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Nucleosome structure completely inhibits *in vitro* cleavage by the V(D)J recombinase

Amit Golding, Simon Chandler[1], Esteban Ballestar[1], Alan P.Wolffe[1] and Mark S.Schlissel[2,3]

Johns Hopkins University School of Medicine, Department of Medicine, Department of Molecular Biology and Genetics, 1068 Ross Building, 720 Rutland Avenue, Baltimore, MD 21205 and [1]Laboratory of Molecular Embryology, National Institute of Child Health and Human Development, NIH, Bethesda, MD 20892, USA

[2]Present address: Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720-3200, USA

[3]Corresponding author
e-mail: mss@uclink4.berkeley.edu

Lineage specificity and temporal ordering of immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangement are reflected in the accessibility of recombination signal sequences (RSSs) within chromatin to *in vitro* cleavage by the V(D)J recombinase. In this report, we investigated the basis of this regulation by testing the ability of purified RAG1 and RAG2 proteins to initiate cleavage on positioned nucleosomes containing RSS substrates. We found that nicking and double-strand DNA cleavage of RSSs positioned on the face of an unmodified nucleosome are entirely inhibited. This inhibition was independent of translational position or rotational phase and could not be overcome either by addition of the DNA-bending protein HMG-1 or by the use of hyperacetylated histones. We suggest that the nucleosome could act as the stable unit of chromatin which limits recombinase accessibility to potential RSS targets, and that actively rearranging gene segments might be packaged in a modified or disrupted nucleosome structure.

Keywords: accessibility/chromatin/nucleosome/V(D)J recombination

Introduction

Antigen receptor genes are assembled from their component gene segments by a regulated series of site-specific DNA recombination reactions known as V(D)J recombination. Highly conserved recombination signal sequences (RSSs) flank all rearranging gene segments (Tonegawa, 1983). The lymphoid-specific components of the V(D)J recombinase, RAG1 and RAG2, recognize pairs of RSSs and introduce double-strand DNA breaks precisely at the RSS-coding segment borders (Gellert, 1997). With the involvement of the cellular DNA break repair machinery, these broken ends are then joined to form genes with the capacity to encode either immunoglobulin or T-cell receptor (TCR) chains (for a review, see Lieber *et al*., 1997).

Seven complex genetic loci undergo V(D)J recombination in developing B or T cells, i.e. immunoglobulin (Ig) heavy chain (μ) and light chain (κ and λ) loci and TCR α, β, γ and δ chain loci. Despite the conserved nature of the RSS and the identical involvement of RAG1 and RAG2 in all rearrangements, the assembly of antigen receptor genes shows striking developmental regulation (for reviews, see Willerford *et al*., 1996; Papavasiliou *et al*., 1997). Ig genes rearrange fully only in B cells and TCR genes only in T cells (lineage specificity). Furthermore, during B- and T-cell development, Ig heavy chain and TCR β chain gene rearrangement precedes Ig light chain and TCR α chain gene rearrangement, respectively (ordered assembly). Finally, an individual B or T cell generates only one productive heavy chain or β chain rearrangement (allelic exclusion). In each of these cases, a common recombinase recognizing a conserved target sequence is nonetheless regulated in a locus-specific fashion.

In an attempt to explain these observations, Alt and colleagues suggested the accessibility hypothesis: the ability of a locus to undergo V(D)J recombination depends on its developmentally regulated accessibility within chromatin structure (Yancopoulos and Alt, 1985). This notion is consistent with the observed correlation between transcription of unrearranged gene segments (germline transcripts) and their activation for recombination (Yancopoulos and Alt, 1985; Blackwell *et al*., 1986; Schlissel and Baltimore, 1989; Sleckman *et al*., 1996). Targeted disruption of transcriptional regulatory sequences has been reported to decrease both germline transcription and gene rearrangement, although exceptions to this observation exist (for a review, see Sleckman *et al*., 1996).

The development of an *in vitro* system that recapitulates the earliest steps of V(D)J recombination has made it possible to study directly mechanisms which target the recombinase (McBlane *et al*., 1995; van Gent *et al*., 1995). Purified recombinant RAG1 and RAG2 core domains can recognize a single RSS on an oligonucleotide and introduce a precise double-strand DNA break at the RSS-coding segment junction. Recently, this assay system was modified in order to study the initiation of V(D)J recombination of endogenous gene segments within chromatin (Stanhope-Baker *et al*., 1996). Recombinant RAG1 core domain protein supplemented by a thymocyte nuclear extract could recognize and cleave a variety of Ig and TCR RSSs in purified genomic DNA. However, when B, T or non-lymphoid nuclei were used as templates, RSSs were cleaved in a developmentally regulated fashion. For example, Ig light chain RSSs could be cleaved *in vitro* using B-lineage nuclei, but not within T-lineage nuclei, while TCR α chain RSSs showed the opposite pattern. Neither locus could be cleaved in non-lymphoid nuclei. These findings suggested that RSSs may be inaccessible in non-lymphoid cells due to some stable feature of

chromatin structure and that the accessibility of specific RSSs is a regulated property of lymphocyte chromatin.

Perhaps the simplest hypothesis to explain the regulated inaccessibility of certain loci to the V(D)J recombinase is that assembly into the basic unit of chromatin structure, the nucleosome, prevents either the recognition or cleavage of RSSs by RAG1 and RAG2. By analogy with transcriptional repression which is correlated with stable chromatin (for review, see Lu *et al*., 1994), it is possible that simply packaging rearranging loci into nucleosomes might inhibit RAG activity and that nucleosome disruption or modification may be necessary for recombinase targeting. However, the packaging of DNA into a nucleosomal context can enhance the activity of certain DNA cleavage enzymes such as human immunodeficiency virus (HIV) integrase (Pruss *et al*., 1994a,b). A number of transcription factors have been tested for their ability specifically to bind target DNA assembled into a mononucleosome *in vitro* (for a review, see Owen-Hughes and Workman, 1994). Such studies have revealed two categories of DNA-binding proteins: factors that are unable to bind their sites assembled into nucleosomes (e.g. HSF, RAR and NF1) and factors that can bind their sites, albeit with lower affinity (e.g. GAL4 derivatives, TFIIIA and steroid receptors). For proteins in both categories, binding affinity can be increased by physiological and non-physiological histone modification (acetylation and trypsin digestion, respectively) (Lee *et al*., 1993; Vettese-Dadey *et al*., 1994, 1996; Lefebvre *et al*., 1998).

To determine whether nucleosomal packaging permits or restricts recognition and cleavage by the V(D)J recombinase, we assembled a variety of Ig κ light chain gene segments into mononucleosomes *in vitro* and compared their susceptibility to cleavage by RAG1 and RAG2 with the susceptibility of the identical free DNA substrate. We report that incorporation into a mononucleosome completely inhibits RAG cleavage *in vitro* and that histone acetylation does not relieve this inhibition.

Results

RSS-12-containing sequences assembled into mononucleosomes

A 209 bp sequence containing an RSS-12 (heptamer–12 bp spacer–nonamer) from VκL8 was amplified from the recombination reporter construct pJH200 (Hesse *et al*., 1987) and labeled uniformly by incorporation of [α-^{32}P]dCTP. We mixed this labeled substrate with an excess of oligonucleosomes prepared from chicken erythrocytes (Wolffe and Hayes, 1993). After assembly into mononucleosomes by octamer transfer, limited micrococcal nuclease digestion followed by deproteinization and polyacrylamide gel electrophoresis yielded a characteristic 146 bp fragment which had been protected from the nuclease due to its association with a histone octamer (Figure 1A, lane 2). This DNA fragment subsequently was eluted from the gel and analyzed by restriction enzyme digestion and gel electrophoresis (Figure 1A, lanes 3–6). *Dde*I digestion resulted in predominant fragments of 60, 49 and 37 nucleotides, and *Sal*I digestion resulted in fragments of 124 and 22 nucleotides. Additional faint bands indicate the existence of less abundant species which have adopted alternative translational positions. These data show that the majority of nucleosomes were in a preferred position on the 209 bp substrate with the RSS near the dyad center of the nucleosome core, leaving ~30 bp of free DNA on either end of the substrate (Figure 1B). In other experiments, similarly labeled templates were assembled into mononucleosomes either by this technique or a very similar one using purified chicken octamers and carrier DNA (Côté *et al*., 1995).

To determine the rotational phasing of the DNA with respect to the nucleosomal surface, the RSS-12 substrate was labeled at one end and assembled into nucleosomes. Hydroxyl radical footprinting yielded multiple minor groove nicks which were resolved by denaturing polyacrylamide gel electrophoresis (Figure 2A). Whereas there is no base preference for hydroxyl radical attack on free substrate (lanes labeled F), the nucleosomal substrate exhibits the ~10.5 bp periodicity expected for the DNA helix wrapped around an octamer (lanes labeled N). Recent studies have revealed the nucleotide contacts between a complex of RAG1 and RAG2 and RSS DNA (Swanson and Desiderio, 1998). The VκL8 RSS-12 substrate (Figure 2A, lanes 1–3, and shown schematically in B) was rotationally phased so that the face of the helix containing the majority of recombinase contacts is exposed to solvent, facing away from the nucleosomal surface.

Assembled substrates contained an excess of donor chicken erythrocyte chromatin (primarily di- and trinucleosomes). In the presence of this higher molecular weight, unlabeled DNA which might compete for RAG1 and RAG2 binding, *in vitro* RSS cleavage was severely inhibited (data not shown). In addition, assembly reactions typically left ~15% free radiolabeled substrate (Figure 3, Starting Material) whose RAG-mediated cleavage product could not be discriminated from that of the nucleosomal substrate. Thus, following octamer transfer assembly, mononucleosomes were purified further on a 5–25% sucrose gradient (Figure 3). This separated nucleosomal from free substrate (compare sucrose gradient fractions 10 and 15) and eliminated unlabeled, higher molecular weight chicken DNA (data not shown). The presence of two nucleosomal bands in sucrose gradient fraction 8 most likely results from two translational positions (Meersseman *et al*., 1992); however, the most abundant species places the RSS at the dyad center (see Figure 1). After sucrose gradient purification, aliquots of fractions containing only trace amounts of free substrate (indicated by asterisks) were tested in an *in vitro* RAG-mediated RSS cleavage assay (McBlane *et al*., 1995; van Gent *et al*., 1995).

A nucleosomal RSS-12 is inaccessible to the V(D)J recombinase

In order to test whether nucleosome assembly interferes with RSS cleavage by RAG1 and RAG2, we first had to make certain that the nucleosome preparation was not generally inhibitory. We devised a strategy for assessing whether RAG1 and RAG2 retained their RSS cleavage activity when exposed to sucrose gradient fractions containing nucleosomal substrate (Figure 4A). By mixing approximately equal amounts of free and nucleosomal substrates labeled at opposite ends, cleavage products from the two could be discriminated based on their length. Whereas recombinase activity could be detected on the free substrate in the presence of purified mononucleo-

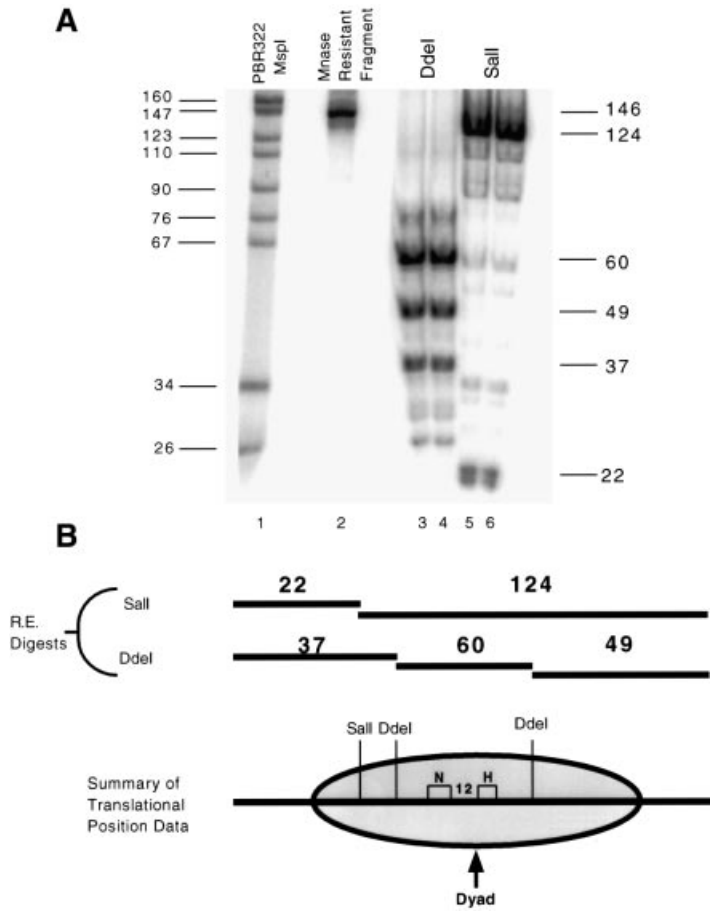

Fig. 1. Translational positioning of a nucleosomal RSS substrate. (**A**) A uniformly labeled 209 bp substrate containing the RSS-12 from VκL8 was assembled into a nucleosome by octamer transfer with chicken erythrocyte chromatin. Limited micrococcal nuclease digestion followed by deproteinization and polyacrylamide gel electrophoresis yielded a 146 bp protected fragment (lane 2), which subsequently was eluted from the gel and digested with either *Dde*I (lanes 3 and 4) or *Sal*I (lanes 5 and 6). (**B**) Summary of the most abundant fragments resulting from restriction enzyme digestion of the 146 bp protected fragment. The preferred translational position places the heptamer (H)–12 bp spacer–nonamer (N) at the dyad of the nucleosome.

somes, there was no evidence for recombinase activity on the nucleosomal substrate (Figure 4B, lanes 3, 6 and 8). Nucleosome assembly had completely inhibited RSS cleavage by RAG1 and RAG2.

Addition of HMG-1 does not alter the inaccessibility of a nucleosomal substrate

High mobility group (HMG) proteins are ubiquitous, non-histone components of chromatin, and some of these proteins have the ability to bind to nucleosomes assembled *in vitro* (Bustin and Reeves, 1996). Interestingly, two recent reports demonstrate that HMG proteins can improve the efficiency of *in vitro* cleavage by RAG1 and RAG2, especially on RSS-23 substrates (Sawchuk *et al*., 1997; van Gent *et al*., 1997). Therefore, we tested the ability of full-length, recombinant human HMG-1 (HMG-1) to rescue inaccessible nucleosomal RSS substrates.

The addition of HMG-1 improved the efficiency of cleavage by RAGs on a free substrate which contains both RSS-12 and RSS-23 sequences, especially at the RSS-23 (data not shown). Furthermore, HMG-1 greatly improved the cleavage of free single RSS-23 substrates (see below). In contrast, HMG-1 did not stimulate cutting of the nucleosomal VκL8 substrate (Figure 4C, compare lanes 5 and 6 with lanes 7 and 8).

Nucleosomal RSS-12 substrates are inaccessible to recombinase cleavage regardless of rotational phase or translational position

The first substrate tested (Figures 1–4) contained an RSS-12 from the murine VκL8 gene as well as partial sequence from the pJH200 plasmid (Hesse *et al*., 1987). It was possible that rotational phasing, specific to this particular RSS-12 fragment, might interfere with cleavage. Since rotational phasing is influenced by intrinsic DNA curvature (Simpson, 1991), two additional, distinct RSS-12 substrates were assembled into nucleosomes and tested for recombinase cleavage. These RSSs were amplified by PCR from the Vκ21c and Vκ24 genes (Figure 5). The hydroxyl radical footprints of these substrates are shown in Figure 2A, and their rotational phases are shown schematically in Figure 2B. In contrast to VκL8 (Figure 2A, lanes 1–3), the Vκ24 substrate (lanes 7–9) adopted a preferred position which oriented the majority of key RAG-1 contacts towards the nucleosomal surface, although the scissile bond at the heptamer-coding juction is solvent-exposed. The Vκ21c substrate (Figure 2A, lanes 4–6) adopted an intermediate rotational phase.

Both the nucleosomal Vκ21c and Vκ24 substrates were inaccessible to cleavage by RAG-1 and RAG-2 *in vitro*, and this inhibition could not be relieved by the addition

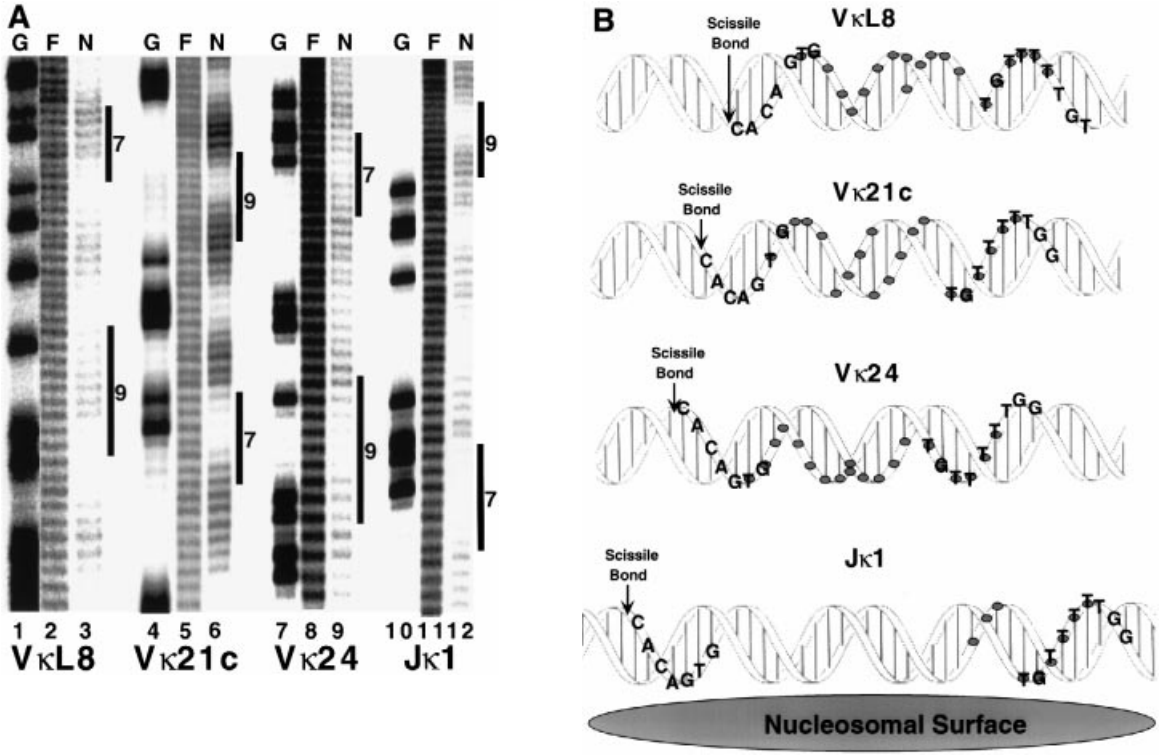

Fig. 2. Rotational phases of nucleosomal RSS substrates. After octamer transfer assembly, an end-labeled RSS substrate was subjected to hydroxyl radical footprinting. (**A**) Free (F) and nucleosomal (N) substrate were exposed to HO·, and nicked products were resolved on an 8% denaturing polyacrylamide gel alongside a DNA sequence G track (G) of the same fragment. Heptamer (7) and nonamer (9) sequences are indicated by solid bars. The footprint shown for Jκ1 (lanes 10–12) resulted from the 187 bp substrate, and identical results were obtained for the other Jκ1 substrates used in this study. (**B**) Schematic representation of rotational phases for RSS-12 and RSS-23 substrates. Heptamer and nonamer sequences are indicated in their position along the DNA helix as it is wrapped around a nucleosome. Filled ovals indicate backbone residues in free DNA contacted by RAG1 in the presence of RAG2 as shown by ethylation interference (Swanson and Desiderio, 1998).

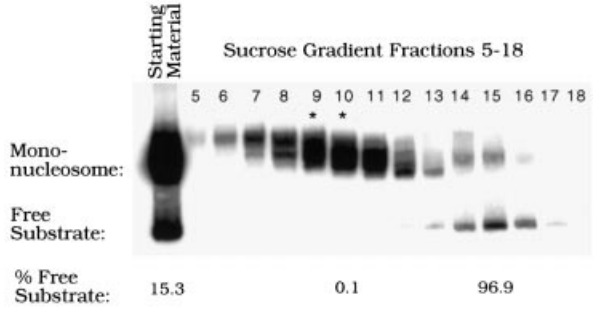

Fig. 3. Sucrose gradient centrifugation of a nucleosomal RSS substrate. Substrates assembled by octamer transfer were purified by centrifugation through a 5–25% sucrose gradient to separate mononucleosomes from excess chicken chromatin and free substrate. Aliquots of the starting material and fractions 5–18 collected after centrifugation were analyzed on a 4% native polyacrylamide gel to distinguish nucleosomal and free substrate. Fractions from left to right were collected from regions of the gradient containing higher to lower density sucrose. The asterisks indicate the sucrose gradient fractions (9 and 10) that were tested subsequently in an *in vitro* recombinase assay (see Figure 4).

of HMG-1 (Figure 5A and B). While free substrate was cleaved readily *in vitro* (Figure 5A and B, lanes 1–4), assembled substrate was not (Figure 5A and B, lanes 7 and 8). As was the case in the experiments shown in Figure 4, the nucleosomal preparation was not generally inhibitory to the recombinase (Figure 5A and B, lanes 5 and 6). Thus, regardless of the rotational phasing of the assembled nucleosome, RSS cleavage is inhibited by this basic unit of chromatin structure.

The influence of translational positioning on accessibility was tested by shifting the VκL8 RSS-12 away from the dyad axis of the mononucleosome (Figure 5C). This translational shift did not alter the rotational phase from that shown in Figure 2 (confirmed by hydroxyl radical footprinting, data not shown). Free substrate was cleaved readily (Figure 5C, lanes 1–4), and the nucleosomal preparation was not generally inhibitory (lanes 5 and 6). In contrast, the RSS-12 positioned away from the dyad was still inaccessible to the recombinase (lane 7) and this inhibition was not relieved by the addition of HMG-1 to the reaction (lane 8).

Nucleosomal RSS-23 substrates are inaccessible, regardless of translational position

To test the ability of the recombinase to cut a nucleosomal RSS-23, we PCR amplified the Jκ1 RSS and assembled it into a mononucleosome as described above. Two translational positions were achieved by using 151 bp fragments with the RSS-23 located centrally (Figure 6A) or with the nonamer oriented towards one end (Figure 6B). A third, 187 bp substrate (Figure 6C) adopted a preferred translational position away from the dyad, with the heptamer oriented closest to the free end (mapped as in Figure 1; data not shown). We then determined by hydroxyl radical footprinting that both the scissile bond at the heptamer-coding junction and a number of key recombinase contacts in the nonamer were exposed in the minor groove away from the nucleosomal surface (187 bp Jκ1 substrate shown

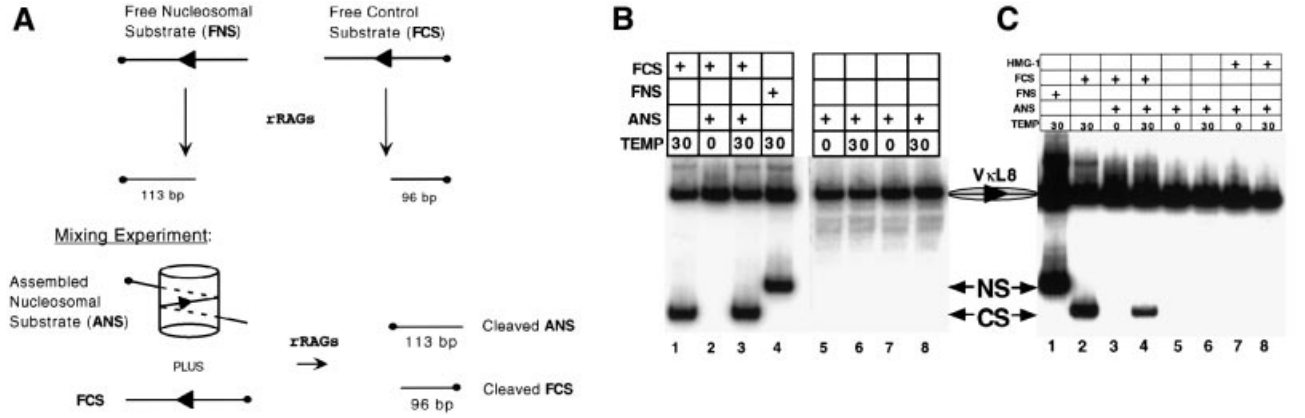

Fig. 4. Nucleosomal RSS substrates are inaccessible in an *in vitro* V(D)J recombinase assay. (**A**) Strategy for discriminating between cleavage products from free and nucleosomal substrates. The labeled end of the substrate is indicated by a filled circle, and the RSS is indicated by a black triangle. In this and subsequent figures, the substrate which is assembled into a nucleosome is labeled either FNS (free nucleosomal substrate) or ANS (assembled nucleosomal substrate), and the free control substrate used in mixing experiments is labeled FCS. (**B**) DNA was purified from *in vitro* recombinase reactions (using Mn^{2+}) and cleavage products were resolved on a 6% polyacrylamide gel: control cleavage of an RSS-12 substrate (VκL8) end-labeled to yield either a 96 (CS) or 113 bp (NS) product, respectively (lanes 1 and 4); mixing experiment with approximately equal amounts of FCS and ANS incubated at 0 or 30°C (lanes 2 and 3); ANS alone incubated at 0 or 30°C—sucrose gradient fractions 9 (lanes 5 and 6) and 10 (lanes 7 and 8). (**C**) HMG-1 does not stimulate cleavage of a nucleosomal RSS. A 6% polyacrylamide gel analysis of cleavage products from the VκL8 substrate: FNS (lane 1); FCS (lane 2); mixing experiment of FCS plus ANS (lanes 3 and 4); ANS alone (lanes 5 and 6); ANS plus 2 μg of HMG-1 (lanes 7 and 8). The highest sensitivity (using ImageQuant) could not detect a cleavage product from the nucleosomal substrate (data not shown). His-tagged, full-length, recombinant human HMG-1 protein (HMG-1) was produced in *Escherichia coli* and purified by nickel affinity chromatography.

in Figure 2A and B, and 151 bp Jκ1 substrates, data not shown).

HMG-1 stimulated double-strand DNA cleavage of the 151 bp RSS-23 as free DNA (Figure 6A and B, lanes 1–4), and the recombinase was not inhibited in the presence of a nucleosomal preparation (Figure 6A and B, lanes 5 and 6). No cutting was observed on nucleosomal RSS-23 substrates, however, regardless of translational position (Figure 6A and B, lane 7), and HMG-1 could not overcome this inhibition (Figure 6A and B, lane 8).

Previous studies have shown that in reactions using Mn^{2+} as the divalent cation, RAG1 and RAG2 can generate a double-strand break in a substrate containing a single RSS. When Mg^{2+} is used, however, substrates containing only one RSS are nicked at the signal-coding junction, but these nicks do not go on to form double-strand breaks (van Gent *et al*., 1996). Efficient nicking of the 187 bp RSS-23 substrate as a free DNA molecule was achieved in the presence of Mg^{2+} and with addition of HMG-1 (Figure 6C, lane 2). We conclude that the cleaved product is a nick, because a double-strand break introduced by the recombinase would result in a hairpin end which would run at twice the length of a nicked single strand molecule on a denaturing gel. We then tested the nucleosomal 187 bp substrate, which adopted a third translational position, for nicking *in vitro* (Figure 6C, lanes 4 and 5). As with the first two translational positions (see above), assembly of the Jκ1 RSS into a nucleosome completely inhibited recombinase accessibility, even as assayed by the formation of DNA nicks in the presence of Mg^{2+} and HMG-1.

12/23 pairwise cleavage conditions do not alter nucleosomal inaccessibility

RSS cleavage *in vivo* is thought to occur in a pairwise fashion using synapsed RSS-12 and RSS-23 substrates. This situation can be mimicked *in vitro* by using DNA substrates containing both RSS-12 and RSS-23 sequences (Eastman *et al*., 1996). Such substrates, however, are too long to assemble efficiently into nucleosomal DNA under our conditions. Since it remained possible that double-strand cleavage under the conditions of 12/23 synapsis might alleviate nucleosomal repression, we adopted a recently reported approach to perform 12/23-coupled cleavage on an assembled mononucleosome substrate *in trans*. Hiom and Gellert (1998) showed that an unlabeled oligonucleotide containing an RSS-12 could stimulate double-strand cleavage of a labeled RSS-23 substrate *in trans*. Our strategy involved the study of a labeled 151 bp RSS-23 substrate (either as free DNA or after assembly into a mononucleosome) coupled *in trans* with an unlabeled oligonucleotide containing a wild-type RSS-12, a nonamer mutant or irrelevent DNA. Double-strand cutting of the free 151 bp RSS-23 substrate in the presence of Mg^{2+} and HMG-1 was nearly undetectable (Figure 7, lane 1), but could be stimulated up to 158-fold by addition of a wild-type RSS-12 oligonucleotide (lane 4). This robust stimulation *in trans* was nonamer dependent (Figure 7, lanes 5–7) and RSS specific (lanes 8–10) since the addition of a nonamer mutant RSS-12 oligonucleotide or an irrelevant oligonucleotide showed little or no effect. Similar results were obtained using a free control substrate in the presence of a nucleosomal preparation (Figure 7, lanes 11–20). In contrast, addition of a wild-type RSS-12 oligonucleotide could not stimulate recombinase accessibility to a nucleosomal RSS-23 (Figure 7, lanes 21–24). In the experiment shown in Figure 7, the labeled Jκ1 RSS-23 was positioned away from the dyad as in Figure 6B. Identical results were obtained with the same substrate positioned near the dyad axis (data not shown).

Histone acetylation does not alter the inaccessibility of a nucleosomal substrate

Acetylation at multiple lysine residues in the histone tail domains has been correlated with transcriptionally active

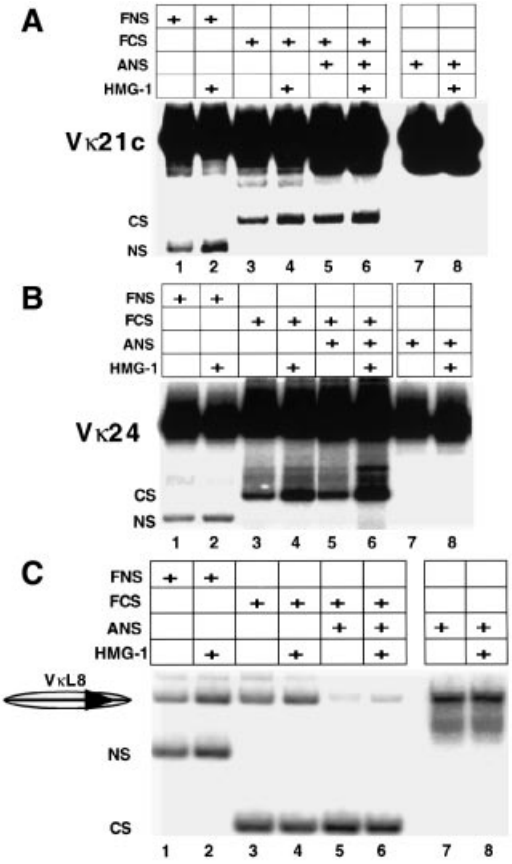

Fig. 5. Inhibition of cleavage of a nucleosomal RSS-12 regardless of rotational phase or translational position. The Vκ21c (**A**) and Vκ24 (**B**) substrates adopted two alternative rotational phases distinct from the VκL8 substrate as shown in Figure 2. The 151 bp VκL8 substrate (**C**) was designed so that the RSS-12 is translationally positioned away from the dyad axis of the nucleosome. The Vκ24 substrate was assembled into a nucleosome by chicken octamer transfer and tested for cleavage using GST–RAGs. The Vκ21c and VκL8 substrates were assembled using purified octamers and carrier DNA (see Materials and methods) and tested for cleavage using MBP–RAGs. (A–C) A 6% polyacrylamide gel analysis of cleavage products (using Mn^{2+}): FNS (lane 1); FCS (lane 3); mixing experiment of FCS plus ANS (lane 5); ANS alone (lane 7). Cleavage products from parallel reactions containing HMG-1 (2 μg for Vκ24 and 200 ng for Vκ21c and VκL8) are shown in lanes 2, 4, 6 and 8.

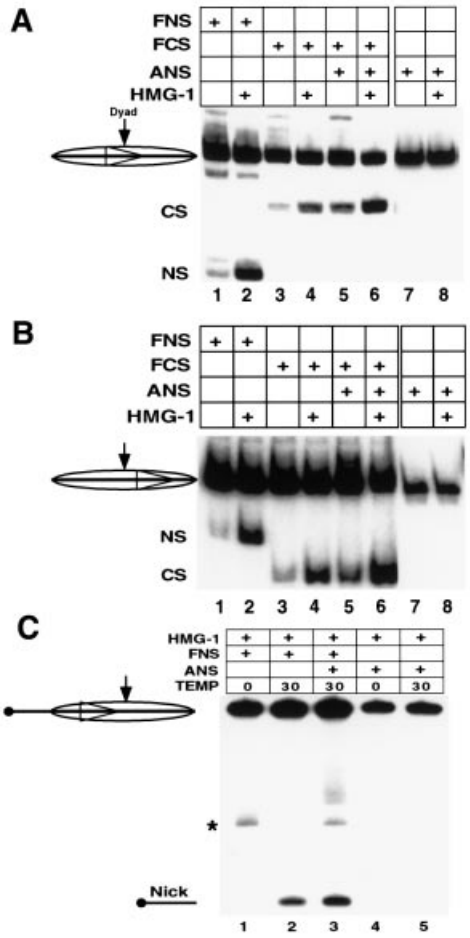

Fig. 6. Cleavage of a nucleosomal RSS-23 substrate is inhibited, regardless of translational position. The 151 bp Jκ1 substrates (assembled with purified chicken octamers and carrier DNA) were designed to position the RSS-23 over the nucleosomal dyad (A) or away from the dyad with the nonamer closest to the free end (B). A 187 bp Jκ1 substrate (C) adopted a third translational position after assembly by octamer transfer with the RSS-23 positioned away from the dyad and the heptamer closest to the free end (data not shown). (**A** and **B**) A 6% polyacrylamide gel analysis of the cleavage products (using MBP–RAGs in Mn^{2+}): FNS (lane 1); FCS (lane 3); mixing experiment of FCS plus ANS (lane 5); ANS alone (lane 7). Cleavage products from parallel reactions containing 200 ng of HMG-1 are shown in lanes 2, 4, 6 and 8. (**C**) No nicking of a nucleosomal RSS-23 (187 bp substrate). A 2 μg aliquot of HMG-1 was added to an *in vitro* cleavage assay containing GST–RAGs in the presence of Mg^{2+} and nicked products were resolved on a 6% denaturing polyacrylamide gel: FNS on ice or at 30°C (lanes 1 and 2); FNS plus ANS (lane 3); ANS alone on ice or at 30°C (lanes 4 and 5). The asterisk indicates a contaminating band which could not be a cleavage product since it is present in the 0°C control lane. Note: since nicked cleavage products cannot be detected for a substrate labeled at the signal end, the nicked products from nucleosomal and free substrate could not be discriminated in the mixing experiment (lane 3).

chromatin (for a review, see Turner and O'Neill, 1995). Furthermore, histone acetylation has been shown to increase the accessibility of various factors to nucleosomal DNA sequences (Lee *et al*., 1993; Vettese-Dadey *et al*., 1996; Lefebvre *et al*., 1998).

Based on these observations, an RSS-12 substrate was assembled into a mononucleosome by octamer transfer with hyperacetylated chromatin as the histone donor. Figure 8A shows the Triton–acid–urea gel analysis of histones from untreated HeLa cells (lane 1) or from cells treated with the histone deacetylase inhibitor sodium butyrate (lane 2). As seen most prominently for histone H4, deacetylase treatment resulted in the altered gel mobility characteristic of hyperacetylation (Zweidler, 1978). H1-depleted nucleosome cores from the butyrate-treated HeLa cells were used as the source for acetylated histones in an octamer transfer assembly of the VκL8 RSS-12 (209 bp) substrate. After purification on a sucrose gradient, aliquots of hyperacetylated nucleosomal substrate were tested for recombinase accessibility. As shown in Figure 8B (lane 5), assembly of an RSS-12 substrate into acetylated mononucleosomes did not result in any change in its inaccessibility to the recombinase.

Removal of histone tails allows partial access to the recombinase

Studies with several specific DNA-binding proteins have shown that inhibition of binding to nucleosomal targets can be partially overcome by proteolytic removal of

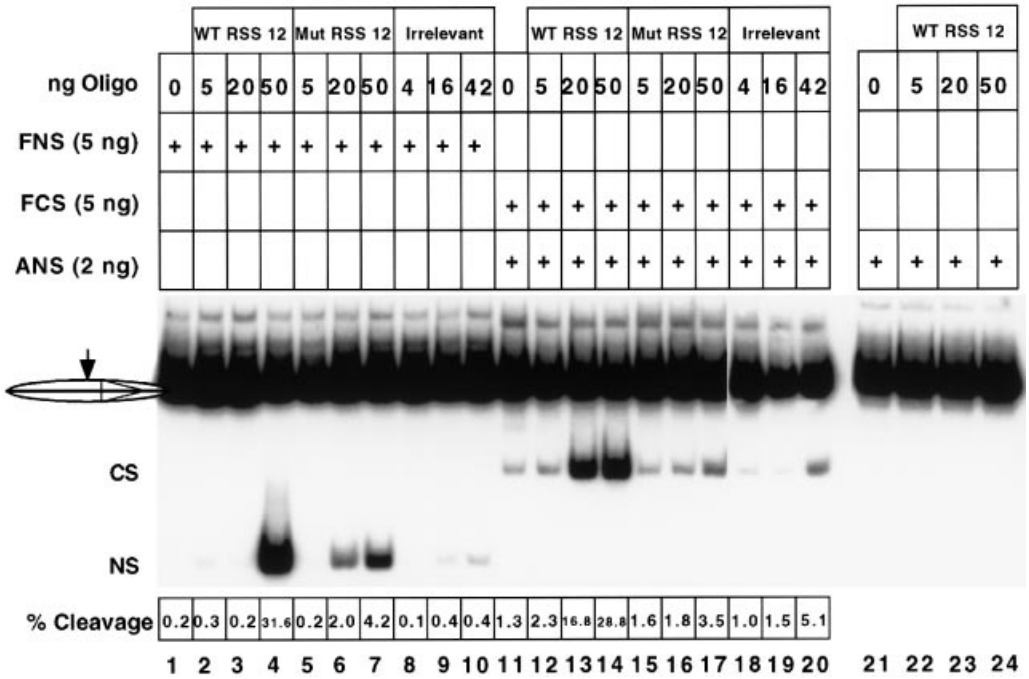

Fig. 7. Cleavage of an RSS-23 in Mg^{2+} cannot be stimulated *in trans* by an RSS-12 if the RSS-23 is assembled in a nucleosome. The Jκ1 RSS-23 substrate was assembled using purified chicken octamers and carrier DNA. Assembled and free substrate were tested for cleavage in Mg^{2+} using an excess of MBP–RAGs (co-expressed in 293T cells, ~1 μg each), 200 ng of HMG-1 and various amounts of an RSS-12 oligo (50mer), a nonamer mutant RSS-12 oligo (50mer) or an irrelevant oligo (42mer). The phosphorimage of a 6% polyacrylamide gel is shown. ANS alone lanes (21–24) contain DNA purified from three identical reactions. ANS and control reactions included the indicated amount of unlabeled oligo for *trans*-paired cleavage. The percentage V(D)J cleavage was calculated using ImageQuant software. At the highest sensitivity, a faint cleavage band could be detected in lanes 21–24. This was quantitated as <0.1% cleavage, which most likely represents cutting of the 1.5% free substrate still present in the nucleosomal preparation (as determined by native gel; data not shown).

histone tails (Lee *et al*., 1993; Vettese-Dadey *et al*., 1994; Lefebvre *et al*., 1998). In addition to removing a potential barrier to access by factors to the nucleosomal surface, removal of the tails may also cause general alterations of the nucleosome. To test whether such a modification would overcome the inaccessibility of nucleosomal RSS substrates, we subjected our assembled VκL8 RSS-12 (209 bp) substrate to limited tryptic digestion followed by addition of excess trypsin inhibitor. As shown in Figure 9A, trypsin digestion led to the accumulation of proteolyzed histone products lacking the tail domains (Bohm and Crane-Robinson, 1984). After sucrose gradient purification, the trypsin-treated nucleosomal RSS substrate remained protein bound, as assessed on a native polyacrylamide gel, though the mobility of the shifted complex does appear somewhat distinct from nucleosomal substrate prior to trypsin treatment (Figure 9B, compare lanes 1 and 2). Furthermore, this complex remained intact throughout an *in vitro* recombination reaction without any detectable accumulation of free substrate (Figure 9B, lanes 3–5). Hydroxyl radical footprinting of the trypsinized nucleosomal substrate showed that the overall rotational periodicity remained distinct from that of the free substrate; however, minor groove accessibility was generally increased (data not shown). As shown in Figure 9C, lane 5, limited proteolysis to remove the histone tails from a nucleosomal RSS-12 substrate resulted in a partial relief of inhibition of cleavage. The recombinase demonstrated an ~15-fold preference for free substrate over the tailless nucleosomal substrate (Figure 9C, compare lanes 1 and 5).

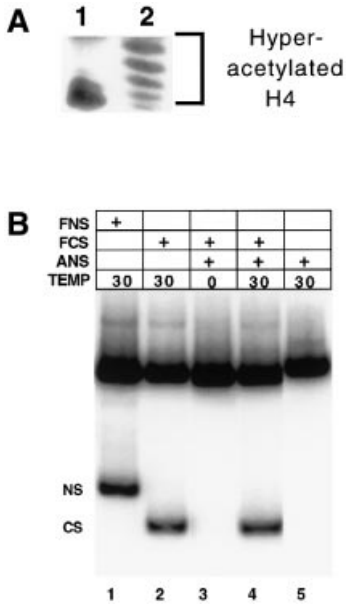

Fig. 8. Histone acetylation does not overcome the inhibition of cleavage of a nucleosomal RSS-12 substrate. (**A**) Coomassie Blue-stained Triton–acid–urea gel analysis of histones prepared from chromatin of untreated HeLa cells (lane 1) or HeLa cells grown overnight in the presence of the deacetylase inhibitor sodium butyrate (lane 2). (**B**) A 6% polyacrylamide gel analysis of *in vitro* cleavage products from VκL8 RSS-12 (209 bp substrate): FNS (lane 1); FCS (lane 2); mixing experiment of FCS plus ANS (lanes 3 and 4); ANS alone (lane 5).

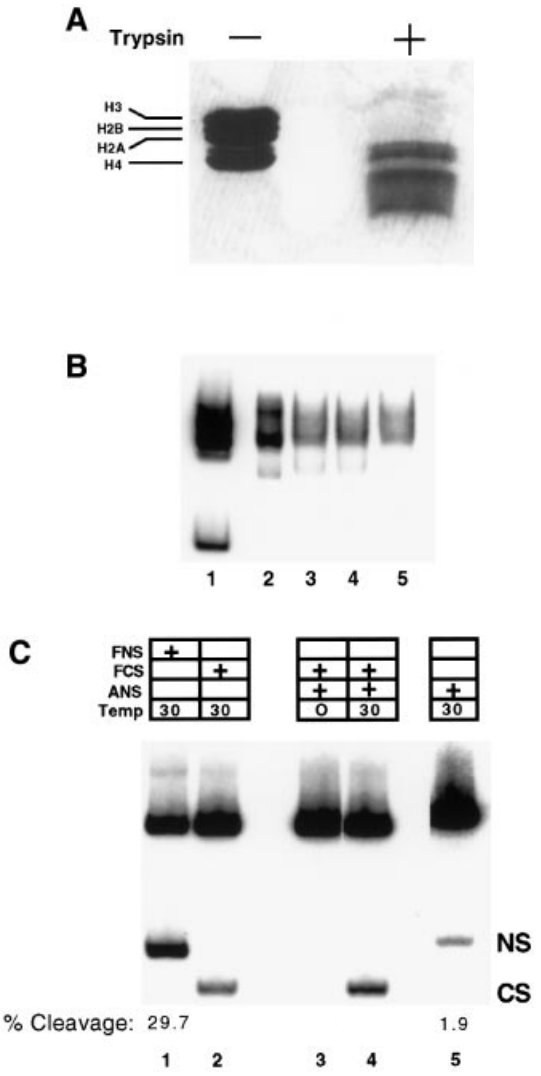

Fig. 9. Limited trypsin digestion of a nucleosomal RSS-12 substrate partially relieves the inhibition of cleavage. (**A**) Coomassie Blue-stained 18% SDS–polyacrylamide gel analysis of histones from initial octamer transfer (–) and after limited proteolysis (+). (**B**) A 4% native polyacrylamide gel anlaysis of nucleosomal complexes by gel shift: lane 1, starting material after octamer transfer; lane 2, protein–DNA complex after trypsin treatment and sucrose gradient purification; lanes 3–5, same material as in lane 2 after exposure to the following *in vitro* recombinase reaction conditions: lane 3, Mn^{2+} buffer on ice; lane 4, Mn^{2+} buffer at 30°C; lane 5, Mn^{2+} buffer plus RAGs at 30°C. (**C**) A 6% polyacrylamide gel analysis of cleavage products from the VκL8 RSS-12 (209 bp substrate): FNS (lane 1); FCS (lane 2); mixing experiment of FCS plus ANS (lanes 3 and 4); ANS alone (lane 5). Note: in order to detect the cleavage product from the trypsinized ANS, DNA purified from three identical reactions was pooled and loaded in lane 5, and the image generated by ImageQuant software was set at maximum sensitivity. In contrast, DNA purified from only a single mixing experiment was loaded in lane 4, the image was set at intermediate sensitivity, and the FCS had a higher specific activity than the ANS. These technical constraints may explain why the cleaved ANS detected in lane 5 is not detected in lane 4.

Discussion

The experiments described here lead us to suggest that accessibility of the V(D)J recombinase to potential target sequences in chromatin might be limited by assembly of RSSs into a nucleosomal structure. Each mononucleosome length DNA fragment we analyzed contained a single RSS-12 or RSS-23 efficiently assembled around a histone octamer in a preferred translational and rotational position. We found that recombinase activity, assessed either by site-specific DNA nicking or double-strand DNA cleavage, was undetectable on the nucleosomal substrate, while the same target DNA, free of protein, was efficiently nicked or cleaved. This inhibition was independent of translational position or rotational phase and could not be overcome either by addition of the DNA-bending protein HMG-1 or by histone hyperacetylation. Similarly, performing V(D)J cleavage under 12/23 pairwise conditions could not overcome the inhibition of a nucleosomal RSS. Only proteolytic removal of histone tails made the nucleosomal substrate partially accessible to RAG-mediated cleavage.

A nucleosomal RSS is inaccessible, regardless of translational position or rotational phase

One explanation for the inhibition of cleavage at a nucleosomal RSS is that residues essential for recombinase recognition might be inaccessible due to their apposition to the nucleosomal surface. As shown in Figure 2, wrapping of DNA around a histone core results in a helical repeat pattern of accessibility and inaccessibility to hydroxyl radical footprinting (Hayes *et al.*, 1990). Recent studies have elucidated the key base and phosphate backbone contacts made by a complex of RAG1 and RAG2 on a free RSS (Swanson and Desiderio, 1998). The majority of these contacts were shown to be on one face of the DNA helix. This led the authors to predict that backbone residues on the opposite side of the helix would be available for interactions with chromatin components and that nucleosome and RAG binding might not be mutually exclusive. This prediction would have been strongest for two of our nucleosomal substrates (VκL8 RSS-12 and Jκ1 RSS-23) in which the residues most exposed to hydroxyl radical attack are almost identical to the residues contacted by purified RAG1 and RAG2 on free DNA as shown by ethylation interference (Swanson and Desiderio, 1998). The fact that these nucleosomal substrates could not be cleaved implies that the recombinase must have access to residues on all faces of the DNA helix. Consistent with this conclusion is the finding that assembly of nucleosomal RSS substrates in alternative rotational positions, with different faces of the RSS potentially accessible to the recombinase, did not overcome the inhibition of cleavage (Figure 5).

Assessing the role of histone tails in the inhibition of recombinase activity on a nucleosomal RSS

An alternative explanation for the inhibition of recombinase accessibility to nucleosomal RSS substrates relies not on the apposition of contacts to the nucleosome surface, but rather on interference by N-terminal histone tails. The recently reported high resolution nucleosome structure (Luger *et al*., 1997) shows that histone tails make multiple contacts with DNA in the minor groove as it wraps around the nucleosome core every 20 bp and that the tails make additional contacts with neighboring nucleosomes. These findings suggest potential roles for histone tails either in stabilizing higher order chromatin structure or in maintaining the structure of a core nucleosome. It was possible that our nucleosomal RSS substrates were inactive because histone tails interfered with essential RAG–RSS contacts.

Many reports have correlated chromatin activity with

the acetylation of multiple lysine residues in histone tails. Hyperacetylated histones are enriched at active loci, whereas hypoacetylation has been strongly correlated with inactive genes (for review see Turner and O'Neill, 1995). In fact, a growing number of transcriptional co-activators have been reported to contain histone acetylase activity, while certain repressors have deacetylase activity (for review see Kadonaga, 1998). Acetylation is thought to mask the positive charge of histone tails, perhaps weakening their association with nucleosomal DNA (Bauer *et al*., 1994; Mutskov *et al*., 1998). Removal of N-termini as well as histone hyperacetylation have been shown to increase binding of transcription factors to nucleosomes (Lee *et al*., 1993; Vettese-Dadey *et al*., 1994, 1996; Lefebvre *et al*., 1998). Based on these observations, we asked whether assembly of nucleosomal RSS substrates with hyperacetylated histones might allow access by the recombinase. Our data suggest that acetylation alone does not relieve inhibition of cleavage of a nucleosomal substrate (Figure 8).

The only modification which resulted in detectable cleavage of a nucleosomal RSS substrate was limited proteolysis to remove the histone tails entirely (Figure 9), although cleavage of the trypsin-treated nucleosomal substrate was still significantly inhibited relative to free substrate. Hydroxyl radical footprinting of the nucleosomal substrate after exposure to limited trypsin digestion indicated a general increase in exposed residues while overall rotational phasing was maintained (data not shown). This is consistent with a less stable nucleosomal structure which might allow greater access to binding (Lilley and Tatchell, 1977; Whitlock and Simpson, 1977). These results do not rule out a role for acetylation in improving accessibility by disfavoring higher order, more compact chromatin (Felsenfeld, 1996). Additionally, the fact that removal of the tails could allow some degree of access by the recombinase may implicate a role for covalent modifications of histone tails other than acetylation (e.g. phosphorylation or ubiquitination, reviewed in Davie, 1998).

A nucleosomal RSS is inaccessible under 12/23 pairwise cleavage conditions

Functional V(D)J rearrangement of antigen receptor loci depends on coupled cleavage of a pair of RSSs and subsequent joining of coding and signal ends. Enforcement of the so-called 12/23 rule has been recapitulated *in vitro* by using Mg^{2+} rather than Mn^{2+} as the divalent cation. A putative synaptic complex containing RAG1, RAG2, HMG-1 and both an RSS-12 and RSS-23 has been purified with substrates containing both RSSs *in cis* (Agrawal and Schatz, 1997) or with separate RSS substrates *in trans* (Hiom and Gellert, 1998). Such a synaptic complex may result in significant conformational changes in the recombinase which not only enforce the 12/23 rule, but which also cooperate so that cleavage is altered qualitatively and quantitatively from that catalyzed by RAG1 and RAG2 on a single RSS. To address the possibility that cooperativity in binding a pair of RSSs might overcome nucleosomal inaccessibility, we performed a coupled cleavage experiment with free and/or nucleosomal RSS-23 and free RSS-12 substrates on separate molecules (*in trans*; Figure 7). This strategy allowed us to test the ability of an unlabeled RSS to stimulate cleavage of labeled free and nucleosomal substrates in the same experiment. While addition of an RSS-12 showed a striking stimulation of cleavage of a free RSS-23, no such stimulation was observed on the nucleosomal RSS-23 in the same reaction (Figure 7, lanes 11–20). This is compelling evidence that a functional synaptic complex cannot form if one of the potential RSS partners is wrapped up in a nucleosome.

Nucleosomal RSS inaccessibility cannot be reversed by HMG-1

In agreement with previously published results, we found that purified recombinant HMG-1 can stimulate cutting of a single RSS using either Mn^{2+} or Mg^{2+} as the divalent cation or cutting of an RSS-23 paired with an RSS-12 using Mg^{2+} as the divalent cation. However, in the same reaction in which HMG-1 is clearly able to affect V(D)J cleavage of a free substrate, it has no influence on the inaccessibility of a nucleosomal RSS (Figures 4–7). It has been suggested that one role of a DNA-bending protein may be to bring the heptamer and nonamer sequences in an RSS-23 closer together so that the distance bridged by the bound RAG proteins would be reduced to resemble an RSS-12 (van Gent *et al*., 1997). This model requires that the RSS substrate be able to make multiple, perhaps simultaneous, contacts with RAGs and accessory factors. Our findings suggest that such contacts are masked by positioning the RSS on the surface of a nucleosome and that any role played by HMG-1 in the stimulation of V(D)J rearrangement would be inhibited by incorporation of RSSs into stable nucleosomes.

While this manuscript was under review, a similar study was published addressing the question of accessibility of a nucleosomal RSS (Kwon *et al*., 1998). In contrast to our findings, these authors concluded that inaccessibility of a nucleosomal RSS could be overcome by addition of HMG-1 and that stimulation by HMG-1 was dependent on rotational phase for an RSS-12 or on translational position for an RSS-23. Interestingly, however, the effects of rotational phase disappeared when the RSS-12 was shifted away from the dyad axis of the nucleosome. In addition, it is possible that RSSs positioned very near the end of a mononucleosome may be uniquely susceptible to cleavage. Such 'end effects' might not be relevant when considering nucleosome structure *in vivo*. Whereas we utilized similar conditions for nucleosomal assembly and V(D)J cleavage, our strategy incorporated the additional step of sucrose gradient purification to remove all but a trace amount of free substrate prior to reaction with purified RAG1 and RAG2 (Figure 3). In the experiments reported by Kwon and colleagues, a crude assembly reaction containing both nucleosomal and free DNA was used as substrate and the nucleosome-associated DNA was purified subsequently by native gel electrophoresis. One possible explanation for the difference between our results and those of Kwon *et al*. is that cleaved free substrate may associate with HMG-1 and contaminate the native gel band used by these workers to distinguish free and nucleosomal DNA. Consistent with this interpretation, we have found that purified HMG-1 can shift free DNA to a mobility almost overlapping with that of the assembled mononucleosome (data not shown). In contrast to the

model based on our data, which predicts that substrate inaccessibility could be achieved by positioning an RSS anywhere on a nucleosome, the results of Kwon *et al*. would suggest that accessibility is governed by more subtle shifts in rotational phase or translational position of a nucleosomal RSS. Further experimentation will be required to reconcile these two sets of data fully.

Establishing and overcoming RSS inaccessibility

Only gross modification of nucleosome structure by proteolytic cleavage could overcome the inhibition of recombinase activity observed in our *in vitro* experiments. Therefore, we conclude that stable positioning of an RSS on the surface of a nucleosome makes it inaccessible to cleavage by the V(D)J recombinase. Since we used recombinant RAG1 and RAG2 core domains, we cannot exclude the possibility that the non-core domains of RAG1 and RAG2 might prove particularly important for the cleavage of nucleosomal substrates. It has been shown, however, that the core domain of RAG1 is sufficient for recognition and cleavage of RSSs in purified nuclei (Stanhope-Baker *et al*., 1996) and the core domain of RAG2 is sufficient to rescue chromosomal D-to-J recombination in a RAG2-null pro-B cell line (Kirch *et al*., 1998). Our experiments do not formally prove that the recombinase is unable to recognize a nucleosomal RSS. However, even if binding is not inhibited, stimulation of enzymatic activity could only be achieved in our experiments by proteolytic disruption of the nucleosome.

One can envisage several ways in which regulated RSS inaccessibility could contribute to the developmental regulation of V(D)J recombinase activity. Based on this study, we propose a model in which the primary barrier to accessibility is the nucleosome. In the most extreme case, actively rearranging loci might be devoid of nucleosomes while non-rearranging loci might exist in an array of stable associated nucleosomes. Examples exist of nucleosome-free regions that play a role in the developmental regulation of gene expression (for reviews, see Elgin, 1990; Boyes and Felsenfeld, 1996). Alternatively, nucleosomes need not be removed, but rather positioned (phased) so that key sites for factor binding are located in linker regions (Schild *et al*., 1993; for a review, see Lu *et al*., 1994). In these cases, repression is probably mediated by linker histones, and transient removal of linker histones could increase accessibility. As a test of this model, we are currently engaged in experiments to evaluate the presence and map the distribution of nucleosomes at silent and actively rearranging antigen receptor loci *in vivo*.

Regardless of whether the inhibition of RSS cleavage is overcome by the removal or the specific positioning of nucleosomes, establishment of RSS accessibility might depend on an energy-dependent chromatin remodeling activity. Various multiprotein complexes have been shown to possess such activity *in vitro* or *in vivo* (e.g. NURF, Swi/Snf and others; for review see Varga-Weisz and Becker, 1998). These activities may promote the sliding of nucleosomes along the DNA or the disruption of core nucleosome structure. Similarly, histone acetylases could increase nucleosome mobility by disrupting interactions between nucleosomes within higher levels of chromatin organization (Luger and Richmond, 1998).

Transcriptional enhancers and other *cis*-acting transcriptional regulatory sequences have been shown to play a role in targeting the V(D)J recombinase. Targeted disruption of these sequences results in diminished recombinase activity at the targeted locus (for review see Sleckman *et al*., 1996). Factors which bind these sequences may be responsible for recruiting histone acetylases or chromatin remodeling complexes to induce local changes in chromatin structure which are permissive for the V(D)J recombinase. We will address these possibilities by studying longer DNA substrates with RSSs positioned in nucleosomal arrays where accessibility might be enhanced by chromatin-modifying factors.

Materials and methods

RSS substrates

Cleavage substrates were generated by PCR under standard conditions using either pJH200 (Hesse *et al*., 1987), pVJG (Lewis *et al*., 1984), Vκ24pBSK (Malipiero *et al*., 1987) or pRBJcK (Lewis *et al*., 1982) as plasmid templates for amplification of the murine VκL8, Vκ21c, Vκ24 or Jκ1 RSSs, respectively. The substrates were designed to accommodate only one nucleosome. The length of one of the VκL8 substrates and of the Vκ21c and Vκ24 substrates was 209 bp, and 187 bp for one of the Jκ1 substrates. The three remaining substrates (VκL8 Shift, Jκ1 Dyad and Jκ1 Shift) were 151 bp in length. For uniform incorporation of radiolabel, [α-^{32}P]dCTP was included in the PCR; for end-labeling, one of each pair of primers was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Note that the Jκ1 substrates contain only genomic sequence whereas all RSS-12 substrates include flanking plasmid sequences.

The following primers were used: VκL8 (209), 5′-AACAATTTCACACAGGAAACAGC and 5′-AAGTTGCTGCGATTCTCACCAAT; VκL8 Shift, 5′-CGACGGATCCGCGCTAAGGAG and 5′-AAGTTGCTGCGATTCTCACCAAT; Vκ21c, 5′-CAGTGGGTCTAGGACAGACTTCACCCTC and 5′-CATGGCGACCACACCCGTCCTGTGGAT; Vκ24, 5′-TCTCAGACCGGTTTAGTGGCAGTGGGTCAGGAAC and 5′-ATTGGGTACCGGGCCCCCCCTCGAGGTCG; Jκ1(187), 5′-GCCCAAGCGCTTCCACGCATGCTTGGAG and 5′-ACGGAAGAAAGAGACTTTGG; Jκ1 Dyad, 5′-GGAGAGGGGGTTAAGCTTTCG and 5′-GACTTTGGATTCTACTTACGTTTG; Jκ1Shift, 5′-GCTCTGTTCCTCTTCAGTGAG and 5′-GAAGCCACAGACATAGACAAC.

Unlabeled oligos were used for *trans*-paired cleavage (see below). The 50mer wild-type RSS-12 oligo was made by annealing DAR39 to DAR40 (Ramsden *et al*., 1996), and the 50mer mutant RSS-12 was made by annealing the same oligos containing a nonamer substitution of 5′-ACAAAAACC with 5′-AGTCTCTGT (Ramsden *et al*., 1996). An irrelevant 42mer oligo was used as a non-specific control.

Nucleosome assembly by octamer transfer

Polyacrylamide gel-purified RSS substrates (~500 ng; ~3×10^6 c.p.m.) were incubated with ~100-fold molar excess of linker histone-depleted nucleosome particles from chicken as described previously (Wolffe and Hayes, 1993; Hayes and Lee, 1997), with the following modifications. The 200 µl octamer transfer mix was made 1.0 M in NaCl and dialyzed for 4 h against 1.0 M NaCl, followed by 4 h dialysis against 0.75 M NaCl, and then overnight dialysis against 0 mM NaCl. All dialysis solutions contained 10 mM Tris pH 7.6, 1 mM EDTA and 0.25 mM phenylmethylsulfonyl fluoride (PMSF). Alternatively, nucleosomes were assembled by salt dilution using 200 ng of end-labeled fragment, 5 µg of sheared salmon sperm DNA (0.2–2.5 kb, 5 Prime to 3 Prime, Inc.) and 5.2 µg of core histone octamers, purified from chicken erythrocytes as described (Côté *et al*., 1995). Unless indicated otherwise, nucleosomal assembly incubations and the resulting assembled RSS substrates were always kept on ice or at 4°C.

For octamer transfer with hyperacetylated histones, nucleosome particles were purified from HeLa cells grown overnight in media containing 10 mM sodium butyrate as described (Lee *et al*., 1993). To analyze the acetylation status of histones, they were resolved on a Triton–acid–urea–15% polyacrylamide gel [(Zweidler, 1978) with modifications added later by other investigators (Lee *et al*., 1993)].

To generate trypsinized nucleosomal structures, assembled RSS substrates (40 µl of an octamer transfer mix) were incubated with 8 µg/ml trypsin (Sigma) for 10 min at room temperature followed by addition of

80 μg/ml trypsin inhibitor (Sigma). Use of these concentrations of trypsin and trypsin inhibitor was determined by adding increasing amounts to separate samples of the assembled substrate. Histones were analyzed in SDS–18% polyacrylamide gels and stained with Coomassie Blue. Trypsinized nucleosomal structures were purified by sucrose gradient centrifugation (see below) before addition to an *in vitro* recombination assay.

To purify mononucleosomal RSS substrates away from unassembled free substrate and from oligonucleosomal-length chicken DNA, octamer transfer was followed by 5 ml, 5–25% sucrose gradient centrifugation according to previously published methods (Côté *et al*., 1995). Mononucleosomal fractions (containing ~15% sucrose, 10 mM HEPES pH 7.6, 1 mM EGTA, 0.1% NP-40) were used directly in subsequent reactions.

Analysis of nucleosome structure

Translational mapping of uniformly labeled nucleosomal substrate and hydroxyl radical footprinting of end-labeled nucleosomal substrate were carried out as described previously (Hayes and Lee, 1997). For analysis of protein–DNA complexes present in nucleosomal substrates, aliquots were analyzed on a native 4% polyacrylamide, 0.5× TBE gel at 150 V for 2–2.5 h (Côté *et al*., 1995).

Cleavage reactions

Core RAG1–GST or RAG1–maltose-binding protein (MBP) and core RAG2–GST or RAG2–MBP proteins were purified separately from transfected 293T cells as described previously (Spanopoulou *et al*., 1995; Li *et al*., 1997). Histidine-tagged, full-length recombinant human HMG-1 was purified from a bacterial strain harboring the plasmid pET-HMG as described previously (Ge and Roeder, 1994). Cleavage reaction conditions were essentially as described (Eastman *et al*., 1996). The total volume of the reaction was 20 μl, consisting of 2 μl each of purified RAG1 and RAG2 (~0.1 mg/ml, unless otherwise indicated), 2 μl of HMG-1 (1 or 0.1 mg/ml, as indicated), ~5–25 ng of free or nucleosomal RSS substrates, 35 mM HEPES pH 7.5, 82 mM KCl, 2 mM dithiothreitol (DTT) and 2% glycerol. All reactions were carried out in 1 mM $MnCl_2$, except for nicking experiments, which were carried out in 4 mM $MgCl_2$, and *trans*-paired cleavage, which was carried out in 25 mM MOPS pH 7.0, 30 mM K-glutamate, 4 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mg/ml bovine serum albumin (BSA), 2 mM DTT and 2% glycerol. All components were added on ice and pre-incubated on ice for 30 min, followed by incubation on ice or at 30°C for 1 h. The reactions were terminated by the addition of 80 μl of 10 mM Tris pH 8.0, 1 mM EDTA, 0.1% SDS and 20 μg of proteinase K and then digested at 50°C for 2 h. This was followed by extraction with phenol/chloroform/isoamyl alcohol, precipitation with ethanol and 20 μg of glycogen and resuspension in TE. Samples were run on 6% polyacrylamide gels alongside an end-labeled pBR322 *Msp*I digest as a molecular weight marker. In order to detect rare cleavage products, two or three identical reactions containing only nucleosomal substrate were combined and terminated after the 1 h incubation at 30°C.

All images were generated using a PhosphorImager (Molecular Dynamics) and quantitated using ImageQuant software (version 4.1). For the experiments shown in all figures other than Figure 7, cleaved nucleosomal substrate could not be detected even at the highest sensitivity. For the experiment shown in Figure 7, lanes 21–24, a faint band could be detected at the highest intensity (quantitated at $<0.1\%$ cleavage). The percentage cleavage of nucleosomal substrates is only reported if it is higher than the percentage of free substrate which remains in the nucleosomal preparation.

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