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The RAG1 and RAG2 Proteins Establish the 12/23 Rule in V(D)J Recombination

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Summary

V(D)J recombination requires a pair of signal sequences with spacer lengths of 12 and 23 base pairs. Cleavage by the RAG1 and RAG2 proteins was previously shown to demand only a single signal sequence. Here, we establish conditions where 12- and 23-spacer signal sequences are both necessary for cleavage. Coupled cutting at both sites requires only the RAG1 and RAG2 proteins, but depends on the metal ion. In Mn^{2+}, a single signal sequence supports efficient double strand cleavage, but cutting in Mg^{2+} requires two signal sequences and is best with the canonical 12/23 pair. Thus, the RAG proteins determine both aspects of the specificity of V(D)J recombination, the recognition of a single signal sequence and the correct 12/23 coupling in a pair of signals.

Introduction

In lymphoid cells, mature immunoglobulin and T-cell receptor genes are assembled from separate gene segments by V(D)J recombination (Gellert, 1992; Lewis, 1994). This process is directed by recombination signal sequences (RSSs), which flank the coding segments. An RSS is made up of conserved heptamer and nonamer motifs, separated by a spacer with nonconserved sequence but a relatively conserved length of 12 or 23 base pairs (bp).

V(D)J recombination can be divided into two stages. First, double-strand breaks (DSBs) are made at the coding/signal borders. Such DSBs have been detected at T-cell-receptor (Roth et al., 1992a) and immunoglobulin loci (Schlissel et al., 1993). Later, pairs of coding ends and signal ends are joined. Signal ends have been found in all rearranging cells and shown to be intermediates leading to signal joints (Ramsden and Gellert, 1995). Coding ends were initially only detected in mice carrying the severe combined immunodeficiency (*scid*) mutation (Roth et al., 1992b), but have also recently been found in a non-*scid* background (Ramsden and Gellert, 1995).

DSBs are now known to be made by the RAG1 and RAG2 proteins in a two-step reaction (McBlane et al., 1995; van Gent et al., 1995). First, a nick is introduced at the 5′ end of the RSS heptamer, leaving a 3′-OH on the coding side, and a 5′ phosphate on the signal side. This 3′-OH is then used to attack the phosphodiester bond in the other strand opposite the initial nick by direct transesterification (van Gent et al., 1996), resulting in a hairpin coding end and a blunt, 5′ phosphorylated signal end.

After DSB formation, the hairpin coding ends are opened by an as yet unknown mechanism and coupled to form a coding joint, and the signal ends are joined in a head-to-head fashion (signal joint). The joining reactions require several factors that are also involved in general DSB repair (Jackson and Jeggo, 1995).

We have shown previously that the RAG1 and RAG2 proteins are able to cleave oligonucleotide substrates containing a single RSS, resulting in the formation of a hairpin coding end and a blunt, 5′ phosphorylated signal end. In vivo, recombination takes place between one RSS with a 12 bp spacer (12-signal) and one with a 23 bp spacer (23-signal); this is the so-called 12/23 rule. DSB formation in vivo also depends on the presence of such a pair of RSSs (S. B. Steen, L. Gomelsky and D. B. Roth, personal communication), indicating that the 12/23 rule is linked to the initial cleavage event. However, with Mn^{2+} as divalent cation, cleavage by the purified RAG1 and RAG2 proteins did not demand a second RSS, nor did the presence of a partner signal stimulate this reaction. Here we show that with Mg^{2+} as divalent cation, efficient cleavage requires the presence of a 12-signal and a 23-signal but does not occur at a single RSS, thus recapturing the 12/23 rule in vitro with only the RAG1 and RAG2 proteins.

Results

Effect of Divalent Cation on Cleavage

As shown before, a single RSS is cleaved by the purified RAG1 and RAG2 proteins (McBlane et al., 1995). The two products, the nicked species resulting from the first step and the hairpin resulting from the second, can be seen in Figure 1 (lane 2). Efficient cleavage of such an oligonucleotide substrate requires Mn^{2+} as divalent cation; in the presence of Mg^{2+}, only the nicked species is made (Figure 1, lane 3).

In the presence of Mn^{2+}, DNA substrates containing two RSSs (one with a 12 bp spacer and one with a 23 bp spacer) were found to be cleaved at either RSS, independent of the other (McBlane et al., 1995; van Gent et al., 1995). We investigated whether substitution of Mg^{2+} for Mn^{2+} might restore the need for a second RSS. To allow the two RSSs to be aligned without any hindrance by limited DNA flexibility, we inserted a 0.9 kb fragment between the signals (pDVG42; see Figure 2A). This plasmid was linearized with the restriction enzyme AatII and incubated with RAG1 and RAG2 in the presence of either Mn^{2+} or Mg^{2+}. Reaction products were analyzed by Southern blotting, using the 0.9 kb insert as probe. Cleavage at both signals generates a 1 kb product; cleavage at only the 12-signal or only the 23-signal will yield 6 kb or 3 kb products, respectively. In the presence of Mn^{2+}, high levels of the 6 kb and 3 kb products were observed (29% and 8% of total substrate, respectively), showing that cuts were made efficiently at one RSS without cleavage at the other (Figure 2B, lane 2). The 1 kb product, arising from cleavage at both signals, was also observed (7%), but at a level that was not significantly higher than expected for two independent cleavage events.

In Mg^{2+}, however, the majority of products was cut at both signals (13%), with only a small minority cleaved just at the 12-signal (3%) and barely detectable single

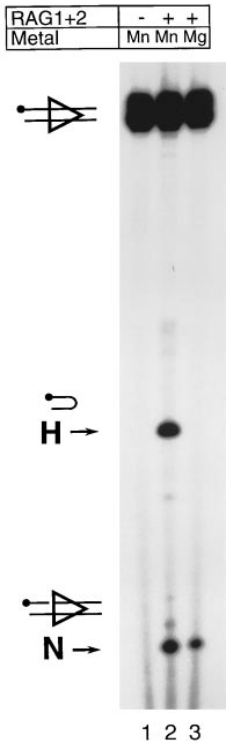

Figure 1. The Effect of the Divalent Cation on Cleavage of Oligonucleotides

End-labeled oligonucleotide substrates containing a 12-signal sequence and 16 bp of flanking ("coding") DNA were incubated without (lane 1) or with (lanes 2 and 3) RAG1 and RAG2 proteins in the presence of Mn^{2+} (lanes 1 and 2) or Mg^{2+} (lane 3). Reaction products were separated by electrophoresis in a 12.5% Tris–borate–EDTA/urea gel and visualized by autoradiography. The positions of the nicked (N) and hairpin (H) species are marked.

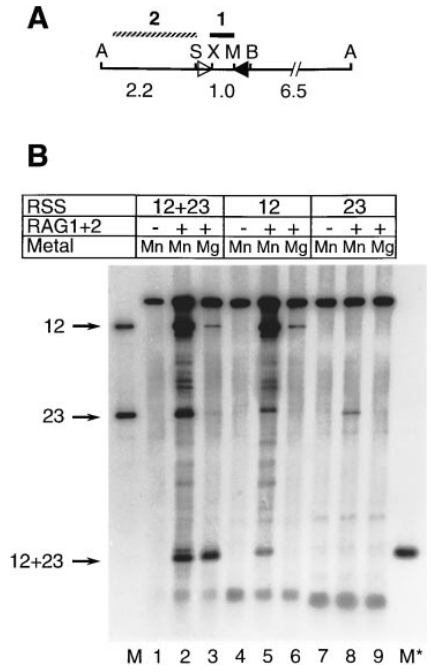

Figure 2. Coupled Cleavage in Mg^{2+}

(A) Schematic representation of recombination substrate pDVG42. The RSSs are depicted by triangles (open for the 12-signal and closed for the 23-signal), and the probes used for detection of cleavage products by bars above the plasmid map. Probe 1 was used in Figures 2B, 3, and 4, and probe 2 was used in Figure 5. A, AatII; B, BamHI; M, MluI; S, SalI; X, XhoI. The distance between the AatII, SalI, and BamHI sites are given below the schematic plasmid map.

(B) Analysis of cleavage products from reactions with pDVG42 (lanes 1–3), pDVG47 (lanes 4–6), or pDVG46 (lanes 7–9). RAG1, RAG2, and divalent cations were present as indicated. A mixture of pDVG42 cut with AatII and BamHI, and pDVG42 cut with AatII and SalI (M; arrows labeled 12 and 23) was used as marker for cleavages at one RSS only, and pDVG42 cut with BamHI and SalI was used as marker for cleavage at both RSSs (M with asterisk; arrow labeled 12+23). Southern blots were developed with probe 1 (see [A]).

cleavage at the 23-signal. Cutting at both RSSs is clearly coordinated under these conditions (Figure 2B, lane 3). When only a 12-signal or a 23-signal was present in the plasmid, much less efficient cleavage was observed in Mg^{2+} than in Mn^{2+} (4% versus 30% cleavage at the 12-signal, <0.3% versus 3% at the 23-signal; Figure 2B, lanes 6 versus 5, and 9 versus 8, respectively). These results show that a second RSS needs to be present for efficient DSB formation in Mg^{2+}. The second RSS has to be on the same DNA molecule; we have not found cooperation between signals on different DNAs (data not shown). Note also that cleavage at secondary sites is frequent in Mn^{2+}, but very low in Mg^{2+}. (With the 12-signal plasmid, the bands near the position of a 23-signal cleavage or a 12/23 cleavage arise from such a secondary site. Both their positions are slightly above those resulting from authentic 23-signal cleavage. A similar product can also be seen as the upper band of a doublet in lane 2.)

Kinetics of Coordinated Cleavage

After 1 hr of incubation in Mg^{2+}, most substrate molecules are either cleaved at both RSSs, or not cut at all. Are the cuts made simultaneously, or sequentially? As shown in Figure 3A, coupled cleavage at both RSSs can be observed from the earliest time point on, showing that both signals are cleaved at or near the same time. The low level of cleavage in Mg^{2+} at one RSS without cutting at the other appears to be a side reaction, because similar cleavage levels can be observed in substrates containing only one RSS (see Figure 2B, lanes 6 and 9), and the singly and doubly cleaved species appear with similar kinetics (Figure 3A). In contrast to the reaction in Mg^{2+}, cleavage in Mn^{2+} shows mainly cleavage at one RSS without cleavage at the other, with the doubly cleaved fragment appearing later, presumably as a result of two independent events (Figure 3B).

The 12/23 Rule

The experiments above show that RAG-mediated cleavage in the presence of Mg^{2+} requires a pair of RSSs. Does this pair have to contain the canonical combination of 12-spacer and 23-spacer signals? We addressed this question by using substrates with two 12-signals or two 23-signals (Figure 4). These substrates were cleaved efficiently in Mn^{2+}, but cleavage occurred at each site independently of the other. In Mg^{2+}, however, the substrate with two 23-signals only showed a very low level

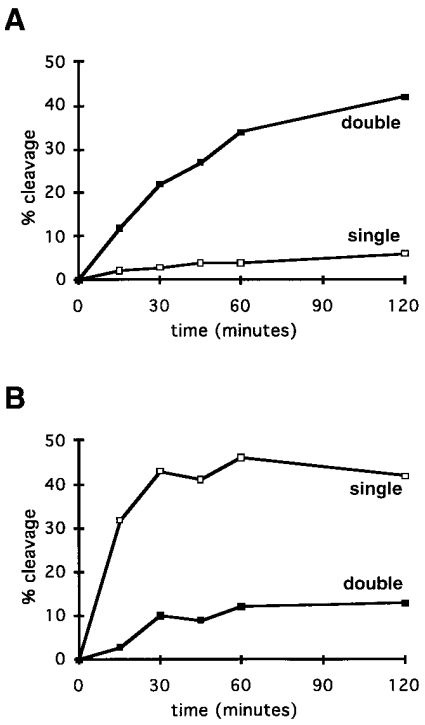

Figure 3. Time Course of Cleavage

The recombination substrate pDVG42 was cleaved with Mg^{2+} (A) or Mn^{2+} (B), and samples were taken at 15, 30, 45, 60, and 120 min. The percentage of total substrate cleaved at one RSS only (open rectangles) and at both RSSs (closed rectangles) is plotted against time.

of cleavage at either one of the two signals (lane 6), and the substrate with two 12-signals was cut less efficiently than the substrate with two different RSSs (4% for two 12-signals versus 13% for the 12/23 pair) (lane 3). In other words, cleavage at a 23-signal requires the presence of a 12-signal, and a 12-signal is cut most efficiently when linked to a 23-signal, although a low level of coordinated cleavage could be observed between two 12-signals.

We also asked whether the 23-signal has to be functional for cleavage at the 12-signal to take place. We therefore constructed a substrate with a mutation in the first base of the 23-signal. The 12-signal of this substrate was cut at the same low level as a substrate with only a 12-signal (Figure 4, lane 9).

In summary, we have reproduced the 12/23 rule with purified RAG1 and RAG2 proteins, showing that these proteins alone can make a complex in which cleavage at both signals is carried out in a coupled fashion.

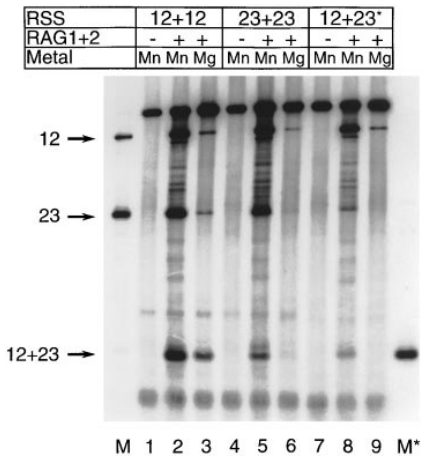

Figure 4. Cleavage of Noncanonical RSS Pairs

Analysis of cleavage products from reactions with pDVG44 (lanes 1–3), pDVG48 (lanes 4–6), and pDVG50 (lanes 7–9) with RAG1, RAG2, and divalent cations as indicated; 23 with asterisk, 23-signal with a C1→A mutation in the RSS heptamer. Further labeling is as in Figure 2. These samples and those shown in Figure 2 are derived from the same experiment and run on different gels.

Effects of Coding Flank Sequence

As shown before, recombination in cells with a mutant form of RAG1 (called D32) displays a strong preference for certain coding flank sequences over others (Sadofsky et al., 1995). Surprisingly, a very similar coding flank preference has been observed in cleavage at a single RSS by nonmutant RAG1 and RAG2 proteins. "Good" flanks are efficiently converted into hairpins, whereas "bad" flanks only support the initial nicking step (Ramsden et al., 1996). The results were taken to suggest that RAG1 may interact with the DNA at the signal/coding border, and that this interaction may not have been reproduced in the cleavage reaction on a single RSS. As recombination in cells expressing wild type RAG1 and RAG2 does not show these same preferences, we investigated whether the coding flank preference might be altered in coupled cleavage. For this purpose, we made substrates containing a coding flank that was very inefficiently converted to hairpins (a bad flank) next to either the 23-signal only, or both signals, and incubated them with RAG1 and RAG2 in the presence of either Mn^{2+} or Mg^{2+}. As shown before, cleavage in Mn^{2+} was very sensitive to the coding flank sequence: RSSs flanked by such a sequence were not efficiently cleaved (Figure 5, lanes 5 and 8). Several secondary cleavage sites were cut even more efficiently than the canonical RSSs. In contrast, cleavage in Mg^{2+} did not show any preference for one coding flank over the other, just as has been observed in cells. (The extent of double cleavage varies by no more than 3% among lanes 3, 6, and 9 of Figure 5.) When only one RSS had a bad flank, cleavage at this signal was inhibited in Mn^{2+}, but not in Mg^{2+}, as expected.

Structure of the Coding Ends

We have shown before that cleavage by RAG1 and RAG2, with Mn^{2+} as divalent cation, results in formation of a hairpin structure at the coding end (McBlane et al., 1995). However, with Mg^{2+} very few hairpins were formed in an oligonucleotide with a single RSS (see above). We therefore investigated the structure of the coding ends produced after coupled cleavage, using two-dimensional gel electrophoresis. In this method, the DNA sample is first separated by native gel electrophoresis, then a gel slice containing the sample is placed

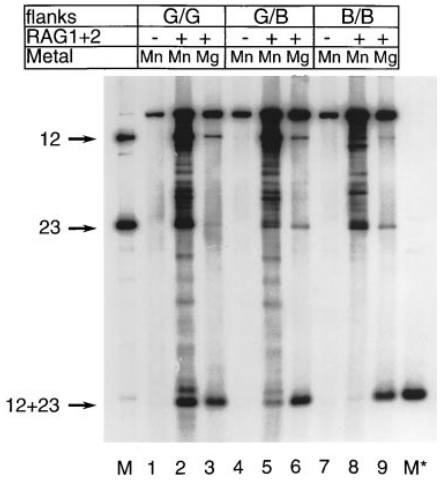

Figure 5. Effect of Flanking Coding DNA Sequence on DSB Formation

Analysis of cleavage products from reactions with pDVG42 (lanes 1–3), pDVG51 (lanes 4–6), and pDVG53 (lanes 7–9). Flanking DNA was identified as "good" (G) when it was cleaved efficiently in an oligonucleotide cleavage assay in the presence of Mn^{2+}, and "bad" (B) when cleaved inefficiently in that assay (as described in the text). Further labeling is as in Figure 2.

perpendicularly on a denaturing gel and electrophoresed under denaturing conditions in the second dimension. As previously described, cleavage by the RAG proteins in Mn^{2+} results in formation of hairpin coding ends (see, for example, Figure 6C). Hairpin coding ends were also observed with Mg^{2+}-containing buffer (Figure 6D), showing that hairpin formation is a general characteristic of V(D)J cleavage, and not a peculiarity of the reaction in Mn^{2+}.

In addition to hairpin coding ends at the 12-signal, several other species were observed. Indeed, the major species, a nick at the 12-signal, is 9-fold more abundant than the full-length substrate (Figure 6D). A similarly high frequency of nicks at the 12-signal is observed when only the 12-signal is present (data not shown), arguing that formation of DSBs in Mg^{2+} requires a partner signal only for the second step, which forms hairpins. (The uncoupling of cleavage in Mn^{2+} also permits the detection of a large amount of 23-signal ends with this probe [Figure 6C].)

In Mn^{2+}, formation of hairpins from substrates with bad coding flanks has been shown to be very inefficient (Ramsden et al., 1996). When the plasmids used here contained bad coding flanks, hairpins were also formed inefficiently when Mn^{2+} was used as divalent cation. Does cleavage in Mg^{2+} still yield hairpins at such coding flanks, or is there another mechanism to deal with these sequences? As shown in Figure 6E, the coding ends were found in a hairpin structure irrespective of their sequence, indicating that the coding flank sequence does not influence the products of cleavage.

We conclude that cleavage with Mg^{2+} as divalent cation has the same mechanistic properties as cleavage in Mn^{2+}. However, its requirements are very different: Mn^{2+} promotes cleavage at a single RSS, whereas Mg^{2+} demands the presence of both a 12-signal and a 23-signal for efficient cutting (Figure 7).

Discussion

It was shown earlier that RAG1 and RAG2 are both required and sufficient for cleavage at RSSs. Here we describe conditions under which the RAG1 and RAG2 proteins require two RSSs for efficient cleavage. The 12/23 rule is in effect: one signal with a 12 bp spacer, and one with a 23 bp spacer must be present for optimal cleavage, as has been found in cells for both recombination (Tonegawa, 1983; Hesse et al., 1989) and cleavage (S. B. Steen, L. Gomelsky, and D. B. Roth, personal communication).

The preference for a 12/23 pair over a 12/12 pair is not as pronounced as previously observed in vivo. Cutting of a 12/23 pair is enhanced by 3- to 4-fold over a 12/12 pair, whereas recombination in lymphoid cells can display as much as a 50-fold preference (Hesse et al., 1989). However, the preference for a 12/23 signal pair is not always so extreme even in vivo (reviewed in Lewis, 1994). While the conditions controlling the exact degree of preference will need to be studied further, it is evident that the RAG proteins alone recapture the essential coupling between 12 and 23 signals.

The divalent cation is very important for the process of cleavage. In Mg^{2+}, a single RSS directs efficient nicking but not hairpin formation. Hairpin formation requires another RSS (of different spacer length) to be present on the same DNA molecule. However, in Mn^{2+} both nicks and hairpins are readily formed on a single RSS, without the need for, or stimulation by, a second one. These reactions are summarized in Figure 7. A similar relaxation of the requirement for coordination in Mn^{2+} has been observed for other recombination reactions (Bushman and Craigie, 1991). In addition to the lack of coordination in Mn^{2+}, cleavage also occurs at several secondary sites, presumably at sequences that resemble heptamers. Such relaxed recognition requirements in Mn^{2+} have also been described for several other nucleases (Roberts and Halford, 1993). As Mg^{2+} is the predominant divalent cation in nuclei, we interpret cleavage in Mn^{2+} as a relaxation of the normal requirement for coordination of two RSSs before V(D)J recombination can be initiated.

A Complex for Coupled Cleavage?

The requirement for two RSSs of different spacer length suggests that there is a coordinated complex containing both RSSs and the RAG proteins. Although we have not yet been able to detect such a complex by direct physical means, one can imagine that such a defined conformation can only be made with a 12-signal and a 23-signal.

Coordinated cleavage not only requires two RSSs of different spacer length, but both RSSs have to be functional; a mutation that blocks cleavage at the 23-signal (first C of the heptamer mutated to A) also blocks cleavage at the 12-signal. Competition of oligonucleotide cleavage by other oligonucleotides (in Mn^{2+}) indicated that binding of RAG proteins to an RSS mainly depends on the nonamer (Ramsden et al., 1996). Mutation of the first three base pairs of the heptamer in the competing oligonucleotide did not influence its ability to act as a competitor. It is thus likely that initial binding of the RAG

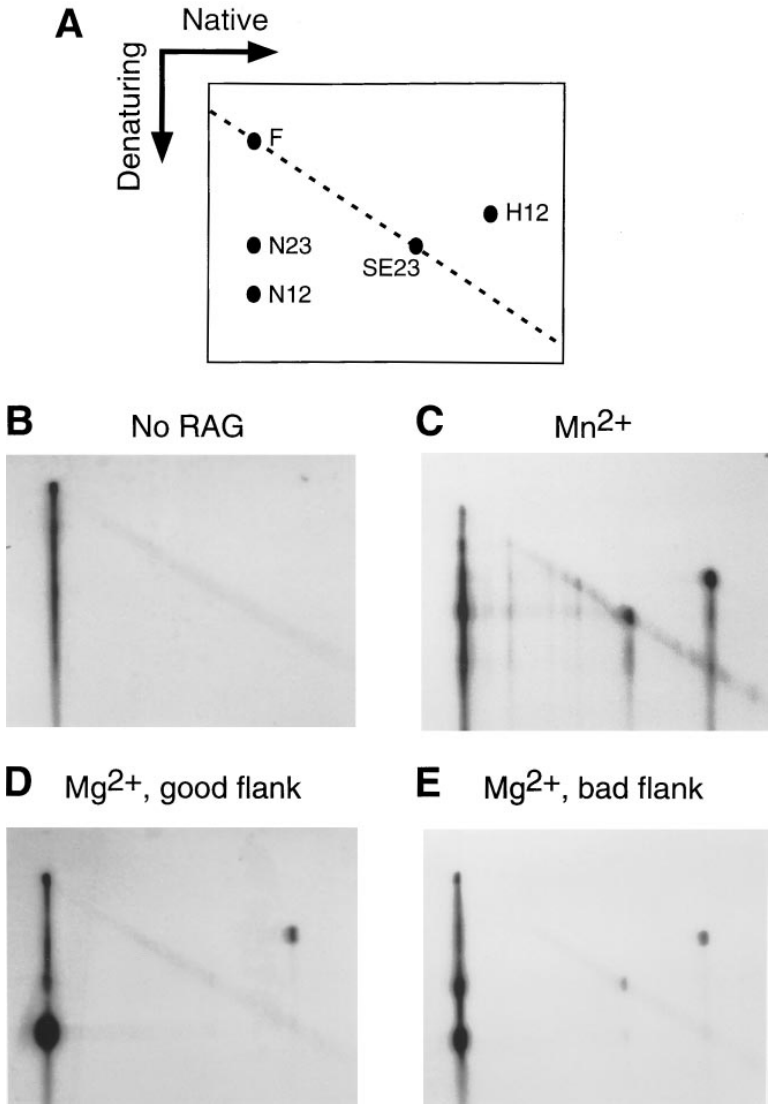

Figure 6. Identification of Cleavage Products by Two-Dimensional Agarose Gel Electrophoresis

(A) Schematic representation of the expected cleavage products. The DNA was electrophoresed under native conditions in the first dimension and under denaturing conditions in the second. The expected positions of full length (unreacted) substrate DNA (F), species nicked at the 12-signal (N12) and 23-signal (N23), the signal end at the 23-signal (SE23), and hairpins at the 12-signal coding end (H12) are indicated.

(B–E) Analysis of products from reactions without RAG proteins (B), and with RAG proteins in Mn^{2+} (C), or Mg^{2+} (D) and (E). The recombination substrates pDVG42 (B–D) and pDVG53 (E) were used, and probe 2 (see Figure 2A) was used for detection of reaction products.

proteins to both the wild-type and the C1→A mutant RSS is normal. We therefore expect that this mutation may either block formation of the (as yet) hypothetical synaptic complex, or that it may cause more subtle differences in the exact architecture of such a complex, thus blocking cleavage at the other RSS as well. In any case, it seems likely that coordinated cleavage involves additional protein–protein and protein–DNA contacts, potentially leading to a more stable complex. Such a stable synaptic complex has been characterized for a related DNA recombination reaction, bacteriophage Mu transposition (Baker et al., 1993), where four MuA transposase molecules form a stable complex with two phage DNA ends. It is to be expected that V(D)J recombination involves formation of a similar complex, containing at least two molecules of both RAG1 and RAG2, and two RSSs.

Effect of Coding Flank Sequence

We previously found that the sequence of the DNA flanking the RSS (the coding flank) was very important for DSB formation in Mn^{2+} (Ramsden et al., 1996). This was puzzling, since the same preference had not been observed for V(D)J recombination in vivo. Interestingly, a mutant version of RAG1 (called D32) had shown a very similar preference for coding flanks in vivo (Sadofsky et al., 1995). However, this mutation is not present in the RAG1 protein used in the cleavage assays. The present experiments may help explain these apparently contradictory observations. We find that coupled cleavage (in the presence of Mg^{2+}) does not show a similar distinction between good and bad coding flanks, suggesting that this preference is a peculiarity of the (Mn^{2+}-dependent) cleavage at a single RSS. However, this still leaves the question why the Mn^{2+}-dependent cleavage in vitro has the same coding flank preference as RAG1-D32 in vivo. We have shown earlier that substrates containing one or a few mismatched bases at the coding flank are good substrates for DSB (and hairpin) formation, even if both strands contain a bad coding flank sequence (Ramsden et al., 1996), suggesting that flexibility at the coding/signal border is the main determinant of its ability to form a hairpin. We thus considered two possible explanations for efficient DSB formation on bad coding flanks

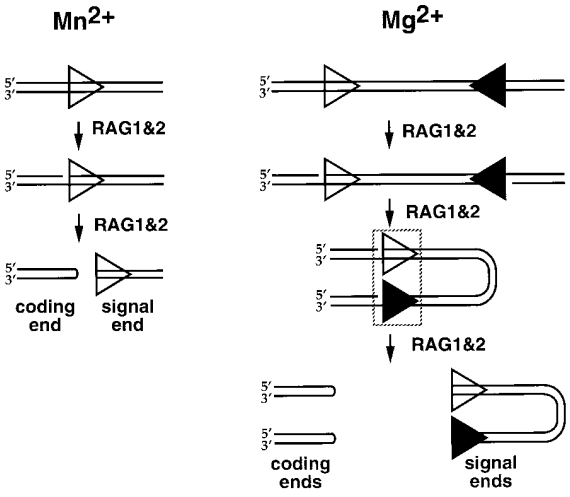

Figure 7. Model for Cleavage in Mn^{2+} and Mg^{2+}

The 12-signal is depicted as an open triangle, and the 23-signal as a closed triangle. Formation of a synaptic complex in the Mg^{2+}-reaction is indicated by a hatched box. The relative orientation of the RSSs is not known; they are arbitrarily drawn in parallel orientation.

in Mg^{2+}: there may be another way to make a DSB, not involving formation of a hairpin structure on the coding end, or, alternatively, formation of a synaptic complex may help to distort the DNA at the coding/signal border in such a way that even bad flanks are cleaved efficiently. We excluded the first possibility by showing that hairpins are formed even from bad coding flanks. We therefore propose that additional protein–DNA interactions in the synaptic complex help to make coding flanks competent for hairpin formation. A possible mechanistic explanation for this observation involves a role for a RAG1 molecule bound to one RSS in making the coding/signal border of the other RSS competent for hairpin formation, and vice versa. This would explain why bad flanks cannot be cleaved in an isolated RSS, and why hairpin formation requires the presence of a second RSS in Mg^{2+}. Cleavage of a single RSS in Mn^{2+} would then be the exception to the rule that a second RSS is needed for efficient cleavage. In this scenario, the RAG1-D32 mutant may have lost its ability to act in this way on the coding flank.

Together, our results show that 12/23 signal coupling in V(D)J recombination is established immediately at the start of the process and only requires the action of the RAG1 and RAG2 proteins. The specificity of V(D)J recombination requires the correct recognition of a single RSS and the correct 12/23 coupling in an RSS pair. Both these properties are determined by the RAG1 and RAG2 proteins.

Experimental Procedures

DNA Techniques

The signal sequences used here have been previously described (Hesse et al., 1989). They are derived from Jκ1 (23-signal) and VκL8 (12-signal, with one base of the nonamer altered to match the consensus sequence). The recombination substrate pDVG42 was made by substituting the 0.9 kb XhoI–MluI fragment of pET16B (Novagen) for the 0.2 kb XhoI–MluI fragment of pMS365, which has the same sequence as pMS319 (Sadofsky et al., 1995), except for an additional XhoI site next to the 12-signal nonamer. Substrates containing only one RSS were made from pDVG42 by cleavage with XhoI/SalI (23-signal plasmid pDVG46) or MluI/BamHI followed by fill-in of the 5′ protruding ends with Klenow enzyme (12-signal plasmid pDVG47), and ligation in a large volume. The other substrates were made by cloning double-stranded oligonucleotides containing the desired sequence into the HincII/XhoI or the MluI/BamHI sites for substitution of the 12-signal or 23-signal, respectively. The following oligonucleotides were used: DG90 (5′-CGCGTGGTTTTTGTTCCAGTCTG TAGCACTGTGCAGGTG-3′) and its complement DG91 for pDVG44 (two 12-signals); DG94 (5′-TCGAGGGTTTTTGTACAGCCAGACAGT GGAGTACTACCACTGTGTAAGTC-3′) and its complement DG95 for pDVG48 (two 23-signals); DG98 (5′-CGCGTGGTTTTTGTACAGCCA GACAGTGGAGTACTAC CACTGTTCAGGTG-3′) and its complement DG99 for pDVG50 (C1→A mutation in the 23-signal). Substrate pDVG51, containing a bad coding flank at the 23-signal side, was made by cloning the SalI/MluI fragment of pDVG42 into the SalI and MluI sites of pMS325, which has the same sequence as pMS319 (Sadofsky et al., 1995), except for a 5 bp deletion in the DNA flanking the 23-signal, resulting in the coding flank sequence 5′-TCC reading into the 23-signal heptamer; pDVG53, with bad coding flanks at both RSSs, was generated by replacing the 12-signal of pDVG51 with oligonucleotides DG102 (5′-TCGACCCGGGTTTTTGTTCCAGT CTGTAGCACTGTGGTC-3′) and its complement DG103.

Gel Electrophoresis

Cleavage products of oligonucleotide substrates were separated by electrophoresis in 12.5% Tris–borate–EDTA/urea gels as described (McBlane et al., 1995).

Reaction products of plasmid cleavage were separated by electrophoresis through 1% agarose/Tris–acetate–EDTA gels (20 V, overnight). Gels were then equilibrated in 0.4 M NaOH for 10 min, and DNA was transferred onto GeneScreen Plus hybridization transfer membranes (Dupont NEN Research Products) in this buffer, using a Posiblotter (Stratagene). The DNA was cross-linked to the membrane by UV irradiation, and blots were hybridized with the 0.9 kb XhoI–MluI insert of pDVG42. Reaction products were visualized by autoradiography and quantified by phosphorimaging (using a Molecular Dynamics Phosphorimager with ImageQuaNT 4.1 software).

Two-dimensional agarose gel electrophoresis was done as described (Roth et al., 1992b). Southern blots from two-dimensional gels were probed with the 1.9 kb XmnI/SalI fragment containing the coding flank sequence on the 12-signal side (see Figure 2A), and products were visualized as described above.

Cleavage Reactions

The RAG proteins used in these reactions were coexpressed in insect cells as fusions to maltose-binding protein and purified as described (MR1 and MR2 [McBlane et al., 1995]). Standard reactions were done in 10 μl containing 25 mM MOPS-KOH (pH 7.0), 5 mM Tris–HCl, 30 mM KCl, 30 mM potassium glutamate, 2.2 mM dithiothreitol, 2% (v/v) glycerol, 1 mM $MnCl_2$ or 1 mM $MgCl_2$ (including components from the protein preparations), 50 ng of plasmid substrate (linearized with AatII), and 50 ng of both the RAG1 and RAG2 proteins. Reaction mixtures were incubated for 1 hr at 37°C (unless stated otherwise). Then SDS was added to 0.1%, and 40 ng of DNA was used for analysis on Tris–acetate–EDTA/agarose gels or two-dimensional agarose gel electrophoresis. The expected amount of cleavage at both RSSs for entirely uncoupled cutting was calculated as the fraction cleaved at the 12-signal (cleavage at the 12-signal only plus cleavage at both signals) times fraction cleaved at the 23-signal (cleavage at the 23-signal only plus cleavage at both signals).

Oligonucleotide cleavage assays were done under the same reaction conditions, but 0.2 pmol of double-stranded oligonucleotide substrate DAR39/40 (McBlane et al., 1995) was used instead of plasmid substrate.

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Note Added in Proof

Coupled cleavage of V(D)J recombination substrates in a cell extract has recently been reported (Eastman, Q. M., Leu, T. M. J., and Schatz, D. G., [1996]. Initiation of V(D)J recombination in vitro obeying the 12/23 rule. Nature *380*, 85–88).