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Nicking is asynchronous and stimulated by synapsis in 12/23 rule-regulated V(D)J cleavage

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ABSTRACT

The first step in DNA cleavage at V(D)J recombination signals by RAG1 and RAG2 is creation of a nick at the heptamer/coding flank border. Under proper conditions *in vitro* the second step, hairpin formation, requires two signals with spacers of 12 and 23 bp, a restriction referred to as the 12/23 rule. Under these conditions hairpin formation occurs at the two signals at or near the same time. In contrast, we find that under the same conditions nicking occurs at isolated signals and hence is not subject to the 12/23 rule. With two signals the nicking events are not concerted and the signal with a 12 bp spacer is usually nicked first. However, the extent and rate of nicking at a given signal are diminished by mutations of the other signal. The appearance of DNA nicked at both signals is stimulated by more than an order of magnitude by the ability of the signals to synapse, indicating that synapsis accelerates nicking and often precedes it. These observations allow formulation of a more complete model of catalysis of DNA cleavage and how the 12/23 rule is enforced.

INTRODUCTION

Functional immunoglobulin and T cell receptor genes are assembled during lymphoid development by a series of DNA rearrangements collectively known as V(D)J recombination. The protein coding segments to be joined together are flanked by recombination signals that are defined by conserved heptamer and nonamer motifs (1). The heptamer and nonamer sequences are separated by a non-conserved spacer of either 12 or 23 bp and efficient recombination requires two signals with spacers of different lengths. In cells substrates containing a recombination signal with a 12 bp spacer (12 signal) and a signal with a 23 bp spacer (23 signal) undergo V(D)J recombination ~50 times more efficiently than substrates with two 12 signals or two 23 signals (2,3). This helps to restrict rearrangement to events that could be biologically useful, because the immunoglobulin and T cell receptor loci are arranged so that similar gene segments at a given locus are flanked by signals with spacers of the same length (4). For example, all of the V and J segments in the IgH locus have nearby 23 signals, while the D segments are flanked by 12 signals. The 12/23 rule prevents unproductive V–V, D–D, J–J or V–J recombination events.

The *RAG1* and *RAG2* genes are expressed together only in lymphoid tissues and are essential for the first step of V(D)J recombination, cleavage of DNA adjacent to the signals. The RAG1 and RAG2 proteins are sufficient to catalyze cleavage at V(D)J recombination signals *in vitro* (5). Cleavage is a two-step process (Fig. 1A; 5). First, a nick is introduced at the 5′-border of the heptamer, between the heptamer and the coding flank. The exposed 3′-hydroxyl then directly attacks the other strand (6), creating a hairpin on the coding side and a blunt 5′-phosphorylated signal end. These two DNA species have been detected in pre-B cell lines and in lymphoid tissues (7–12). The end result of V(D)J recombination is an often imprecise joint between the protein coding segments and a usually precise head-to-head fusion of the signals. Factors involved in double-strand break repair, such as the Ku proteins (13–15) and the catalytic subunit of the DNA-dependent protein kinase (16,17), are necessary for rejoining of the broken DNA.

The 12/23 rule is imposed at the cleavage step *in vivo* (18) and can be reproduced in crude extracts containing truncated RAG proteins in the presence of magnesium (19). Under these conditions a 12/23 substrate is cleaved ~25 times more efficiently than a 12/12 substrate and at least 50 times more efficiently than a 23/23 substrate (19), ratios which closely approximate those observed *in vivo* (2). Cleavage events at the two signals are tightly coupled temporally and efficient cleavage requires the ability of the two signals to synapse. Thus, coupled cleavage is thought to require formation of a complex involving both signals. Purified recombinant RAG proteins will display coupled cleavage activity, but their ability to discriminate between a 12/23 substrate and a 12/12 substrate is substantially attenuated compared with that observed with crude extract, down to ~4-fold (20). This suggests that the RAG proteins by themselves have the ability to recognize recombination signals and form a synaptic complex, but that other factors aid in complex formation or in the destabilization of inappropriate complexes. Interestingly, the non-specific DNA bending proteins HMG1 and HMG2 enhance cleavage by the RAG proteins at 23 signals (21,22), but together with the RAG proteins are not sufficient to reconstitute the 12/23 rule (21).

In the presence of manganese, purified RAG proteins will efficiently cleave an isolated 12 signal. This has allowed a dissection, using oligonucleotide substrates, of the structural and DNA sequence requirements for nicking and hairpin formation at

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an isolated signal (23,24). These studies have found that the requirements for hairpin formation are more strict than the requirements for nicking. Some sequences immediately flanking the heptamer, as well as some mutations at the first two positions of the heptamer, will substantially inhibit hairpin formation without impairing nicking. It has also been reported that with purified RAG proteins in the presence of magnesium, nicking occurs readily at an isolated 12 signal, although hairpin formation does not (20).

The nicking phase of the reaction has been examined *in vitro*, but not under conditions where the 12/23 rule is tightly regulated. Hence, it has remained unclear if, and to what extent, interaction between a 12/23 pair of signals facilitates nicking. We report here that nicking is substantially less tightly regulated than hairpin formation, with nicks occurring at isolated signals and asynchronously at 12/23 pairs of signals. However, if the 12 and 23 signals can synapse, accumulation of substrates nicked at both signals is accelerated >13-fold compared with a situation in which they cannot synapse. These results reveal a role for synapsis in the nicking reaction and have implications for the mechanism of early steps in V(D)J recombination and for the generation of nicks in the vertebrate genome.

MATERIALS AND METHODS

Substrates

Cleavage substrates were generated by PCR under the following conditions: 50 mM KCl, 20 mM Tris–HCl, pH 8.4, 2 mM $MgCl_2$, 50 µM each dNTP, 1 mCi/ml [α-^{32}P]dCTP, 160 ng/ml plasmid template linearized with *Xba*I and 25 U/ml *Taq* DNA polymerase (Gibco); 3 min at 95°C, 21 cycles of 45 s at 95°C, 30 s at 58°C and 75 s at 72°C, then 10 min at 72°C. In Figures 2 and 3 substrates were generated using 2 µg/ml LE1 and cit4a primers. For Figures 2 and 5–8 substrates were generated using the same concentrations of primers CD1 and cit4a.

Primers: CD1, 5′-GCTCACATGTTCTTTCCTGC-3′; LE1, 5′-GGAATTGTGAGCGGATAAC-3′; cit4a, 5′-GCAACTGACT-GAAATGCCTC-3′. Note that the sequence of cit4a published in Eastman *et al.* (19) is the (incorrect) complement.

Cleavage extracts

Whole cell extracts were prepared as described in Agrawal and Schatz (25) with minor modifications. Frozen cell pellets from heat-shocked cells (recovered for 5.5 h, then washed twice with cold PBS and frozen at –70°C) were extracted twice with 3 vol 350 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 25 mM Tris–HCl, pH 7.5, 10% (w/v) sucrose, 5 mM DTT, 0.5 mM PMSF. Extracts were spun at 30 000 *g* for 30 min at 4°C and then ammonium sulfate was added to the supernatant to 75% saturation. Protein was recovered by centrifugation for 30 min at 15 000 *g*, resuspended (doubling the volume of the protein pellet) in dialysis buffer and then dialyzed against 50 mM NaCl, 25 mM Tris–HCl, pH 7.5, 1 mM EGTA, 2 mM DTT, 20% glycerol for 16 h at 4°C. Extracts were spun at 30 000 *g* for 30 min to remove precipitated proteins and lipids. Protein concentrations varied between 12 and 16 mg/ml.

Cleavage reactions

The standard volume for a single cleavage reaction was 50 µl. Each contained, including contributions from the extract, 150 mM sodium acetate, 20 mM NaCl, 25 mM Na HEPES, pH 7.5, 10 mM Tris–HCl, pH 7.5, 10 mM magnesium acetate, 1 mM EGTA, 2.5 mM DTT, 8% glycerol and 200 000 c.p.m. ^{32}P-labeled cleavage substrate. The concentration of extract was 40% (v/v). Reactions were prepared by mixing all components on ice and were incubated on ice for a minimum of 10 min before being brought to 37°C. Reactions were stopped by addition of 300 µl 100 mM Tris, pH 8.5, 10 mM EDTA, 0.2% SDS and 35 µg proteinase K and then digested at 55°C for at least 1 h. Reactions were extracted with phenol/chloroform/isoamyl alcohol, precipitated with isopropanol and 8 µg yeast tRNA and then resuspended in TE containing 100 ng/ml RNase A.

Gels

Native 4% polyacrylamide gels were run in 1× TBE until the bromphenol blue had migrated 80% of the length of the gel. Agarose gels with 1% SeaKem/1.2% NuSieve (FMC), 1× TBE were used to isolate ‘uncut’ DNA, which was visualized by ethidium bromide staining and compared for reference against a 123 bp ladder. DNA was isolated from agarose blocks with QIAquick spin columns (Qiagen), but eluted with 200 µl 10 mM NaOH, precipitated with isopropanol and 8 µg yeast tRNA and resuspended in 95% formamide, 10 mM EDTA plus dyes. Samples were heated to 95°C and then plunged into ice before being loaded onto 4% polyacrylamide, 8 M urea, 1× TBE gels, run until the xylene cyanol had migrated 60% of the length of the gel. All gels had equal counts loaded in the sample lanes, except for that shown in Figure 3.

Quantitation

The percentages of DNA nicked at the signals were determined by scanning dried gels with a BioRad Molecular Imager and using Molecular Analyst software. A rectangle was placed surrounding the ^{32}P signal in a lane and the ‘Extract Profile’ feature was used to integrate over the long dimension of the lane. The signal was corrected for background and the areas under each peak were determined. The intensity of each band was divided by a correction factor (based on the number of cytidines in that fragment) and then the amount of unnicked top strand (which is only part of the highest ‘uncut’ band) was determined by subtracting the calculated intensity of the bottom strand. Percentage nicking is calculated by dividing the corrected c.p.m. of the fragment by the total c.p.m. of the top strand.

RESULTS

We have adapted our system for studying DNA double-strand cleavage by truncated forms of the RAG1 and RAG2 proteins in crude extracts to the study of nicking (19). As in our previous study, the prototype substrate (Fig. 1B), fr12×23 (fragment derived from p12×23) is a product of PCR and uniformly labeled with ^{32}P. It has a 12 signal and a 23 signal, with the heptamer/coding flank borders separated by 279 bp (3). We incubated this substrate, alongside other substrates having different combinations of 12 and 23 signals, with extract containing the RAG proteins. Analysis of the cleavage products on native polyacrylamide gels confirmed that, as reported previously, efficient cleavage requires a 12 and a

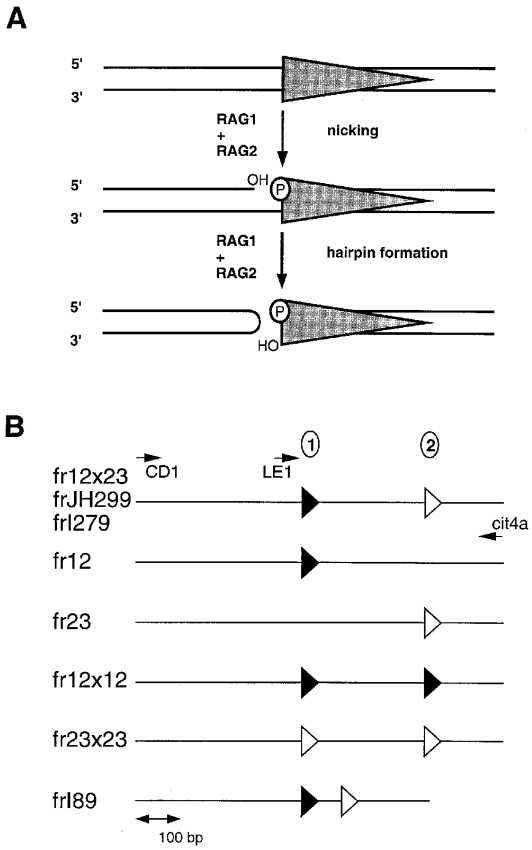

Figure 1. (**A**) Mechanism of RAG-mediated cleavage at a single V(D)J recombination signal. The introduction of a nick at the heptamer/coding flank border is followed by nucleophilic attack of the 3′-hydroxyl on the bottom strand phosphate to yield a hairpin coding end and blunt signal end. Derived from McBlane *et al.* (5). (**B**) Substrates used in cleavage reactions. Black triangles represent 12 signals and white triangles represent 23 signals. Small arrows indicate PCR primers. The fr12×23, frJH299 and frI279 substrates are essentially identical except for the sequence of the nucleotides immediately flanking the heptamers of the recombination signals. The longer substrates, made using primer CD1, were used in the experiments shown in Figures 2 and 5–8 and have 379 bp to the left of the 12 signal. The shorter substrates, made using primer LE1, were used for the experiments shown in Figures 3 and 4 and have 79 bp to the left of the 12 signal.

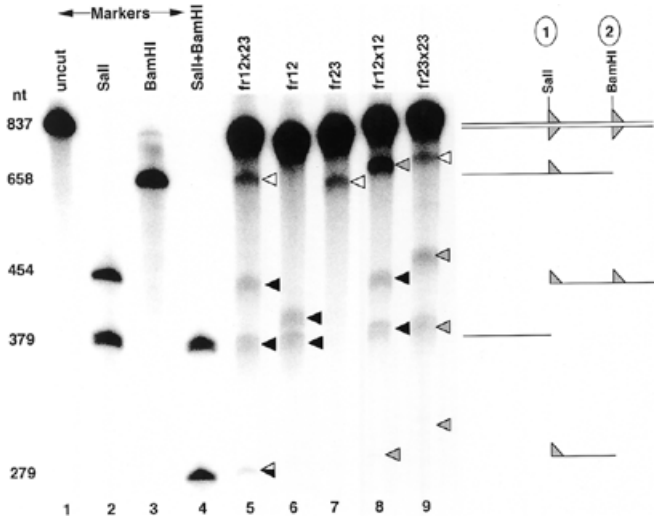

Figure 2. Nicks occur efficiently at isolated recombination signals. Cleavage reactions were performed with the indicated substrates and then analyzed for nicking as described in Materials and Methods. Markers were obtained by restriction digestion of the fr12×23 substrate (lanes 2–4; lane 1 shows the undigested substrate and the size of the markers in nucleotides is shown on the left). The structure of the fragments of DNA produced by nicking and subsequent denaturation is indicated on the right. Note that the highest and most prominent band for each substrate is actually composed of varying proportions of unnicked top strand as well as the bottom strand, which is not normally nicked by the RAG proteins. White arrowheads indicate fragments produced by nicks at signal 2 only. Black arrowheads indicate fragments resulting from nicks at signal 1 only (the 470–440 nt bands) and fragments representing both DNA nicked at signal 1 by itself and at both signals (the 379 nt bands). The black/white arrowhead indicates the central fragment resulting from nicks at both signals in fr12×23. Gray arrowheads indicate fragments resulting from nicks at (substituted) partner signals: at signal 2 in fr12×12, signal 1 in fr23×23 and the central fragment resulting from nicks at both signals in fr12×12 and fr23×23.

Table 1. Quantitation of nicking data presented in Figure 2

substrate	name	configuration		% of substrate nicked at:				
		signal 1	signal 2	1 only	2 only	1 and 2	1 total	2 total
	fr12x23	12	23	6.8%	4.5%	2.9%	9.7%	7.4%
	fr12	12	no	3.3%			3.3%	
	fr23	no	23		4.4%			4.4%
*	fr12x12	12	12*	5.3%	12.9%	1.2%	6.5%	14.1%
*	fr23x23	23*	23	9.4%	2.5%	1.8%	11.2%	4.3%

*, Substituted (non-standard) partner signal.

23 signal and, in particular, was not observed with fr12, fr23 or fr23×23. Cleavage at the 12 and 23 signals occurs synchronously (data not shown).

Nicking at isolated signals

We established a two-step nicking assay to be able to analyze nicking without interference from products that had undergone double-strand cleavage. In the first step, the products of a cleavage reaction are fractionated on a native agarose gel and the band running at the size expected for the full-length substrate is purified from the gel. This band contains substrate molecules that have been nicked at one or both signals, but not substrates that have undergone double-strand cleavage. In the second step, the purified material is analyzed for nicking by fractionation on a denaturing polyacrylamide gel.

We had previously observed that with fr12×23, coupled cleavage occurs most rapidly between 30 and 60 min after being brought to 37°C (19). Reactions were therefore stopped after 30 min at 37°C and subjected to the two step nicking assay (Fig. 2). With fr12×23 (lane 5) DNA fragments resulting from nicks at the 12 signal alone (black arrowheads), at the 23 signal alone (white arrowhead) and at both signals (black and white arrowhead) are visible, in roughly similar amounts (6.8, 4.5 and 2.9% respectively). DNA nicked at both signals is more abundant than one would expect if nicking events at the two signals were completely independent (9.7% × 7.4% = 0.7%, which is <2.9%). However, the major species are those nicked at one signal and not the other. This suggests that coupling between nicking events is not strong and that nicks at the two signals can occur in either

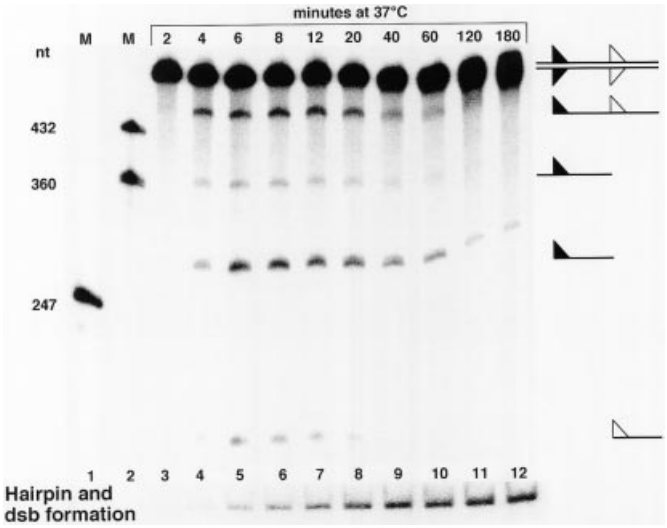

Figure 3. Nicks can occur asynchronously preceding coupled hairpin formation. Large scale cleavage reactions were performed with frJH299 and fr12×23 and aliquots were stopped at the indicated times. A native polyacrylamide gel, with equal counts loaded in each lane, was run with part of each sample to determine the percentage of doubly cleaved DNA (shown at bottom). The remaining DNA in each sample was separated on an agarose gel as described in Materials and Methods and counts proportional to the fraction of 'uncut' DNA remaining at that time point were loaded on the denaturing polyacrylamide gel. Markers were obtained by restriction digestion of frJH299 (lanes 1 and 2; the size of the markers in nucleotides is shown on the left). The structure of the nicked and denatured DNA is indicated on the right, with black and white triangles representing the two strands of the 12 and 23 signals respectively.

order. Nicks are also visible at the isolated signals of fr12 and fr23 and the extent of nicking is reduced <2-fold compared with fr12×23 (6.5 versus 9.7% total at the 12 signal, and 4.4 versus 7.4% total at the 23 signal), where the two signals can synapse and coupled double-strand cleavage does occur. Similarly, the percentage of DNA nicked at a given signal does not change drastically when that signal is paired with a signal with a spacer of the same length. Nicking at the non-substituted signal stays almost the same in substrates fr12×12 (lane 8, black arrowheads, 6.5%) and fr23×23, (lane 9, white arrowhead, 4.3%), compared with fr12×23. A summary of the amounts of nicking in each substrate is displayed in Table 1. It appears that the extent of nicking after 30 min is only weakly affected by interaction between signals. It is important to note, however, that at this time point a significant fraction of nicked fr12×23 has gone on to double cleavage (~20%; cleavage levels off at ~30% of total substrate), resulting in an underestimation of the extent of nicking of this substrate. This can be overcome by analysis of nicking at earlier time points, before accumulation of significant amounts of cleaved product (see below).

Kinetics of appearance and disappearance of nicked DNA

To determine the kinetics of appearance and disappearance of the various singly and doubly nicked species we performed cleavage reactions with substrates derived from p12×23 and pJH299, a recombination substrate that is similar to p12×23 in the spacing and orientation of its 12 and 23 signals (26). Samples taken at various time points were split into two parts and analyzed to

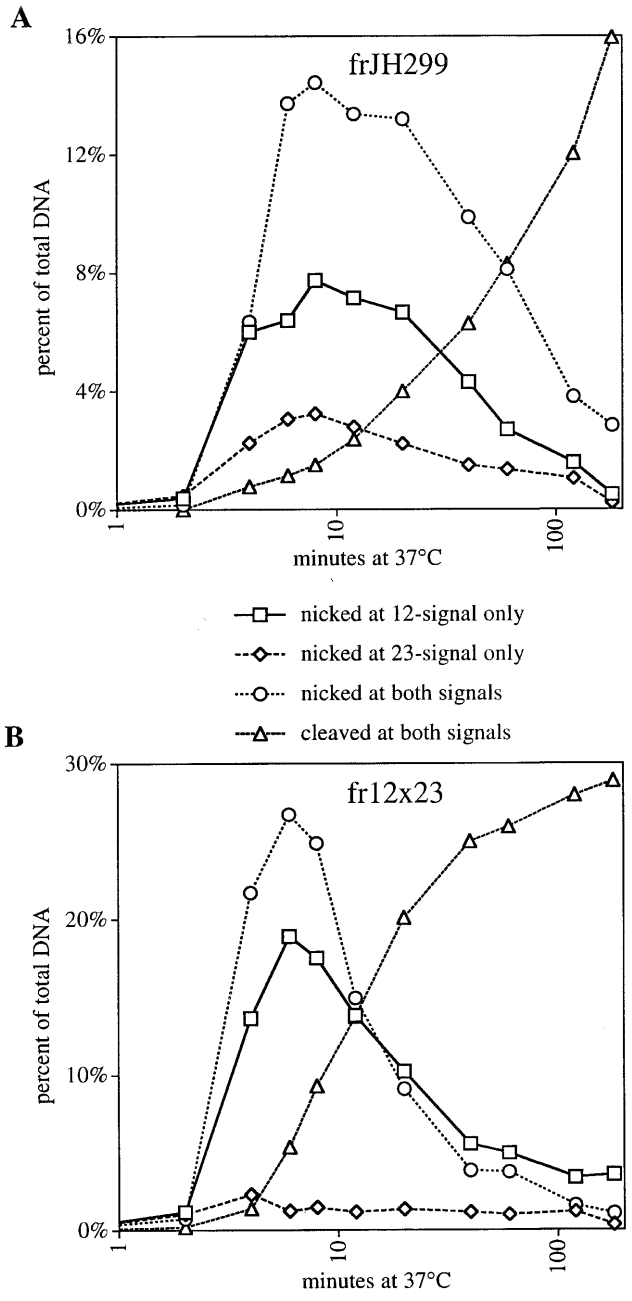

Figure 4. Nicked DNA appears before coupled cleavage and disappears as cleavage occurs. (**A**) Graphical representation of the results shown in Figure 3, after quantitation with a Molecular Imager. The *x*-axis is log scale to show early time points more clearly. (**B**) The experiment described in Figure 3 was repeated with the fr12×23 substrate and is depicted graphically. The legend refers to both (A) and (B). Note that these data were gathered using the shorter version of fr12×23 (see legend to Fig. 1), while those for Figure 2 and Table 1 were gathered (at the 30 min time point) using the longer version of fr12×23. Comparison of the two indicates that the longer version undergoes more nicking at the 23 signal only than the shorter version. The reason for this difference is unclear, but may be related to the distance between the DNA ends and the signals.

detect double-strand cleavage on a native polyacrylamide gel and also to detect nicking of the unbroken DNA as described above. Data for frJH299 are shown in Figure 3 and depicted graphically in Figure 4A; data for fr12×23 are depicted in Figure 4B.

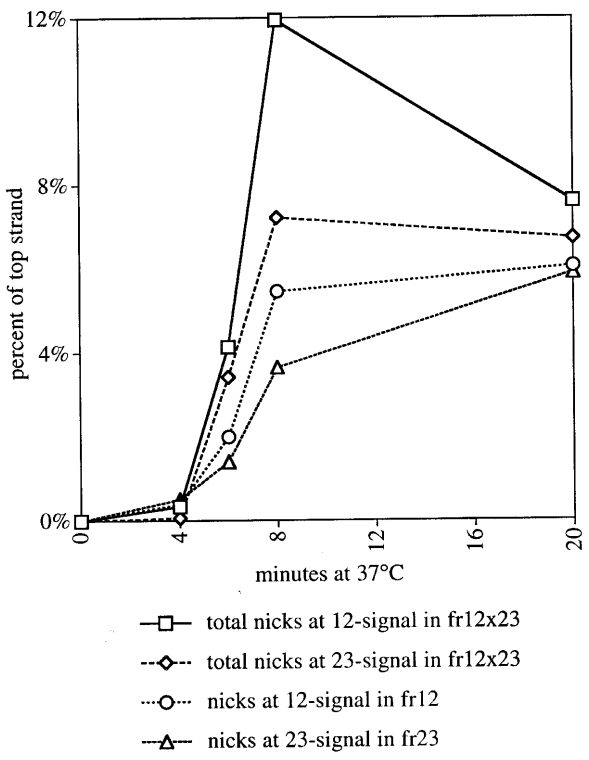

Figure 5. The presence of an appropriate partner signal boosts the rate of nicking at a given signal. Large scale cleavage reactions were performed with substrates fr12, fr23 and fr12×23 and aliquots removed at the indicated times. Samples were separated on an agarose gel and the 'uncut' DNA purified and analyzed for nicking on a denaturing polyacrylamide gel. Quantitation was performed with a Molecular Imager and percent nicking calculated as described in Materials and Methods. The total amount of nicking at a given signal in fr12×23 is the sum of nicking at that signal independently of the other signal and double nicking.

DNA molecules nicked at the 12 signal only (466 nt), at the 23 signal only (364 nt) and at both signals (286 nt) are all most abundant between 6 and 8 min after the cleavage reaction has begun (Fig. 3, top) and begin to disappear thereafter, as the products of coupled cleavage begin to appear (Fig. 3, bottom). The kinetic behavior of the singly and doubly nicked DNA species is thus consistent with their being precursors of the doubly cleaved molecules (Fig. 4). It is possible that some of the nicked DNA is degraded by nucleases rather than being cleaved by RAG proteins. A small fraction of the singly nicked DNA never goes on to be cleaved at both signals (Fig. 3, lane 12, and Fig. 4). The least abundant nicked species is that with a nick at the 23 signal only. A clear example of this is seen with fr12×23, which is seldom nicked at the 23 signal only (Fig. 4B). In contrast, fr23 is nicked efficiently (Fig. 2, lane 7, and Fig. 5). This indicates a strong preference to nick the 12 signal first when there is an interacting 12/23 pair of signals. For both substrates the most abundant nicked species after 6–8 min is that with nicks at both signals. This species appears without a substantial delay compared with DNA molecules nicked at the 12 signal alone and is substantially more abundant at all time points than would be expected if nicking events at the two signals were completely independent, further suggesting that interaction between the two signals stimulates nicking, even though it does not enforce a tight coupling between nicking events.

Effect of partner signal on the rate of nicking

To test the hypothesis that interaction between signals enhances nicking, we analyzed separate cleavage reactions containing fr12, fr23 and fr12×23 and measured nicking at early time points as described above. We compared the rate of appearance of nicks at the 12 signal in fr12×23 (adding the amount nicked at the 12 signal alone to the amount nicked at both signals) to the rate of nicking at the 12 signal of fr12 (Fig. 5). The amount of nicking levels off with fr12 after 8 min and does not decline substantially after 60 min, indicating that in the absence of double-strand cleavage DNA nicked by the RAG proteins is stable. Until 8 min, when accumulation of nicks in fr12×23 peaks, the rate of nicking at the 12 signal in fr12×23 (1.5%/min) is approximately double that in fr12 (0.7%/min). Similarly, the initial rate of nicking at the 23 signal in fr12×23 (0.9%/min) is about double the rate of nicking at the 23 signal in fr23 (0.5%/min) (Fig. 5). Therefore, it can be concluded that the presence of an appropriate partner signal boosts the rate of nicking at a given signal by a factor of two.

A similar comparison was made between the rates of nicking at the two signals in frI279 and frI89. These two substrates have their signals oriented like fr12×23 and frJH299, but in frI89 the 12 and 23 signal are moved closer together. Double-strand cleavage with frI89 is greatly reduced compared with frI279, presumably because the synaptic complex required for hairpin formation is destabilized by the high energetic cost of bending the DNA between the signals (19,27). The rate of nicking with frI279 is approximately twice that of frI89 (2.9 versus 1.6%/min for the 12 signal and 2.1 versus 0.9%/min for the 23 signal; Fig. 6A), indicating that, again, interaction between the two signals increases the rate of nicking. Importantly, the proportion of DNA nicked at both signals in frI89 after 8 min is >13 times lower than the amount of DNA nicked at both signals in frI279 (compare Fig. 6B and C). The amount nicked at both signals in frI89 closely approaches the amount expected if nicking events at the two signals are independent of each other (Fig. 6C), but this is not the case for frI279 (Fig. 6B). These results strongly argue that interaction between the 12 and 23 signals strongly stimulates the appearance of DNA nicked at both signals and boosts the initial rate of nicking at a given signal.

Effect of signal mutations on nicking

We wanted to test the effect of specific mutations of the recombination signals on nicking, to compare our results with those obtained using purified RAG proteins, Mn^{2+} and oligonucleotide substrates. We used a panel of recombination substrates based upon pJH299 in which a single base pair in a highly conserved part of one (or in one case both) of the recombination signals is mutated (2). Identical cleavage reactions with each substrate were stopped after 8 min, when nicking is expected to reach a maximum with the wild-type substrate frJH299, and after 30 min, to look for double-strand cleavage as well as nicking. The only substrates which are detectably cleaved at both signals are the wild-type (Fig. 7, lane 2) and those with changes of the fifth position of the nonamer (Fig. 7, lanes 11 and 13). In addition, when the first C of the heptamer is changed to T, cleavage at the unmutated signal only is visible (lanes 3 and 6, white arrowheads; notice that a small amount of DNA cleaved at one signal only is

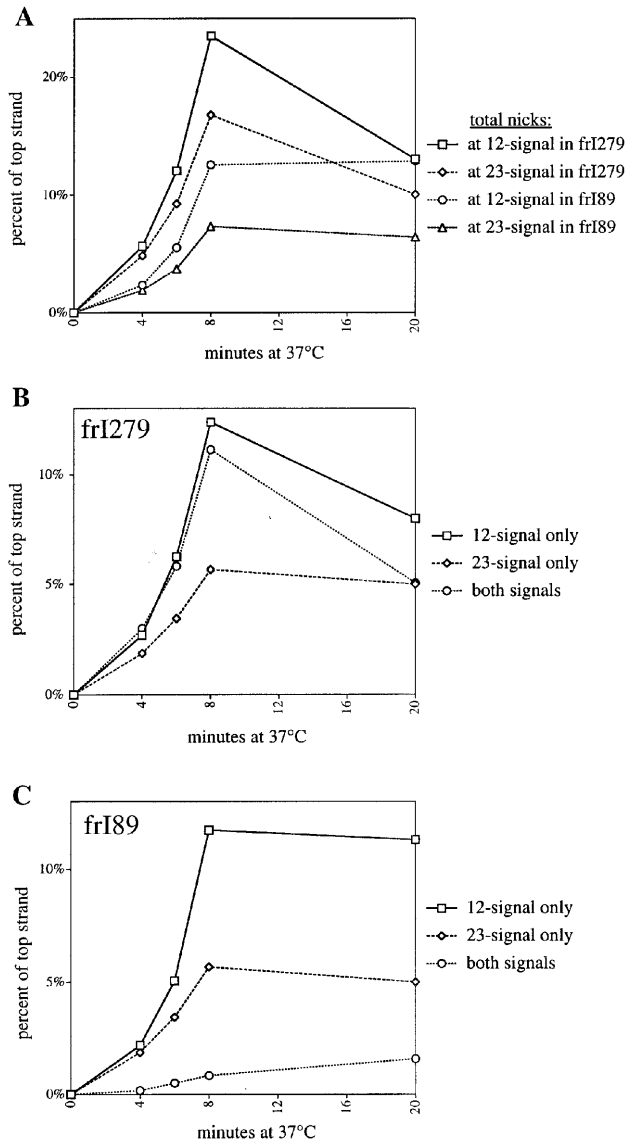

Figure 6. The ability of two signals to synapse stimulates nicking at both signals. Large scale cleavage reactions were performed with the substrates frI279 and frI89 and aliquots removed at the indicated times. Samples were separated on an agarose gel and the 'uncut' DNA was purified and analyzed for nicking on a denaturing polyacrylamide gel. Quantitation was performed with a Molecular Imager and percent nicking calculated as described in Materials and Methods. (**A**) Comparison of nicking of frI279 and frI89. The total amount of nicking at a given signal in frI279 and frI89 is the sum of nicking at that signal independently of the other signal and double nicking. (**B**) The amount of DNA nicked at both signals in frI279 is compared with the amount of DNA nicked at the 12 signal or the 23 signal independently. (**C**) The amount of DNA nicked at both signals in frI89 is compared with the amount of DNA nicked at the 12 signal or the 23 signal independently. Note that double nicking with frI89 is much less efficient than with frI279.

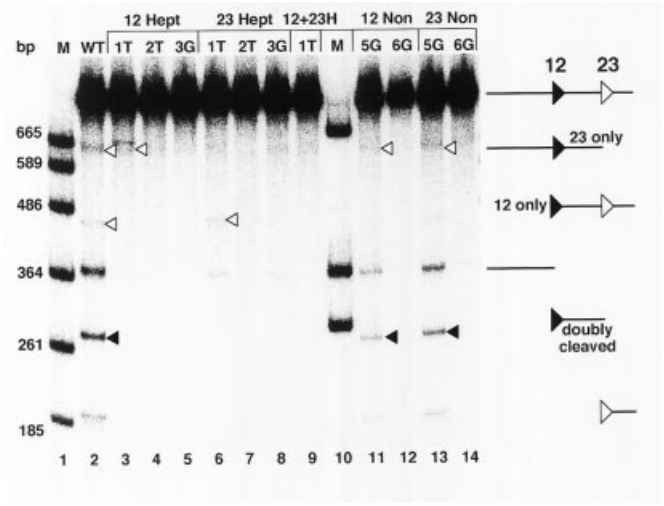

Figure 7. Mutations at one signal impair cleavage at both signals. Cleavage reactions were performed with substrates described in Hesse *et al.* (2) and stopped after 30 min, processed and then loaded on a native 4% polyacrylamide gel (lanes 2–9 and 11–14). Markers were obtained by digesting the frJH299 substrate with various restriction enzymes and mixing the digested products together: lane 1, *Ban*I, *Hin*dIII and *Pvu*II; lane 10, *Sal*I and *Sma*I + *Sal*I. The sizes of the markers in base pairs are shown on the left. The substrates are variants of frJH299 and the parts of the signal indicated at the top are mutated, with the specific mutation shown immediately above each lane. For example, the substrate used in lane 5 has a mutation '12 Hept: 3G' that changes the third base in the heptamer element of the 12 signal from C to G. Only a single nucleotide in each mutant substrate differs from the control frJH299, except for the substrate used in lane 9, which has the first base of both heptamers changed to T. The structures of the fragments generated by double-strand cleavage are indicated on the right, with abbreviations as in Figure 1B. White and black arrowheads indicate products of single and double cleavage respectively.

visible in lane 2 as well, white arrowheads). Therefore, as observed previously (19), mutations at a given signal reduce the amount of double-strand cleavage at both signals.

A similar effect is not seen when the same substrates are analyzed to detect nicking after 8 min (Fig. 8). Studies using purified RAG proteins and Mn^{2+} have established that some mutations at the first and second positions of the heptamer impair hairpin formation without impairing nicking, while other mutations, at the third position of the heptamer and at the nonamer, impair both nicking and hairpin formation (24). In general, when analyzing the effect of a given mutation upon the extent of nicking at the mutated signal, the results obtained here match those previously obtained. The results as visualized on the autoradiogram are complicated, however, by the presence of the unmutated signal and the ability to detect DNA nicked at both signals as well as at a given signal individually. DNA nicked at both signals is uniquely identified by the 279 nt band, but double nicking contributes to the intensity of the 379 (total nicks at the 12 signal) and 175 nt (total nicks at the 23 signal) bands as well. Nicks at the 12 signal only or the 23 signal only are detectable by visualizing the 454 and 658 nt bands respectively.

Given our results that complete elimination of a heptamer and nonamer impairs nicking at the remaining signal by a factor of two, a mutation of the heptamer known to permit nicking in a single signal context (24) would not be expected to drastically change the amount of nicking at either signal. For example, a change of the first C of the heptamer to T leaves unchanged the total extent of nicking at the mutated signal and diminishes slightly the amount of DNA nicked at the mutated signal only (compare the 379 and 454 nt bands in lanes 2 and 3 and the 658 and 175 nt bands in lanes 2 and 6). A similar effect is seen in lane 4 with a change from A to T at the second position of the heptamer

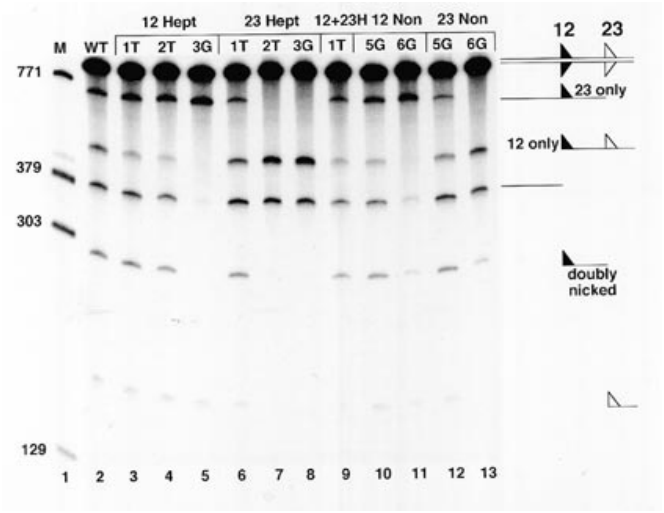

Figure 8. Mutations at one signal do not interfere greatly with nicking at the other signal. Cleavage reactions were performed with the same substrates as in Figure 7 and stopped after 8 min. Each sample was separated on an agarose gel and the 'uncut' DNA was isolated and purified as described in Materials and Methods. The cleavage reaction samples (equal counts) were then loaded onto a denaturing polyacrylamide gel (lanes 2–13). Markers (lane 1) are the same as in lane 10 of Figure 6, but denatured and single stranded. The sizes of the markers in nucleotides are shown on the left. The substrates are variants of frJH299 and the parts of the signal indicated at the top are mutated, as described in the legend to Figure 7. The structures of the fragments generated by nicking and denaturation are indicated on the right, with black and white triangles representing the 12 signal and 23 signal respectively. Black and white arrowheads indicate bands derived uniquely from double cleavage and single cleavage respectively. Note that the highest and most prominent band for each substrate is actually composed of varying proportions of unnicked top strand as well as the bottom strand, which is not normally nicked by the RAG proteins.

in the 12 signal and in lanes 10 and 12 with mild nonamer mutations.

Mutations of the heptamer which substantially lower the rate of nicking in a single signal context (24), such as a change of the second position to T (lane 7) or a change of the third position to G (lanes 5 and 8), virtually abolish initial nicking at that signal. However, the total amount of nicking at the other signal remains unchanged. The decreased amount of doubly nicked product is compensated for by an increase in the amount of substrate nicked at the unmutated signal only (compare the 658 nt bands in lanes 2 and 5 and the 454 nt bands in lanes 2 and 7 or 8). Thus heptamer mutations that impair nicking at one signal leave unchanged the amount of nicking at the other signal. Severe nonamer mutations substantially lower nicking at the mutated signal and weakly affect the total amount of nicking at the unmutated signal (lanes 11 and 13). Comparing our results with those of Ramsden *et al.* (24) and Cuomo *et al.* (23) indicates that changing the divalent cation does not perturb intrasignal DNA recognition by the RAG proteins as much as intersignal interaction.

DISCUSSION

The experiments described here examine the nicking step of DNA cleavage in V(D)J recombination in crude extract. Strict adherence to the 12/23 rule was critical to ensure that the second step of DNA cleavage (hairpin formation) occurred in the context of the appropriate synaptic complex in substrates with a 12/23 pair of signals and did not occur detectably with substrates containing an isolated signal. Cleavage with purified RAG proteins adheres weakly to the 12/23 rule (20) and recently it has been demonstrated that addition of crude extract is sufficient to fully reinstate adherence to the rule (21). Non-specific DNA bending proteins, including HMG1 and HMG2, enhance cleavage by the purified RAG proteins at 23 signals (21,22). However, it appears that these DNA bending proteins are not sufficient to enforce the 12/23 rule, since purified RAG proteins exhibit only a 3-fold preference for a 12/23 pair of signals over a 23/23 pair even in the presence of added HMG1 (21). In contrast, the preference is 50-fold or more *in vivo* or *in vitro* in the presence of crude extract (2,19,21). Currently, therefore, the 12/23 rule can be recapitulated only in the presence of crude extract.

Nicking at one signal can occur independently of another signal

It was observed previously, using purified RAG proteins in buffer containing magnesium, that nicking is approximately as efficient at an isolated recombination signal as with a 12/23 pair of signals (20). This study, however, did not resolve the question of whether nicking at an isolated signal was a result of the inability of the purified RAG proteins to adhere completely to the 12/23 rule. Hence, it remained possible that under conditions where cleavage was restricted to the appropriate synaptic complex, nicking might be similarly restricted. Our data show that this is not the case, because in crude extract under conditions where the 12/23 rule is obeyed strictly an isolated signal is still efficiently nicked. We have also observed that nicking events at the 12 signal and the 23 signal can occur independently. These results imply that during recognition and double-strand cleavage of DNA by the RAG proteins it is possible for one, or possibly even both, of the nicking events to precede formation of the synaptic complex. *In vivo* at an immunoglobulin or TCR locus the RAG proteins might nick the DNA at a given recombination signal even if interaction with signals of dissimilar spacer length is not possible. Our results strengthen the possibility that in actively rearranging cells the RAG proteins have the ability to nick many DNA sequences with sufficient similarity to recombination signals, causing DNA damage (24). It remains to be determined whether *in vivo* the RAG proteins can nick DNA signals preceding synapsis of the 12 signal and the 23 signal or whether some additional chromatin-based regulatory factor inhibits nicking in the absence of synapsis.

Interaction with a partner signal increases the rate of nicking

Despite our observation of nicking at isolated signals, the accumulated evidence does not support the model that nicking events at the two signals are completely independent of one another. We have seen a stimulation (2-fold) of the rate of nicking at a given signal by the presence of an appropriate partner signal on the same substrate. More importantly, our observation that the ability of the 12 signal and the 23 signal to interact stimulates the appearance of DNA nicked at both signals by more than an order of magnitude (Fig. 6B and C) suggests that for most of the substrate molecules in a given cleavage reaction synapsis precedes the second nick. Thus, one pathway followed during a cleavage reaction probably entails: recognition and nicking of the 12 signal, followed by synapsis and a facilitated interaction of the

assembled RAG proteins with the 23 signal. However, because the kinetics of appearance of the singly nicked species (at the 12 signal) and the double nicked species are similar (Fig. 4), both nicking events may occur after synapsis in some cases. Minor pathways would include completely independent nicking events and nicking at the 23 signal first. It is clear, therefore, that the 12/23 rule is enforced at the double-strand cleavage step of the reaction and not at the nicking step. However, in the generation of doubly nicked molecules, the immediate precursors of doubly cleaved products, there is a strong preference for a synapsed 12/23 pair of signals.

Comparison with other systems and mechanistic implications

Others have noted the mechanistic similarities between transposition, retroviral integration and V(D)J recombination (6,28,29). These reactions involve two separate chemical steps: in transposition, donor cleavage followed by strand transfer or, analogously, in V(D)J recombination nicking at the 5′-end of the heptamer followed by hairpin formation. V(D)J recombination is the only system in which the initial chemical step can occur before synapsis. In the Tn*7*, Tn*10* and Mu systems a synaptic complex must form before donor cleavage can occur (30–32). However, in the Tn*7* and Tn*10* systems it is possible to observe asynchronous donor cleavage, showing some similarity to RAG-mediated nicking (33,34). In addition, the two chemical events of donor cleavage in the Tn*7* system are separable by mutations near the transposon ends (31), which is similar to the way mutations at the distal end of the heptamer impair hairpin formation but not nicking.

Why do nicking and hairpin formation have such different requirements, on both the sequence and intersignal interaction levels? A significant distortion in DNA structure would be necessary to have a 3′-hydroxyl on one DNA strand attack a phosphate on the other strand, 16 Å away. Convincing evidence has been provided that the influence of the coding flank upon the efficiency of hairpin formation under conditions where the 12/23 rule is not obeyed is a result of this requirement for deformation of the DNA (20,23,24). Unpaired bases or gaps next to the heptamer restore hairpin formation with oligonucleotide substrates whose coding flank would ordinarily stop hairpin formation. Presumably, deformation of the coding flank is not necessary for nicking. Interestingly, an influence of the coding flank on efficiency of hairpin formation is not seen during coupled cleavage. A reasonable hypothesis is that the positive energetic contributions from DNA-assisted protein–protein interactions (synapsis) and the demonstrated ability of the RAG proteins to recognize single-stranded DNA (23,24) drive the requisite distortion of the DNA, even with coding flanks that prevent this in a single signal context. Thus, hairpin formation is more tightly regulated because it requires deformation of the coding flank.

Recently it was shown that RAG1 and RAG2 catalyze hairpin formation by a direct nucleophilic attack, resulting in an inversion of chirality at the attacked phosphate, exactly as occurs with Mu transposase or retroviral integrase (6). This similarity, and the ability to substitute various alcohols for water as the attacking nucleophile in the nicking reaction, led to the suggestion that nicking and hairpin formation are mediated by the same active site, because strong evidence from the Mu and Tn*10* systems exists that donor cleavage and strand transfer are also mediated by the same active site (35,36). This single putative active site must be able to mediate nicking using water as the attacking nucleophile, but then later exclude water from the active site during attack by the 3′-hydroxyl on the opposite DNA strand. The crystal structure of core Mu transposase reveals the active site residues apparently in an inactive conformation (37). It was suggested that only multimerization of Mu into a tetramer, as is required for donor cleavage and strand transfer to occur, might promote a change of conformation, producing a truly ‘active’ site.

Although residues corresponding to the aspartate or glutamate residues shown to be essential for activity of many transposases have not been identified in RAG1 or RAG2, an allosteric transition similar to that proposed for Mu transposase may explain the coupling between synapsis, DNA deformation and catalytic activity. This model would suggest that the mutated RAG1, defined as ‘D32’ by Sadofsky and Gellert (38), is compromised in its ability to make this allosteric transition. With this mutant, inhibition of recombination is observed with coding flanks thought to resist the unpairing required for hairpin formation. Significantly, a recently identified dimerization domain in RAG1 (amino acids 264–389; 39) is immediately adjacent to the region responsible for nonamer binding (amino acids 384–477; 40,41). There have been some reports that RAG1 self-associates in crude extracts, but so far no evidence exists to confirm that RAG1 exists as a dimer in solution without nucleic acids or that the dimerization of RAG1 is important for its activity (42).

The conformational change in the putative RAG active site that occurs upon interaction between proteins bound at the 12 and 23 signals must be somewhat different from that proposed for Mu transposase. Unlike the RAG proteins, Mu transposase stays completely inactive catalytically until a synaptic complex is formed. The RAG proteins are inactive only for hairpin formation in the absence of synapsis. Thus, the suggestion that the RAG proteins must catalyze DNA cleavage events on the recombination signal to which they are not bound (cleavage *in trans*; 41), an extension of the parallel between Mu transposase and the RAG proteins, is probably incorrect. Eventually, crystallographic analysis and mutagenesis will provide an understanding of how RAG1 and RAG2 interact with the recombination signals and catalyze first nicking and then hairpin formation. Recent advances in the ability to study DNA binding may enable investigation of how complexes involving two recombination signals are constructed (22,25,43).

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