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Stimulation of V(D)J cleavage by high mobility group proteins

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V(D)J recombination requires a pair of signal sequences with spacer lengths of 12 and 23 bp between the conserved heptamer and nonamer elements. The RAG1 and RAG2 proteins initiate the reaction by making double-strand DNA breaks at both signals, and must thus be able to operate on these two different spatial arrangements. We show that the DNA-bending proteins HMG1 and HMG2 stimulate cleavage and RAG protein binding at the 23 bp spacer signal. These findings suggest that DNA bending is important for bridging the longer spacer, and explain how a similar array of RAG proteins could accommodate a signal with either a 12 or a 23 bp spacer. An additional effect of HMG proteins is to stimulate coupled cleavage greatly when both signal sequences are present, suggesting that these proteins also aid the formation of a synaptic complex.

Keywords: DNA bending/HMG proteins/RAG1 and RAG2/V(D)J recombination

Introduction

Immunoglobulin and T-cell receptor genes are assembled from separate gene segments by a process called V(D)J recombination (reviewed by Gellert, 1992; Lewis, 1994). The gene segments to be joined are flanked by recombination signal sequences (RSSs), which consist of conserved heptamer and nonamer motifs, separated by relatively non-conserved spacer regions of 12 or 23 bp. Recombination preferentially takes place between RSSs of different spacer lengths, thus directing joining of the correct types of gene segments.

The V(D)J recombination reaction can be divided into two stages: first, double-strand DNA breaks (DSBs) are made at the border between each RSS and coding segment, followed by ligation of the RSSs in a head–head fashion (signal joint), and joining of the coding sequences (coding joint). The DSB intermediates have been detected both *in vivo* (Roth *et al.*, 1992a; Schlissel *et al.*, 1993; Ramsden and Gellert, 1995) and *in vitro* (van Gent *et al.*, 1995). Coding ends isolated from cells have been found in a hairpin structure (Roth *et al.*, 1992b; Ramsden and Gellert, 1995; Zhu and Roth, 1995), and the same species is produced by *in vitro* cleavage (van Gent *et al.*, 1995). DSB formation *in vitro* requires only the presence of the RAG1 and RAG2 proteins and an RSS (McBlane *et al.*, 1995). The RAG proteins first hydrolyze the phosphodiester bond 5′ of the RSS, and subsequently couple the newly formed 3′-OH to the phosphodiester bond next to the RSS in the other strand, resulting in formation of a covalently closed (hairpin) coding end. Hairpin formation does not require an exogenous energy source, and is accomplished by direct *trans*-esterification (van Gent *et al.*, 1996a).

In vivo, cleavage requires the presence of two RSSs for efficient cleavage (Steen *et al.*, 1996). *In vitro*, the substrate requirements for V(D)J cleavage are dependent on the divalent cation: in Mn^{2+} both nicks and hairpins can be formed on a single RSS, but in Mg^{2+} only nicks are made, unless a second RSS (preferably with the other spacer length) is present on the same DNA molecule (Eastman *et al.*, 1996; van Gent *et al.*, 1996b). As Mg^{2+} is the most abundant divalent cation in the nucleus, we assume that these *in vitro* conditions most closely resemble the *in vivo* situation. The coupled cleavage reaction in Mg^{2+} requires only the RAG1 and RAG2 proteins (van Gent *et al.*, 1996b), showing that these proteins contain all the specificity necessary for V(D)J cleavage.

Recent work from this laboratory has identified a stable complex of RAG1 and RAG2 with an RSS that is active in V(D)J cleavage (Hiom and Gellert, 1997). However, it has not been easy to understand how the RAG proteins recognize both types of RSS, as the difference in spacing between the heptamer and nonamer recognition elements is approximately one turn of the DNA double helix (corresponding to a linear displacement of ~3.5 nm). To allow for the two different spacings, the protein composition in the two complexes could be different, or the spacer DNA in the 23-signal could be much more bent than in the 12-signal.

Several well-studied recombination reactions have been shown to be stimulated by DNA-bending proteins, such as the *Escherichia coli* HU and integration host factor (IHF) proteins (Nash, 1996). For example, the MuA-transposase binding sites in the bacteriophage Mu left end are brought together by the bending action of HU (Lavoie and Chaconas, 1990), and bacteriophage λ requires the site-specific DNA-bending protein IHF for efficient integrative recombination (Nash, 1996). In mammalian cells, the chromatin-associated high mobility group proteins HMG1 and HMG2 are abundant DNA-binding factors that are able similarly to bend DNA. Although the detailed biological functions of these proteins are still being investigated, they have been reported to stimulate transcription (Singh and Dixon, 1990; Shykind *et al.*, 1995; Paull *et al.*, 1996) and are also believed to have a more general function in assembly of nucleoprotein complexes (Grosschedl *et al.*,

1994). It has been shown that HMG1 and HMG2 can functionally replace HU or IHF in some recombination reactions (Paull *et al.*, 1993; Segall *et al.*, 1994), presumably by supplying the necessary DNA-bending function.

We show here that V(D)J cleavage is stimulated by the HMG1 and HMG2 proteins. These proteins stimulate formation of a binding complex and cleavage at the 23-signal, suggesting that a similar array of RAG1 and RAG2 proteins may recognize both types of RSSs, and that the much more severe bending required in the 23 bp spacer can be stabilized by an HMG protein. Coupled cleavage of both the 12- and 23-signal sequences on plasmid substrates is stimulated further by HMG1 or HMG2, suggesting that these abundant chromatin factors may contribute to the assembly of productive RAG1–RAG2 synaptic complexes. We believe that this is the first demonstration of an important role for a DNA-bending protein in a eukaryotic recombination process.

Results

V(D)J cleavage is stimulated by DNA-bending proteins

Recent work has shown that the RAG1 and RAG2 proteins are necessary and sufficient for RSS cleavage (McBlane *et al.*, 1995). To identify accessory factors that might stimulate this activity, we tested mammalian cell extracts. Addition of a small amount of a nuclear extract from the pre-B cell line 103/BCL2 (Chen *et al.*, 1994; van Gent *et al.*, 1995) gave equivocal results but, when the extract was first heated to 65°C for 20 min, cleavage of a plasmid substrate was stimulated greatly (data not shown). As proteins of the high mobility group are among the few proteins that survive this temperature, we asked whether the heated extract could be replaced by the ubiquitous mammalian HMG1 protein. HMG1 is known to introduce a sharp bend into DNA. End-labeled oligonucleotide substrates containing a 12- or a 23-signal were incubated with RAG1 and RAG2 in the absence or presence of HMG1 protein, and reaction products were separated by denaturing polyacrylamide gel electrophoresis (Figure 1). When Mn^{2+} was used as divalent cation, both nicks and hairpins were formed from either the 12- or the 23-signal oligonucleotide upon incubation with RAG1 and RAG2 (Figure 1, lanes 2 and 6). In Mg^{2+}, only nicked species were formed, but much more efficiently on the 12-signal than on the 23-signal (Figure 1, lanes 3 and 7). Addition of HMG1 protein to these reactions stimulated nicking of the 23-signal 7- to 10-fold over that seen with the RAG proteins alone (Figure 1, lane 8). Thus, in the presence of HMG1, nicking at the 23-signal was increased to a level slightly higher than at the 12-signal, and a small amount of hairpins was also made. Hairpin formation at a single RSS in Mg^{2+} is unexpected; perhaps in the presence of HMG1, the complex of 23-signal DNA with the RAG proteins is slightly distorted. Maximum stimulation of 23-signal cleavage was observed with HMG concentrations <0.2 μg/ml, whereas we observed little effect on cleavage at the 12-signal with HMG1 concentrations up to 2 μg/ml (data not shown). At the HMG1 concentration used in Figure 1 (1 μg/ml), there are about two molecules of HMG1 per oligonucleotide.

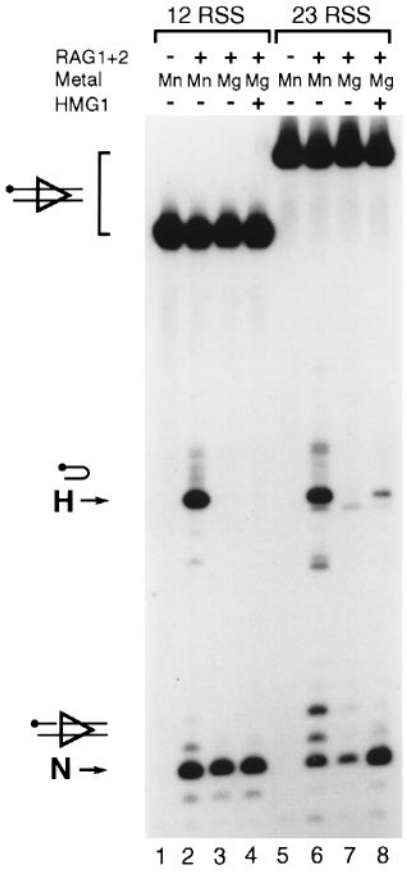

Fig. 1. Effect of HMG1 on oligonucleotide cleavage. An oligonucleotide substrate containing a 12-signal (lanes 1–4) or a 23-signal (lanes 5–8) was incubated without RAG proteins (lanes 1 and 5), with RAG1 and RAG2 (lanes 2, 3, 6 and 7) or with RAG1, RAG2 and 1 μg/ml HMG1 (lanes 4 and 8). The divalent cation in the reactions was either Mn^{2+} (lanes 1, 2, 5 and 6) or Mg^{2+} (lanes 3, 4, 7 and 8). The positions of the 16 nucleotide nicking product (N) and the hairpin (H) resulting from DSB formation are indicated on the left.

Binding of RAG proteins to the 23-signal is enhanced by HMG1

As the HMG1 and HMG2 proteins have been implicated in assembly of various nucleoprotein complexes (reviewed by Grosschedl *et al.*, 1994), we investigated whether formation of a binding complex containing the RAG proteins and an RSS was affected by HMG1. We recently have developed an assay in which binding to an RSS is dependent on the presence of both RAG1 and RAG2 proteins (Hiom and Gellert, 1997). As shown in Figure 2, RAG1 and RAG2 bind to 12-signals and, less efficiently, to 23-signals (lanes 2 and 5). Again, addition of HMG1 protein led to a considerable increase (~10-fold) in the formation of a binding complex on the 23-signal (lane 6), but had little effect on the 12-signal ($<$2-fold; lane 3). No binding complexes were detected when either the 12- or 23-signal substrate was incubated with HMG alone (data not shown). Thus the effect of HMG1 is observed early, at the stage where a complex is assembled. Upon electrophoresis in a lower percentage polyacrylamide gel, the mobility of the 23-signal complex made in the presence of HMG1 was slightly slower (not shown), implying that HMG1 is incorporated into the complex together with RAG1 and RAG2, and does not just facilitate its formation.

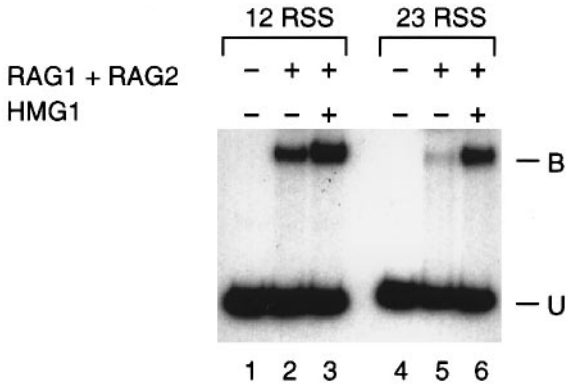

Fig. 2. Stimulation of RSS binding by HMG1 protein. Binding reactions contained oligonucleotide substrates with either a 12-signal (lanes 1–3) or a 23-signal (lanes 4–6), in the absence of RAG proteins (lanes 1 and 4), with RAG1 and RAG2 (lanes 2 and 5) or with RAG1, RAG2 and 1 μg/ml HMG1 (lanes 3 and 6). B = bound complex, U = unbound DNA.

In Mn^{2+}, a stabilization of binding complexes at the 23-signal was also observed, but this did not result in increased cleavage efficiency (data not shown). We presume this reflects a less stringent need for long-lived complex formation in Mn^{2+}.

HMG1 stimulates coupled cleavage

Subsequently, we asked whether HMG1 also has a stimulatory effect on coupled cleavage involving both a 12- and a 23-signal. The linearized plasmid substrate pDVG42, which contains both types of RSS (Figure 3A), was incubated with RAG1 and RAG2 proteins in the absence or presence of HMG1 protein. Cleavage at both RSSs leads to a 1 kb product, whereas cleavage at the 12- or 23-signal alone results in a 7.5 or 3.2 kb fragment. Under the conditions used, the RAG proteins alone induced very low levels of DSBs at either the 12- or the 23-signal, and <0.1% of the input DNA was cleaved at both signals (Figure 3B, lane 2). Addition of HMG1 stimulated an increase in levels of uncoupled cutting of ~4-fold at the 12-spacer RSS, and ~10-fold at the 23-spacer RSS (Figure 3B, lanes 3–8), suggesting once again that there is a more significant effect of HMG1 protein at the 23-signal. However, at higher concentrations of HMG1, coupled cleavage was increased to 10%, a stimulation of 100-fold over that caused by the RAG proteins alone under these conditions (Figure 3B, lanes 7–8). A similar increase was also seen with a substrate containing 'bad' coding flank sequences (Sadofsky *et al.*, 1995) attached to both signals (not shown). The range of HMG1 concentration (from 0.5 to 16 μg/ml) corresponds to one HMG1 molecule per 320 bp at the low end and one per 10 bp at the high end, values compatible with the effective titration of HMG1 in other assays (Paull *et al.*, 1993).

Since this increase is much larger than that at an isolated 23-signal, we conclude that HMG protein must have an additional and separate effect on coupled cleavage, presumably involving synapsis of the two recombination signals. To test this hypothesis, we used a cleavage substrate containing two 12-signals, and no 23-signal. As shown before, this substrate allows a low level of coupled cleavage (van Gent *et al.*, 1996b). Indeed, the same

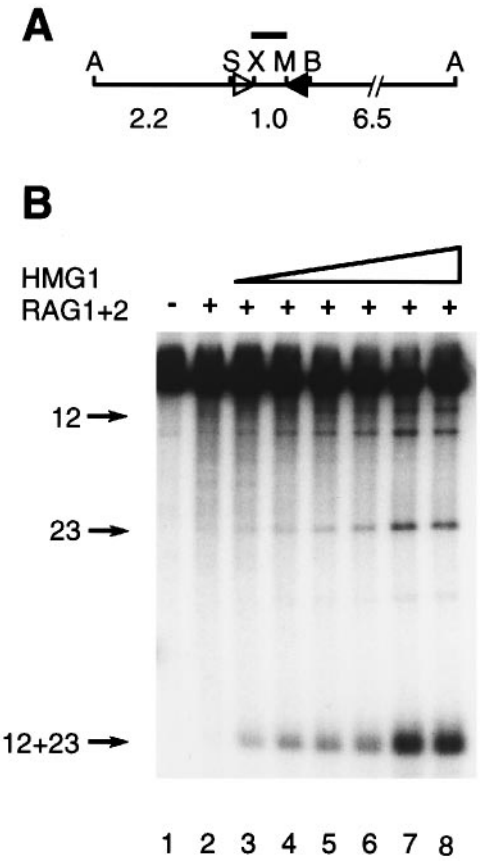

Fig. 3. Stimulation of coupled cleavage by HMG1 protein. (**A**) Schematic representation of the linearized cleavage substrate pDVG42. The RSSs are represented as triangles (open for the 12-signal, closed for the 23-signal), and restriction sites for *Aat*II (A), *Sal*I (S), *Bam*HI (B), *Mlu*I (M) and *Xho*I (X) are indicated. The position of the probe for detection of reaction products by Southern blotting is depicted by a bar above the map. (**B**) Southern blot analysis of pDVG42 incubated without RAG proteins (lane 1), with RAG1 and RAG2 (lane 2) or with RAG1, RAG2 and HMG1 protein (0.5, 1, 2, 4, 8 and 16 μg/ml in lanes 3–8). (The band below the cleaved 12-signal is due to a cross-hybridizing fragment generated by cleavage at the 23-signal, with or without cleavage at the 12-signal.)

concentrations of HMG1 were found to increase the level of coupled cleavage by RAG proteins on a substrate containing only 12-signals by ~10-fold (data not shown), showing that the effects of HMG1 are not limited to the 23-signal.

Effects of other DNA-bending proteins

If the DNA-bending activity of HMG1 is important in stimulating cleavage, one might expect that other DNA-bending proteins will have a similar effect. We found that the related DNA-bending protein HMG2 was as efficient as HMG1 in promoting cleavage of a plasmid substrate (Figure 4, lane 4). Similarly, the yeast non-histone protein 6A (NHP6A), which is more distantly related to HMG1, stimulated both cleavage at the 23-signal and coupled cleavage, but less efficiently than HMG1 or HMG2 (Figure 4, lane 5). In contrast, the bacterial HU protein, which achieves bending by a different mechanism (see Discussion), did not stimulate cleavage (Figure 4, lane 6), even at higher concentrations (data not shown).

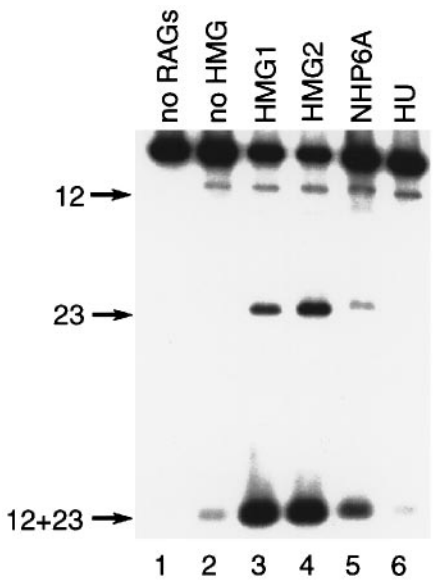

Fig. 4. Effect of DNA-bending proteins on V(D)J cleavage. Cleavage substrate pDVG42 was incubated without RAG proteins (lane 1), with RAG1 and RAG2 (lane 2) or with RAG1, RAG2 and the following DNA-bending proteins (each at 4 μg/ml): HMG1 (lane 3), HMG2 (lane 4), NHP6A (lane 5) or HU (lane 6).

Discussion

HMG1-promoted cleavage of a 23-signal

We have shown previously that the RAG1 and RAG2 proteins are both necessary and sufficient for cleavage at an RSS (McBlane *et al.*, 1995). However, cleavage at an RSS with a 12 bp spacer was more efficient than cutting at a 23-signal, either in Mn^{2+} (Ramsden *et al.*, 1996) or in Mg^{2+} (this report). Here we show that cutting at the 23-signal in Mg^{2+} can be stimulated to a level similar to or greater than 12-signal cleavage by the addition of HMG1 protein. Furthermore the interaction between a 12-signal and a 23-signal is also greatly stimulated by HMG protein in a coupled cleavage assay.

HMG1 and HMG2 have been shown to induce extreme bending of DNA *in vitro* (Paull *et al.*, 1993; Pil *et al.*, 1993). Here, we propose that the bending activity of these proteins stimulates V(D)J cleavage. *In vitro* binding studies show that RAG1 and RAG2 bind both 12- and 23-signals to form similar complexes in a band-shift assay, suggesting that the two types of signals are recognized in a similar way by the RAG proteins (Hiom and Gellert, 1997). Furthermore, binding is dependent on both the heptamer and nonamer elements of the RSS. Since these elements are more distant in a 23-signal, distortion of the intervening DNA may be necessary for stable binding by the RAG proteins on the 23-signal, but not on the 12-signal. Hence, HMG1 might promote binding at the 23-signal by stabilizing a nucleoprotein complex in which the DNA is severely bent (Figure 5).

In addition, it is formally possible that HMG1 interacts with one or both of the RAG proteins in such a way that they become activated for DNA binding and cleavage (e.g. by a conformational change). Such an interaction, however, would have to be more effective on the 23-signal than the 12-signal and, in view of the strong affinity of HMG proteins for DNA, would presumably occur in the context of DNA-bound HMG. More extensive experiments may be able to determine exactly where HMG proteins bind on the RSS, and whether they make close contacts with the RAG proteins.

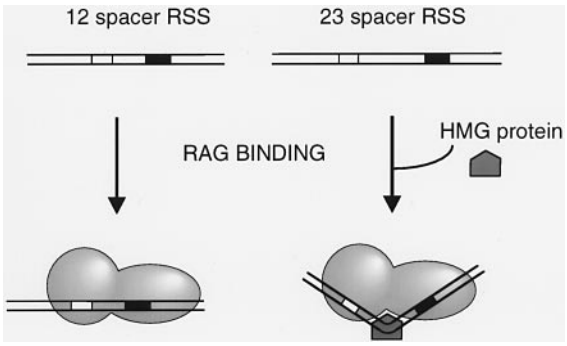

Fig. 5. Model for the role of HMG1 protein in the V(D)J cleavage reaction. This model explains how a very similar array of RAG1 and RAG2 proteins may be able to recognize either a 12- or a 23-signal (compare the left and right panels). The RAG proteins are schematically depicted as rounded shapes, and HMG1 as a pentagonal shape.

Stimulation of coupled cleavage by HMG proteins

HMG1 and HMG2 proteins also stimulate coupled cleavage of both 12- and 23-signals in a plasmid substrate. This activity is probably due in part to the increased cutting of the 23-signal. However, the increase in coupled cleavage exceeds that in 23-signal cutting by an order of magnitude, suggesting that the HMG proteins are inducing an additional effect on a RAG1–RAG2 complex responsible for coupled cleavage. The observation that HMG1 induces a moderate level of coupled cleavage in a substrate containing two 12-signals is in agreement with this view. HMG proteins have been shown to induce and restrain negative supercoils in plasmid DNA (Javaherian *et al.*, 1978; Stros *et al.*, 1994; Paull and Johnson, 1995), suggesting that they may be affecting the topology of the recombination substrate.

Although this is the first report of involvement of DNA-bending proteins in a eukaryotic recombination reaction, the prokaryotic DNA-bending proteins HU and IHF have been shown to stimulate assembly of higher order intermediates in several site-specific recombination reactions. For example, the non-specific DNA-binding protein HU is required to bring two transposase binding sites together in the bacteriophage Mu transposition reaction (Lavoie and Chaconas, 1990; Mizuuchi, 1992), or to stimulate assembly of an intermediate complex in the Hin inversion reaction (Haykinson and Johnson, 1993), and has been shown to substitute for IHF in facilitating bacteriophage λ excision (Goodman and Nash, 1989). Although HMG1 and HMG2 have been shown to substitute at least partially for HU in each of these reactions (Paull *et al.*, 1993; Lavoie and Chaconas, 1994; Segall *et al.*, 1994), the exchangeability evidently does not operate in both directions, because we have found HU to be ineffective in V(D)J cleavage.

Mode of DNA bending in V(D)J cleavage

The importance of DNA bending by the HMG1 protein is also consistent with the observation that the yeast NHP6A protein has a similar effect on cleavage, which can be explained by its DNA-bending capacity (Paull and

Johnson, 1995). However, the fact that HU protein does not induce cleavage of signal sequences by the RAG proteins shows that not all DNA-bending proteins act equivalently. This may reflect the different modes of DNA-binding exhibited by these proteins. The interaction of HMG1 with DNA can be extrapolated from the three-dimensional structure of the sequence-specific HMG-box protein SRY, the male sex-determining factor, bound to DNA (Werner *et al.*, 1995). In this complex, the bulk of the protein is located on the outside of the bend, leaving the inside of the bend potentially available for the RAG proteins. The mode of DNA bending exhibited by HU protein appears to be very different, and can again be extrapolated from the structure of a similar DNA-binding protein, IHF. The three-dimensional structure of the IHF–DNA complex reveals that this protein is located almost exclusively on the inside of the bend (Rice *et al.*, 1996), and might, therefore, interfere with a RAG1–RAG2 complex. Furthermore, analysis of IHF–DNA (Yang and Nash, 1995) and HU–DNA (Lavoie *et al.*, 1996) complexes has shown that ~40 bp are covered by these proteins, which may not be compatible with binding of the RAG proteins to a signal sequence. A less probable explanation for the inactivity of HU protein is that HMG proteins not only bend DNA, but also have specific interactions with the RAG proteins which the non-homologous HU protein does not share.

The ratio of coupled cleavage to uncoupled RSS cleavage in the presence of nuclear extracts *in vitro* has been shown to be very high (Eastman *et al.*, 1996), while the coupled reaction performed by the RAG proteins alone has exhibited significant levels of single cutting (van Gent *et al.*, 1996b). The work presented here demonstrates that HMG1 and HMG2 are two factors present in nuclear extracts which preferentially stimulate the coupled reaction. In addition, it is possible that other proteins could act on V(D)J cleavage in a similar way to HMG1 or HMG2. Even so, in the presence of HMG proteins, the degree of coupling is similar to that observed in V(D)J cleavage *in vivo* (Steen *et al.*, 1996; D.B.Roth, personal communication). Thus the HMG proteins may contribute to the enforcement of the 12/23 rule *in vivo*.

Materials and methods

DNA methods

DNA techniques were used as described (Sambrook *et al.*, 1989). The cleavage substrate pDVG42 was described previously (van Gent *et al.*, 1996b).

Cleavage reactions

The RAG1 and RAG2 proteins used were fusions of maltose-binding protein and amino acids 384–1008 of RAG1 or amino acids 1–387 of RAG2 (described as MR1 and MR2 in McBlane *et al.*, 1995). Plasmid cleavage assays were carried out in a volume of 10 µl, containing 25 mM MOPS, pH 7.0, 5 mM Tris, 30 mM KCl, 30 mM potassium glutamate, 2.2 mM dithiothreitol (DTT), 2% glycerol (v/v), 0.1 mg/ml bovine serum albumin (BSA), 4 mM $MgCl_2$ (or 1 mM $MnCl_2$), 50 ng pDVG42 and 50 ng each of RAG1 and RAG2. In some reactions, HMG1 or other DNA-bending proteins (kind gifts of R.C.Johnson and of H.A.Nash) were added as described in the figure legends. Reaction mixtures were incubated for 1 h at 37°C. SDS was then was added to 0.1% and proteinase K to 100 µg/ml, followed by 30 min incubation at 37°C. Reaction products were analyzed by agarose gel electrophoresis and visualized by Southern blotting as described (van Gent *et al.*, 1996b).

Oligonucleotide cleavage reactions were carried out under very similar conditions, but the plasmid was replaced by 0.2 pmol of end-labeled oligonucleotide substrates DAR39/40 or DG61/62 (McBlane *et al.*, 1995). Cleavage products were separated on TBE-urea polyacrylamide gels and visualized by autoradiography.

DNA binding

Binding experiments contained 0.02 pmol of DNA and 50 ng each of RAG1 and RAG2. Reactions were carried out at 37°C for 10 min in binding buffer (25 mM MOPS pH 7.0, 2 mM DTT, 15 mM potassium chloride, 60 mM potassium acetate, 1 mM magnesium chloride, 100 µg/ml BSA). The samples were then fixed with glutaraldehyde, and products analyzed by polyacrylamide gel electrophoresis. A more detailed description of binding conditions has been given (Hiom and Gellert, 1997).

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