al.*, 1995), with modifications. In brief, in a final volume of 10 μl, reaction mixtures contained 50 mM Tris–HCl (pH 8), 20 mM NaCl and 2.5 mM MgCl₂. The indicated amounts of purified GST–Rep fusion proteins (GST–Rep 78, GST–Rep K340H or GST–Rep 52) were added. Purified HMG1/2 or HMG1 (5–50 ng) or single-stranded DNA (0.2 μg of M13mp18) was added as indicated. The total protein concentration was kept constant by adjusting with BSA. Reactions were incubated on ice for 15 min, 0.25 μCi of [γ -³²P]ATP (3000 Ci/mmol) added and the reaction continued for a further 60 min at 24°C. Reactions were stopped by the addition of EDTA to a final concentration of 20 mM. The reaction products were analyzed by thin-layer chromatography. One μl was spotted onto polyethyleneimine–cellulose F plastic sheets (Merck), and developed by ascending chromatography in 0.375 M potassium phosphate

(pH 3.5). The percentage of ATP hydrolyzed was quantified with a PhosphorImager (Molecular Dynamics).

Detection of protein-protein interactions

All manipulations were carried out between 0 and 4°C. In a microfuge tube, HeLa whole cell perchloric acid extract was made up to final volume of 1 ml with buffer A: 10% glycerol, 25 mM HEPES-KOH, pH 7.5, 120 mM KCl, 1 mM EDTA, 1 mM β -mercaptoethanol. This was followed by centrifuging for 20 min (14 000 r.p.m.) in order to pellet any insoluble material. The supernatant was removed to a new tube and 10 μ l of glutathione-Sepharose beads (Pharmacia Biotech) which had been equilibrated previously with buffer A were added. Incubation with rotation was carried out for 1 h (to clear the solution of proteins which bind to the Sepharose beads themselves), after which time the beads were removed by centrifugation (1 min, 14 000 r.p.m.). The supernatant was divided into aliquots, corresponding to 50 μ g of perchloric extract per sample. To separate aliquots, 20 μ l of Rep 78 beads (GST-Rep 78 immobilized onto glutathione-Sepharose beads) or, as control, GST beads (GST immobilized onto glutathione-Sepharose beads), corresponding in each case to ~20 μ g protein, were added. Incubation with rotation for 1 h was carried out as before and again beads were removed by centrifugation (1 min, 14 000 r.p.m.). They were then washed four times with 1 ml of buffer A and resuspended in 2 $\times$ SDS loading buffer. After boiling, proteins were separated by 15% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes in a buffer containing 25 mM Tris-HCl, 0.192 M glycine, 20% methanol and 0.1% SDS. HMG proteins were detected with a rabbit polyclonal antiserum, which recognizes both HMG1 and HMG2, and a peroxidase-coupled secondary antibody (Dako). Detection was by enhanced chemiluminescence (Amersham) following the protocol of the manufacturer.

Transfections

293T cells were maintained in DMEM supplemented with 10% FCS. For transfection (by calcium phosphate precipitation, Graham and van der Eb, 1973), they were grown to 30–40% confluence. Following a 10–12 h incubation period with the precipitate, cells were washed, the medium changed and they were incubated for a further 48 h. Cells were then washed, lysed in Reporter Lysis Buffer (Promega) and assayed immediately for luciferase activity. The protein concentration of the lysate was determined by Bradford assay (Biorad).

In a typical transfection experiment, 5 μ g of DNA were used per 35 mm dish; 0.5 μ g of reporter plasmid and the indicated amounts of pCHMG1 and pYNRep were used. The amount of DNA was adjusted to 5 μ g with pYN vector DNA. For each plasmid, experiments were done with at least two independent cesium chloride preparations.

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