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Initiation of V(D)J Recombination in a Cell-Free System

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Summary

Cells performing V(D)J recombination make specific cuts in DNA at recombination signal sequences. Here, we show that nuclear extracts of pre-B cell lines carry out this specific cleavage. The products of cleavage are the same as found previously in thymocytes: full-length, blunt, 5′-phosphorylated signal ends, and covalently sealed (hairpin) coding ends. A complete signal sequence is required. Recombinant RAG1 protein greatly increases activity and complements an inactive extract from a *RAG1* (−/−) pre-B cell line. When the extracts are fractionated, cleavage activity correlates with the presence of RAG2 protein. These results suggest that RAG1 and RAG2 are components of the V(D)J recombinase.

Introduction

Mature immunoglobulin and T cell–receptor genes are assembled during the development of lymphoid cells from separate gene segments (for recent reviews, see Gellert, 1992; Lewis, 1994). This process, called V(D)J recombination, requires the presence of recombination signal sequences, consisting of a conserved heptamer sequence and a conserved nonamer sequence separated by a relatively nonconserved 12 or 23 base pair (bp) spacer region. The DNA is cut adjacent to the heptamer, followed by a head-to-head ligation of two signal sequences (a signal joint) and ligation of the two coding ends (a coding joint). Recombination always takes place between one signal sequence with a 12 bp spacer (12-signal) and one with a 23 bp spacer (23-signal) (Tonegawa, 1983). Signal joints are normally exact head-to-head ligations of two heptamers, whereas coding joints often contain deletions or additions of a few nucleotides.

A first indication of possible intermediates in this recombination reaction was obtained by analysis of the TCRδ locus in the thymus of newborn mice (Roth et al., 1992a). Double-stranded breaks (DSBs) were found at the signal sequence borders. Similar DSBs have been found in the immunoglobulin heavy and light chain loci (Schlissel et al., 1993; D. A. R. and M. G., unpublished data) and the TCRβ locus (J. F. M. and M. G., unpublished data), implying that DSBs are generally associated with V(D)J recombination. Almost all signal ends are blunt and 5′-phosphorylated, and they terminate exactly at the heptamer border (Roth et al., 1993; Schlissel et al., 1993). In thymocyte DNA from wild-type mice, only signal ends were found, and no coding ends. However, in mice with the severe combined immunodeficiency (*scid*) mutation, coding ends could also be detected. These coding ends were covalently closed, forming a hairpin structure (Roth et al., 1992b). On the basis of the frequent observation of coding joints with self-complementary (P nucleotide) insertions, it was suggested that these hairpin DNA molecules are intermediates in the V(D)J joining reaction in normal cells as well.

Several genes have been shown to be involved in V(D)J recombination. The recombination-activating genes 1 and 2 (*RAG1* and *RAG2*) are the only lymphoid-specific genes that are required (Schatz et al., 1989; Oettinger et al., 1990). Cotransfection of *RAG1* and *RAG2* expression plasmids together with a recombination substrate into fibroblasts results in recombination of this substrate (Oettinger et al., 1990). Mice lacking either of these two genes do not recombine their immunoglobulin or T cell–receptor genes, and they do not develop mature B or T cells (Mombaerts et al., 1992; Shinkai et al., 1992). Pre-B cell lines derived from these mice do not carry out V(D)J joining on recombination substrates. Upon further investigation, it was found that relatively large parts of the RAG1 and RAG2 proteins could be deleted without losing recombination activity (Sadofsky et al., 1993; Silver et al., 1993; Cuomo and Oettinger, 1994; Sadofsky et al., 1994). Mutations in a specific region of the RAG1 protein lead to a dramatically increased sensitivity to sequence alterations in substrates, within and near the signal heptamer, suggesting a direct interaction of RAG1 at the site of recombination (M. J. S., J. E. H., D. C. v. G., and M. G., unpublished data).

In addition to these lymphoid-specific genes, several DSB repair gene products are required for signal joint formation, coding joint formation, or both (Pergola et al., 1993; Taccioli et al., 1993). These include the *XRCC5* gene, identified as coding for the p80 component of the Ku antigen (Rathmell and Chu, 1994; Smider et al., 1994; Taccioli et al., 1994; Boubnov et al., 1995; Finnie et al., 1995), and the *scid* gene, recently identified as the gene for the catalytic component of the DNA-dependent protein kinase (DNA-PK_{CS}) (Blunt et al., 1995; Kirchgessner et al., 1995). These factors are believed to act late in V(D)J recombination, after the initial specific breaks are made.

A better understanding of the recombination reaction, the components involved in it, and the reaction mechanism requires development of a cell-free system in which the process can be analyzed in detail. Here we report reconstitution of the earliest step of the recombination reaction, the site-specific cleavage at signal sequences, in nuclear extracts of pre-B cell lines supplemented with recombinant RAG1 protein. Fractionation of the extracts also implicates RAG2 protein in the reaction. Both signal and coding ends are detected, and their structures are similar to those found in vivo: full-length, blunt, 5′-phosphorylated signal ends and covalently closed (hairpin) coding ends.

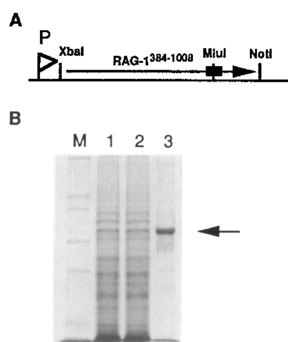

Figure 1. Expression and Purification of RAG1 Protein

(A) Schematic representation of the baculovirus transfer vector pDVG23. The closed box represents the His tag and Myc epitope tag. P, polyhedrin promoter. Only the relevant part of the construct is shown.

(B) SDS–PAGE analysis of total protein in Sf9 cells expressing RAG1 (lane 1), soluble fraction after sonication (lane 2), and RAG1 protein purified by Ni^{2+} chromatography (lane 3). Lane M contains protein size markers; from top to bottom, the molecular masses are 200, 116, 97, 66, 55, 36, and 31 kDa, respectively.

Results

Components of the Cell-Free Reaction

Since the V(D)J recombination reaction is thought to proceed through an intermediate in which DSBs are present at the signal sequences prior to ligation, we set out to reproduce this first step (formation of DSBs) in a cell-free reaction. We used pre-B cell nuclear extracts as the source of recombination factors. To get the highest level of recombination activity, we used a recently described inducible pre-B cell line, 103/BCL-2 (Chen et al., 1994). This cell line harbors a temperature-sensitive v-*abl* oncogene. Upon shift from the permissive to the nonpermissive temperature, expression of RAG1 and RAG2 is up-regulated, and the recombination activity is dramatically increased. Nuclear extracts were made from cells that had been shifted to the nonpermissive temperature for 20–24 hr.

As described in the Introduction, there is reason to assume that the RAG1 protein is directly involved in the recombination reaction. A convenient way to express this protein is to use a baculovirus expression vector in insect cells. However, when we expressed the full-length RAG1 protein in this system, virtually all of it was insoluble (data not shown). Since it was found that a truncated RAG1 protein containing amino acids 384–1008 supported V(D)J recombination in fibroblasts (Sadofsky et al., 1993), we produced this shortened protein in the baculovirus expression system. A His tag was added to the C-terminus for easy purification, and a Myc epitope tag for efficient detection (Figure 1A). This protein was soluble and could be purified on a Ni^{2+}–Sepharose column (Figure 1B).

Recombinant RAG2 protein was produced in Escherichia coli as a fusion of amino acids 1–387 of RAG2 and maltose-binding protein (added for reasons of solubility), with a His tag and a Myc epitope tag added on the C-terminus. The protein was purified on an amylose resin column. When expressed in fibroblasts, this protein (together with RAG1) supported V(D)J recombination (data not shown).

Reconstitution of Cleavage in a Cell-Free Reaction

The cell-free cleavage reaction was carried out by use of nuclear extracts from induced 103/BCL-2 cells, with or without addition of recombinant RAG1 protein. Plasmid pJH200, which acquires a signal joint upon recombination, was used as substrate. DSBs at the recombination signal sequences were detected by the ligation-mediated polymerase chain reaction (LMPCR) as outlined in Figure 2A. LMPCR should produce a 145 bp product at the 12-signal end and a 173 bp product at the 23-signal end. Ligation of the LMPCR linker requires a blunt, 5′-phosphorylated end on the target DNA; ligation to a heptamer sequence creates an ApaLI restriction site. Since virtually all DSBs detected in thymocytes were immediately adjacent to the heptamer (Roth et al., 1992a; Roth et al., 1993; Schlissel et al., 1993), we used ApaLI sensitivity of the PCR product as an additional criterion for bona fide V(D)J cleavage activity. As shown in Figure 2B, a nuclear extract from 103/BCL-2 cells supplemented with recombinant RAG1 protein was able to generate DNA molecules broken at the 12-signal (lane 5), and this 145 bp PCR product was shortened by ApaLI digestion to a 120 bp fragment (lane 6), confirming that it represents a signal end. Treatment of signal ends with T4 DNA polymerase prior to LMPCR did not significantly increase the amount of PCR product, further indicating that most signal ends are blunt (data not shown). Without recombinant RAG1 protein, a low number of DSBs were detected (lanes 3 and 4). The RAG1 protein alone did not have any specific nuclease activity (lanes 7 and 8). Addition of the RAG2 fusion protein did not stimulate the reaction under any conditions tested. This protein preparation was not investigated further (but see the Discussion).

At the 23-signal, similar DSBs were detected (Figure 2C), though the bands were less intense. (In some experiments, a small fraction of the material at the signal end position was not shortened upon ApaLI digestion. We do not yet know whether this unshifted band arises from imprecise cleavage of a fraction of signal ends or whether it is accidentally amplified DNA of the same size.)

Since DSBs could be detected more efficiently at the 12-signal, cleavage was monitored at this position unless otherwise stated. Standard reaction conditions are described in the Experimental Procedures. Substitution of $MgCl_2$ (1–10 mM) for $MnCl_2$ resulted in increased background nuclease activity and low or undetectable cleavage at the signal sequence (Table 1). In the presence of 2 mM EDTA, no specific cleavage was detected.

In the standard reaction, no high energy cofactors were added. Addition of ATP (at 0.5 mM) and the other nucleoside triphosphates (NTPs) and dNTPs (50 μM each) did not affect the level of cleavage (Table 1). Although initial cleavage experiments were done with supercoiled plasmid substrates, a linear fragment (the 3.2 kb EcoRI fragment of pJH200) was cleaved to a similar extent (data not

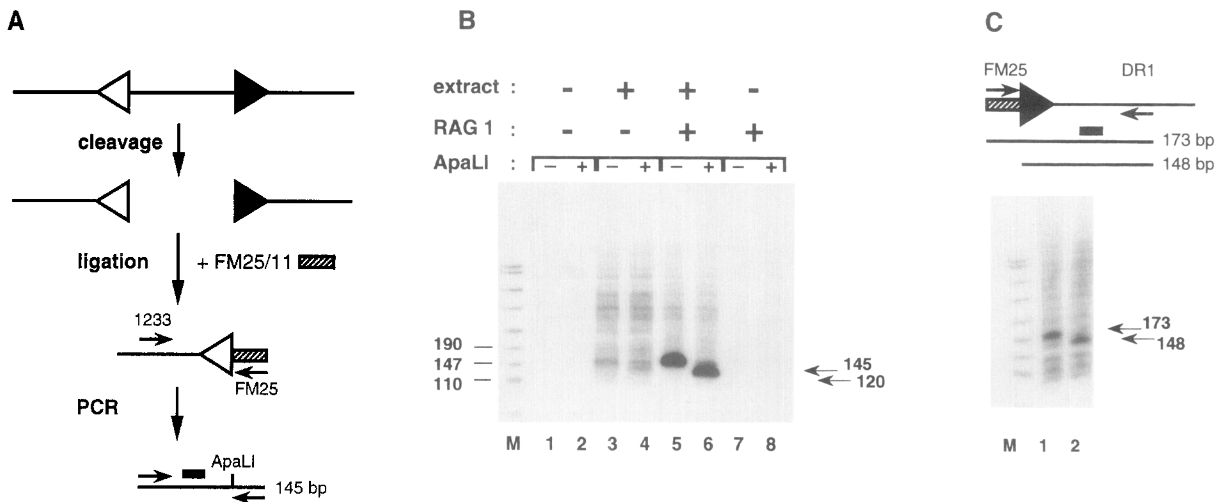

Figure 2. Cleavage of Recombination Signal Sequences

(A) Outline of the LMPCR reaction. Open triangles represent 12-signals, closed triangles 23-signals. The positions of PCR primers are depicted by arrows, the FM25/11 linker is represented by a striped box, and the oligonucleotide probe used for detection on Southern blots is represented by a closed box. Only cleavage at the 12-signal is diagrammed; cleavage at the 23-signal can be detected in a similar way.

(B) Detection of 12-signal ends by LMPCR. Cleavage reactions were done with buffer only (lanes 1 and 2), 103/BCL-2 nuclear extract only (lanes 3 and 4), 103/BCL-2 nuclear extract supplemented with RAG1 protein (lanes 5 and 6), or RAG1 protein only (lanes 7 and 8). PCR products were digested with ApaLI in lanes 2, 4, 6, and 8. Arrows depict the positions of the 145 bp specific PCR product, and the 120 bp product resulting from ApaLI digestion.

(C) Detection of 23-signal ends by LMPCR. The PCR primers and oligonucleotide probe used for detection on Southern blots are shown on the diagram. Cleavage reactions were done with 103/BCL-2 nuclear extracts supplemented with RAG1 protein. PCR products were digested with ApaLI in lane 2. Arrows indicate the positions of the 173 bp specific PCR product and the 148 bp product resulting from ApaLI digestion. Blots were probed with oligonucleotide MS174 (B) or FM30 (C). The marker lane (M) contains a HpaII digest of plasmid pUC19; the sizes of relevant marker fragments are shown on the left.

Table 1. Requirements for V(D)J Cleavage Activity

Reaction Conditions	Activity
Standard reaction	+
No extract	−
No recombinant RAG 1 protein	±
Addition of NTPs and dNTPs	+
$MgCl_2$ instead of $MnCl_2$	−
EDTA instead of $MnCl_2$	−
Linear DNA instead of supercoiled plasmid	+
Extract heated to 65°C for 15 min	−
Extract incubated with RNase	+

Plus, 50%–150% of activity of standard reaction; plus/minus, less than 10% of activity of standard reaction; minus, undetectable activity.

shown), indicating that neither a circular form of DNA nor supercoiling is required for site-specific cleavage.

To get a first indication of the nature of factor(s) contributed by the nuclear extract, we incubated the extract for 15 min at 65°C. This treatment inactivated it, suggesting that the extract factor has a protein component. Preincubation of the extract with RNase (10 U of RNace-It, from Stratagene) for 15 min at 30°C did not result in a significant reduction in cleavage activity, indicating that RNA is not required for this reaction.

Characterization of the Broken DNA

Incubation of substrate DNA with an extract could be anticipated to yield breaks terminating at signal sequences and coding sequences and might conceivably generate completed signal joints or coding joints. As shown above, nuclear extracts of 103/BCL-2 cells can mediate formation of blunt-ended, 5′-phosphorylated DSBs exactly at the border of signal sequences. However, we were unable to detect open coding ends by LMPCR in these samples (data not shown). In addition, neither signal joints nor coding joints were detectable by the appropriate PCR assay (data not shown).

As the coding ends found in *scid* mouse thymus have been shown to be in covalently closed (hairpin) structures (Roth et al., 1992b; Zhu and Roth, 1995), we searched for such DNA molecules. We incubated the BglI–BglII fragment of the coding joint retaining recombination substrate pMS319 (diagrammed in Figure 3A) with nuclear extract and RAG1 protein and analyzed the DNA by two-dimensional agarose gel electrophoresis. On these gels, the DNA is separated in the first dimension under native conditions, and in the second dimension under denaturing conditions. Linear fragments of DNA will appear on a diagonal, except for molecules whose strands are covalently linked; those will run above the diagonal. Nicked linear DNA will appear below the diagonal. As shown in Figure 3C, a product is formed that runs above the diagonal and is at the expected size for a coding end at the 12-signal side. The vertical trail from the spot down to the diagonal is caused by nonspecific nicking activity in the extract. No hairpin DNA was detected in a parallel

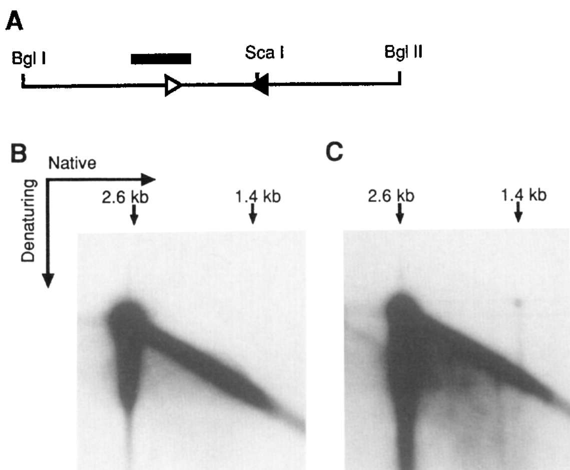

Figure 3. Detection of Hairpin Coding Ends by Two-Dimensional Gel Electrophoresis

(A) Schematic representation of the DNA substrate (the BglI–BglII fragment of pMS319).

(B) and (C) are from an experiment in which the cleavage reaction, electrophoresis, and Southern blot analysis were performed in parallel. In (B), the DNA from a mock cleavage reaction without extract or RAG1 protein is shown, and (C) displays the DNA from a complete cleavage reaction including RAG1 protein and a nuclear extract of 103/BCL-2 cells. DNA was electrophoresed under native conditions in the first dimension (from left to right). The gel slice was then equilibrated in denaturing buffer, and the DNA was electrophoresed under denaturing conditions in the second dimension (top to bottom). The arrows show the expected sizes after native electrophoresis of the BglI–BglII substrate fragment (2.6 kb) and a broken coding end at the 12-signal side (1.4 kb) and were determined by comparison to molecular mass standards. The probe used for analysis of the Southern blot is depicted as a bar in (A). See Experimental Procedures for details.

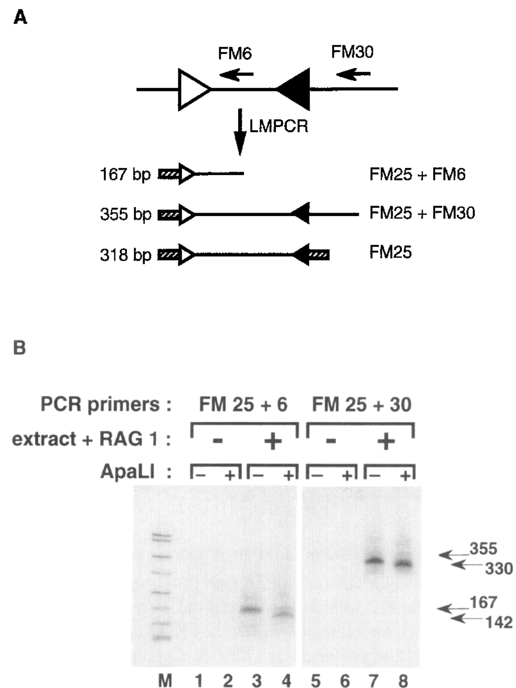

Figure 4. Cleavage at the 12-Signal without Cutting at the 23-Signal

(A) Schematic representation of LMPCR reactions with various primer sets. The length of the expected PCR products is depicted on the left.

(B) LMPCR analysis of the cleavage reaction with different primer sets: FM25 plus FM6 (lanes 1–4) or FM25 plus FM30 (lanes 5–8). Reactions were done without (lanes 1, 2, 5, and 6) or with (lanes 3, 4, 7, and 8) 103/BCL-2 nuclear extract and RAG1 protein. PCR products were digested with ApaLI in lanes 2, 4, 6, and 8. The blot was probed with oligonucleotide DG 38. Lane M contains DNA size markers; see Figure 2 for details. Arrows indicate the specific PCR products before and after ApaLI digestion.

experiment, in which RAG1 protein and the nuclear extract were omitted from the incubation (Figure 3B). It was also possible to detect the hairpin coding end at the 23-signal side, but there was about 10-fold less of this species (data not shown). The presence of hairpin coding ends was also demonstrated in experiments where samples were treated with mung bean nuclease, which specifically cleaves hairpins near their terminus, prior to a standard LMPCR reaction (data not shown).

To investigate whether hairpin formation is coupled to the initial cleavage, or whether any blunt-ended DNA molecules made by endonuclease digestion could also be converted into hairpins, we included a blunt-ended fragment in the cleavage reaction; 0.4 ng of the BglI–ScaI fragment of pMS319 was added to a cleavage reaction containing 40 ng of the BglI–BglII fragment, and this DNA was incubated with extract and RAG1 protein. Only the product formed by cleavage at the V(D)J signal border was converted into a hairpin, and not the control fragment (data not shown), indicating that hairpin formation is linked to the specific cleavage reaction.

In summary, we can detect both signal and coding ends, both at the 12-signal and the 23-signal side. Is cleavage at the two signals coupled? By use of the appropriate primer sets, an LMPCR experiment can answer this question. After cleavage of pMS319 in the cell-free reaction, amplification with FM25 and FM6 should result in a 167 bp product for a 12-signal end, whether or not the 23-signal is cut (Figure 4A). With FM25 and FM30 as primers, a 355 bp product should be formed if the 12-signal is cut, but only if there is no cut at the 23-signal. Both these products can be detected, and they are ApaLI sensitive (Figure 4B), showing that at least a fraction of the substrate is only cut at the 12-signal. If the substrate were cut at both signals, then amplification with only FM25 should have resulted in a 318 bp product. This product was not detected even at long exposures (data not shown), indicating that there is very little if any DNA cut at both signals.

Subsequently, we showed that a second consensus signal sequence is not needed even for recognition. As shown in Figure 5A (lanes 7 and 8), pFM201, which contains only a 12-signal, was cut to about the same extent as a substrate with both signals. However, we cannot exclude the possibility that other sequences in the plasmid might act as cryptic signals. Plasmid pFM203, which contains only a 23-signal, is also a substrate for V(D)J cleavage (Figure 5B, lanes 7 and 8). When the nonamer of the 12-signal is deleted (pMS366), the remaining heptamer is not cleaved (Figure 5C, lanes 7 and 8), showing that the nonamer is important for site-specific cleavage.

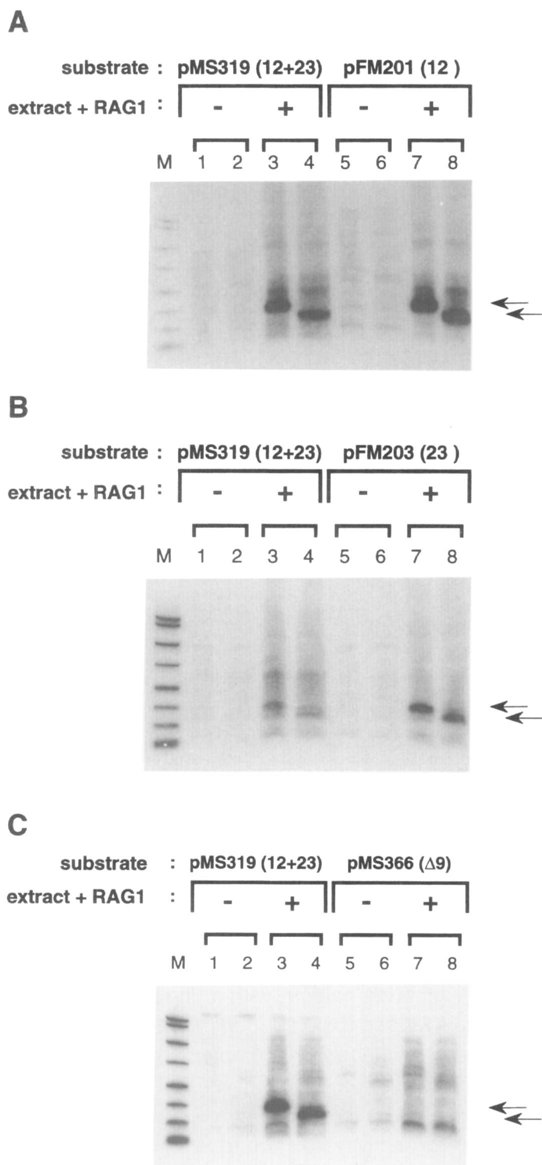

Figure 5. Cleavage of Plasmid Substrates with Various Signal Sequences

(A) Cleavage at the 12-signal of pMS319, which has 12- and 23-signals (lanes 1–4), or pFM201, which has only a 12-signal (lanes 5–8). Lanes 1, 2, 5, and 6 are negative controls; lanes 3, 4, 7, and 8 are cleavage reactions. The PCR products in lanes 2, 4, 6, and 8 were digested with ApaLI.

(B) Cleavage at the 23-signal of pMS319 (lanes 1–4) or pFM203 (which has only a 23-signal; lanes 5–8); incubation conditions were as described for (A). The arrows indicate the PCR product of 174 bp and its ApaLI digestion product of 149 bp.

(C) Cleavage at the 12-signal of pMS319 (lanes 1–4) or at the single heptamer of pMS366 (lanes 5–8); incubation conditions were as described for (A). PCR primers used were FM6 and FM25 in (A) and (C), or FM6B and FM25 in (B). Note that the PCR product in lanes 7 and 8 is not at the correct position for a signal end (which should be 149 bp long) and is not sensitive to ApaLI digestion. The blots were probed with oligonucleotides DG38 (A and C) or DG37 (B). Lane M contains DNA size markers; see Figure 2 for details. Arrows point toward the specific PCR products before and after ApaLI digestion.

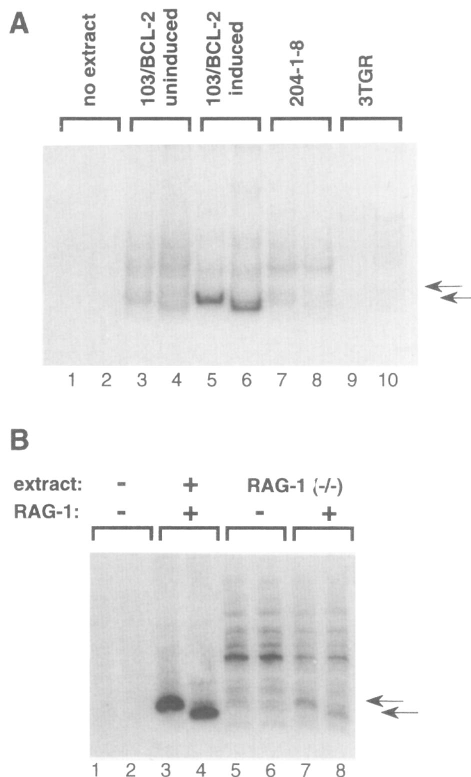

Figure 6. V(D)J Cleavage in Nuclear Extracts from Various Cell Types

(A) Cell type specificity of the V(D)J cleavage activity. Cleavage at the 12-signal of pJH200 without extract (lanes 1 and 2), or with nuclear extracts of uninduced 103/BCL-2 cells (lanes 3 and 4), induced 103/BCL-2 cells (lanes 5 and 6), 204-1-8 cells (lanes 7 and 8), or 3TGR cells (lanes 9 and 10). All assays were done in the presence of added RAG1 protein. PCR products were digested with ApaLI in lanes 2, 4, 6, 8, and 10.

(B) Complementation of a *RAG1* (–/–) extract by purified RAG1 protein. Cleavage at the 12-signal of pJH200 without extract (lanes 1 and 2), or with nuclear extract of induced 103/BCL-2 cells (lanes 3 and 4) or the *RAG1* (–/–) cell line 2A15 (lanes 5–8), without (lanes 1, 2, 5, and 6) or with (lanes 3, 4, 7, and 8) additional recombinant RAG1 protein. PCR products were digested with ApaLI in lanes 2, 4, 6, and 8. Blots were probed with oligonucleotide MS174. Arrows point toward the specific PCR products before and after ApaLI digestion.

A Direct Role for RAG1 and RAG2

Pre-B cell lines have varying levels of V(D)J recombination activity. The pre-B cell line 204–1–8 (McKearn and Rosenberg, 1985) is known to have a low recombination activity; uninduced 103/BCL-2 cells are more active, and upon temperature shift, these cells are induced to a very high level of recombination. The fibroblast cell line 3TGR does not have any recombination activity. The cleavage activity profile of nuclear extracts prepared from these cell lines paralleled the recombination activity of the cells (Figure 6A).

What factors in the extracts are responsible for cleavage? Obvious candidates are the RAG1 and RAG2 proteins, possibly in complexes with as yet unidentified additional factors. As shown above, addition of recombinant RAG1 protein to nuclear extracts from pre-B cell lines enhances cleavage, suggesting that the RAG1 protein is directly involved in the reaction. However, this observation does not exclude the possibility that endogenous RAG1

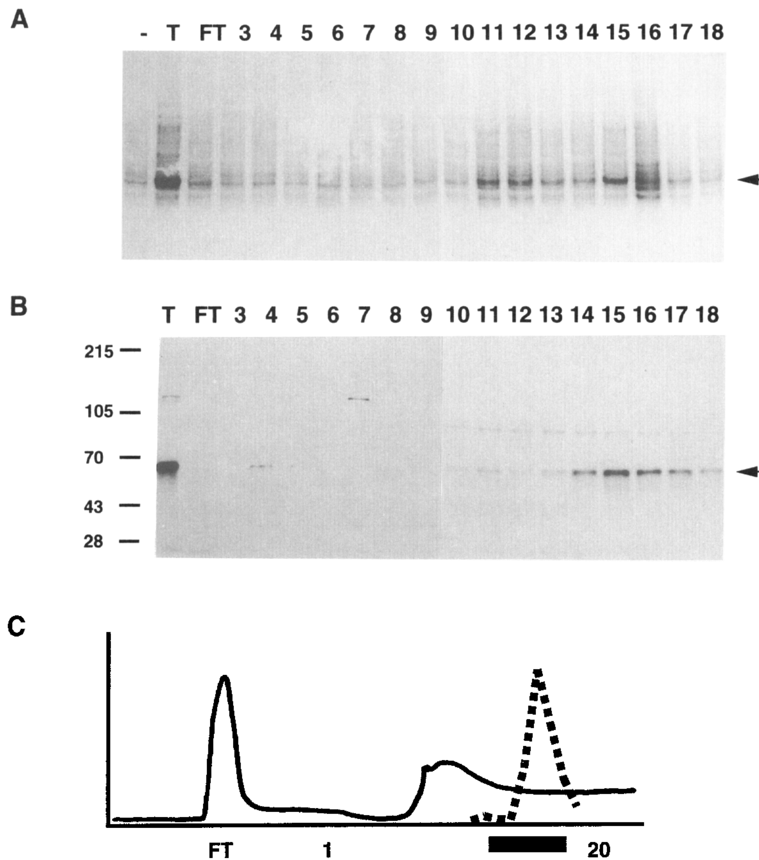

Figure 7. Fractionation of Nuclear Extract of 103/BCL-2 Cells on a Mono S Column

The elution profile of the cleavage activity is shown in (A) and a Western blot of RAG2 protein in (B). (C) shows the elution profile of total protein (solid line) and summarizes the profiles of cleavage activity (closed bar) and RAG2 protein (broken line) from (A) and (B). The positions of the flowthrough (FT) and fractions 1 and 20 are indicated. All assays in part (A) were done with added RAG1 protein. Total extract (T), flowthrough (FT), and fractions 3–18 were assayed. The arrow points toward the specific LMPCR product in (A) and the RAG2 protein in (B). The blot in (A) was probed with oligonucleotide MS174. The molecular masses of marker proteins in (B) are indicated in kilodaltons.

might have contributed to the transcriptional or translational activation of other genes. To resolve this question, we made nuclear extracts from the *RAG1* (−/−) pre-B cell line 2A15. These extracts alone do not support any site-specific cleavage (Figure 6B, lanes 5 and 6). However, after addition of RAG1 protein, DSBs can be observed (Figure 6B, lanes 7 and 8), showing that RAG1 does not need to activate other genes in order to support cleavage at recombination signal sequences. The activity in the RAG1-supplemented 2A15 extract is not as high as in an extract of 103/BCL-2 cells, presumably because 2A15 is more analogous to a standard Abelson murine leukemia virus (AMuLV)-transformed cell line such as 204-1-8.

Defining the role of RAG2 in the cell-free reaction was more difficult. Although RAG2 is required for V(D)J rearrangement in lymphoid cells, it was not initially clear whether it was necessary for the cleavage activity detected here. Recombinant RAG2 protein failed to provide any stimulation (see above). Nevertheless, an extract from a *RAG2* (−/−) AMuLV cell line supplemented with recombinant RAG1 was completely inactive, consistent with a role for RAG2 in cleavage. (This extract could not be complemented by recombinant RAG2 protein either. However, we believe this RAG2 protein preparation may be inactive).

Stronger evidence that RAG2 protein is likely to be directly involved in cleavage came from fractionation of nuclear extracts. Extract proteins were separated on a Mono S cation-exchange column with a linear gradient of KCl (40 mM to 1 M; see Experimental Procedures for details). When these fractions were complemented with recombinant RAG1 protein, the highest level of cleavage activity was detected in fraction 15 (Figure 7A). Lower but detectable cleavage activity was found in fractions 11–17 (approximately 0.5–0.8 M KCl). The specific PCR product is sensitive to ApaLI digestion (data not shown). The products formed with RAG1 protein alone or in fractions 3–10 are not ApaLI sensitive and are therefore considered to be caused by nonspecific nucleases. The active fractions contain only a small proportion of the total protein, approximately 1/20 of the amount applied to the column. As shown in Figure 7C, approximately half of the total protein was found in the flowthrough, and most bound proteins were eluted between 0.2 and 0.5 M KCl. The fractions were also assayed by Western blot for the presence of RAG2 protein (Figure 7B). Most of the cleavage activity was present in the fractions containing the RAG2 protein (fractions 11–17), with the highest level of activity in the fraction with the greatest amount of RAG2 (fraction 15). (Closer inspection of the elution profiles suggests that earlier fractions are relatively more active than later ones. We are currently investigating whether this is significant.)

Extracts were also separated on anion-exchange and gel filtration columns, and in both cases activity was again found in fractions enriched for RAG2 protein (data not

shown). Because activity is recovered in fractions highly enriched in RAG2, these results strongly suggest that the RAG2 protein is directly involved in the cleavage reaction.

Discussion

This cleavage activity faithfully reproduces many features of the first stages of V(D)J recombination in cells. Cleavage is totally dependent on the presence of the RAG1 protein, and probably also the RAG2 protein. DNA is cut specifically at the border of recombination signal sequences, leaving a full-length, blunt, 5′-phosphorylated signal end and a coding end with a hairpin structure. Recombination signal sequences must contain a heptamer and a nonamer in order to be cleaved. The level of specific cleavage activity in nuclear extracts made from different cell lines correlates with the level of recombination carried out by these cell lines (in transfection assays with plasmid substrates). This observation supports the hypothesis that formation of DSBs is the rate-limiting step in V(D)J recombination.

The only significant difference from intracellular events is that cell-free cleavage does not require a pair of signal sequences: cuts at one signal predominate over pairwise cuts, and DNA with a single signal sequence is cut efficiently. By contrast, in vivo the evidence points toward coupled cleavage at both signal sequences. At the TCRδ locus in mouse thymocytes, only pairwise cleavage could be detected on Southern blots (Roth et al., 1992a). Furthermore, the lifetime of coding ends in normal cells is much shorter than that of signal ends (Roth et al., 1992a; Zhu and Roth, 1995). This would not be expected if cleavage were uncoupled. It is also difficult to imagine why the coding end from a 12-signal would always be ligated to the coding end from a 23-signal if cleavage and rejoining of ends did not take place in a coordinated complex. A similar type of uncoupling has been seen in the cell-free form of other recombination reactions, for example, retroviral integration (Bushman and Craigie, 1991). A precedent for single-site cleavage may exist in the rare intracellular V(D)J-mediated events leading to so-called open and shut joints (Lewis and Hesse, 1991).

The observation of hairpin DNA coding ends in the cell-free reaction should be emphasized. Previously, such ends had been found only in DNA from *scid* cells, and it remained possible, though unlikely, that the *scid* defect results in diversion of coding end DNA to this structure. The fact that hairpins are also detected in the present experiments supports the hypothesis that they are on the direct pathway of V(D)J joining.

The formation of the phosphodiester bond between the two strands requires energy, but is carried out without addition of high energy cofactors, suggesting that the hairpin is formed as a result of the cleavage reaction rather than as a later step. The energy of one broken phosphodiester bond could be conserved to form the new bond between strands by either direct *trans*-esterification or through a covalent intermediate with the recombinase. However, we cannot exclude the possibility that the extract provides a separate factor (with a tightly bound high energy cofactor) that forms a hairpin after the original cleavage reaction has taken place. Although blunt DNA ends added to nuclear extracts are not converted into hairpins (showing that hairpin formation is not a general activity of these extracts), one could still envision a separate hairpin-forming factor being recruited to coding ends by the V(D)J recombinase.

The results obtained here provide some information about the role of the RAG1 and RAG2 proteins in V(D)J recombination. It had been observed that these gene products together can induce V(D)J recombination in cells that do not normally have this activity, such as fibroblasts. It was not known, however, how they activate recombination. The main possibilities were the following: first, activation of recombinase expression; second, activation of the recombinase protein; or third, a direct role in recombination. The last possibility seemed the most likely, since the only difference that could be observed between fibroblasts with and without RAG expression was their ability to recombine V(D)J-joining substrates, and mice lacking either RAG1 or RAG2 were normal except for the absence of mature B and T cells. In addition to this, a RAG1 mutant has been identified that is much more sensitive to sequence variations in and next to the heptamer, suggesting that the RAG1 protein interacts with the DNA in that region (M. J. S., J. E. H., D. C. v. G., and M. G., unpublished data). We present here direct proof that the RAG1 protein activates cleavage at recombination signal sequences without transcription or translation: nuclear extracts from pre-B cells without a functional *RAG1* gene can be complemented by recombinant RAG1 protein. Although we cannot exclude the possibility that RAG1 only activates the recombinase, without being part of the complex, our working model is that the RAG1 protein is an integral part of the V(D)J recombinase.

As stated above, RAG2 is required for V(D)J recombination in vivo. Here we have shown that nuclear extracts with the highest level of RAG2 protein have the highest cleavage activity and that a *RAG2* (−/−) cell line is inactive, suggesting that cleavage involves RAG2 as well as RAG1. Upon fractionation of the extract, cleavage activity copurifies with RAG2 protein, showing that RAG2 is probably directly involved in the cleavage reaction. Only a minority of all extract proteins is present in the active fractions, suggesting that the extract provides either only RAG2 protein, or perhaps a complex of RAG2 protein and another factor (or factors). However, we have not been able to stimulate cleavage by addition of recombinant RAG2 protein. It is possible that the form of RAG2 protein we added is not active. Alternatively, one could envision that in addition to its direct function in the cleavage reaction, RAG2 might also be required for activation of another gene (or genes). Further fractionation and characterization of the cleavage activity is in progress.

In conclusion, our current working model is that the RAG1 and RAG2 proteins are directly involved in generation of DSBs at recombination signal sequences, possibly together with another factor (or factors) from pre-B cell nuclear extracts. The products of cleavage are blunt, 5′-phosphorylated signal ends and covalently closed (hairpin) coding ends (Figure 8). Since these are the same

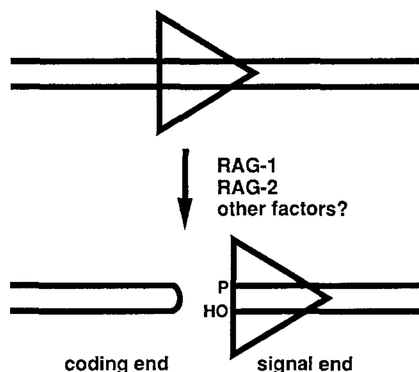

Figure 8. Model for V(D)J Cleavage

The DNA is cleaved at the border of the signal sequence (depicted by a triangle) by a recombinase complex containing RAG1, RAG2, and possibly other proteins. The result of the reaction is a blunt-ended, 5'-phosphorylated signal end and a covalently closed (hairpin) coding end.

products as those found in vivo, we believe we have reconstituted the earliest steps of V(D)J recombination.

Experimental Procedures

Cell Culture

AMuLV-transformed cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and 50 μM β-mercaptoethanol, at 5% CO_2 (Rosenberg et al., 1975). Cell line 103/BCL-2 (a gift from N. Rosenberg) was grown at the permissive temperature (34°C), then transferred to the nonpermissive temperature (39.5°C) to induce higher recombination activity (Chen et al., 1994). AMuLV-transformed cell lines 2A15 and R2-2 were gifts from C. Paige and G. Wu. The 2A15 line is derived from *RAG1*-deficient mice (Mombaerts et al., 1992), and the R2-2 line from *RAG2*-deficient mice (Shinkai et al., 1992). The fibroblast cell line 3TGR (Schatz and Baltimore, 1988) was grown in DMEM supplemented with 10% bovine serum and antibiotics, at 5% CO_2. The insect cell line Sf9 was grown in supplemented Grace's insect medium (Bethesda Research Laboratories) with 10% fetal bovine serum at 27°C. Standard baculovirus techniques were carried out as described (O'Reilly et al., 1994).

DNA Techniques

Standard DNA techniques were used as described (Sambrook et al., 1989). The baculovirus transfer vector pDVG23 was constructed by cloning the 2.1 kb XbaI-NotI fragment of pMSD27B (Sadofsky et al., 1993), containing the *RAG1* open reading frame coding for amino acids 384–1008 fused to a His tag, and a Myc epitope tag, into the transfer vector pVL1393 (Pharmingen). The recombinant baculovirus AcD23 was made by using pDVG23 and the BaculoGold Transfection Kit (Pharmingen) as described by the manufacturer. The *RAG2* expression construct pDVG11 was made by cloning the NcoI–XbaI fragment of pMSE16 (Sadofsky et al., 1994) containing the *RAG2* open reading frame into the XmnI–XbaI-cut pMalc-2 vector (New England Biolabs) together with an adapter oligonucleotide. Recombination substrates used in standard cleavage reactions were signal joint–retaining plasmid pJH200 (Hesse et al., 1987) and coding joint–retaining plasmid pMS319 (M.J.S., unpublished data). Substrates pFM201 and pFM203, each containing only a single consensus signal sequence, were derived from pMS319 by standard methods. The 23-signal of pMS319 was removed to yield plasmid pFM201. Plasmid pFM203 was obtained by removing the 12-signal. Substrate pMS366 is a derivative of pMS319 in which the nonamer sequence has been removed from the 12-signal.

LMPCR

Linker FM25/11 (5'-GCGGTGACTCGGGAGATCTGAAGTG-3' annealed to 5'-CACTTCAGATC-3'; 40 pmol) was ligated to blunt-ended cleavage products with 2.5 U of T4 DNA ligase at 16°C. Following phenol–chloroform extraction and ethanol precipitation, ligation products were amplified by 25 cycles of PCR using linker-specific primer FM25 (5'-GCGGTGACTCGGGAGATCTGAAGTG-3') plus a recombination signal-specific primer. Each cycle comprised 15 s at 94°C, 15 s at 60°C, and 30 s at 72°C (in a PEC 9600 thermal cycler). The following primers were used: NEB#1233 (5'-GCGGATAACAATTTCACACAGGA-3') for 12-signal ends in pJH200; DR1 (5'CAACGGTGGTATATCCAGTG-3') for 23-signal ends in pJH200; FM6 (5'-TGGCTCGATTGGCGCGACAAGT-3') or FM30 (5'-CTCCATTTTAGCTTCCTTAGCTCCTG-3') for 12-signal ends in pMS319; and FM6B (5'-AACTTGTCGCGCCAATCGAGCCATG-3') for 23-signal ends in pMS319. Molecules cut at both the 12-signal and the 23-signal of pMS319 were assayed with primer FM25 alone. In this case, an initial incubation at 72°C for 2 min was carried out to fill in the 5' overhangs of the ligated linkers on both sides of a potential double cleavage product. Samples were analyzed by using 6% polyacrylamide gels (Novex) in TBE and visualized by ethidium bromide staining, Southern blotting, or both. Additional oligonucleotides used as hybridization probes were the following: MS174 (5'-GGGCTGCAGGTCGACGGATCCGCGCTAA-3'), DG37 (5'-GCATTGAGAACTTTGGAATCCAGTCC-3'), and DG38 (5'-GGCAACCGAGCGTTCTGAACAAATCCA-3').

Two-Dimensional Agarose Gel Electrophoresis

Two-dimensional agarose gel electrophoresis was carried out as described (Roth et al., 1992b); 40 ng of fragment was used per analysis. Southern blots were probed with a 285 bp probe spanning the 12-signal; 195 bp of this probe was derived from the coding sequence next to the 12-signal, and the remaining 90 bp include the 12-signal.

Protein Expression and Purification

For a typical RAG1 protein purification, two culture flasks (150 cm^2 each) of Sf9 cells were infected with the recombinant baculovirus AcD23. After 48 hr, the cells were harvested and washed with PBS, and cell pellets were stored at −80°C. The cell pellet was resuspended in 1 × binding buffer (20 mM Tris–HCl [pH 7.9], 0.5 M NaCl, 5 mM imidazole, 2 mM β-mercaptoethanol) and mildly sonicated. Cell debris was removed by centrifugation for 30 min at 10,000 × g. The RAG1-containing supernatant was loaded onto a 1 ml HiTrap chelating column (Pharmacia), which had been charged with $NiSO_4$. The column was washed with 5 ml of 1 × binding buffer and 5 ml of 1 × binding buffer with 60 mM imidazole. The RAG1 protein was eluted with a linear gradient from 60 mM to 1 M imidazole in binding buffer. Fractions containing RAG1 were pooled and dialyzed against buffer R (25 mM Tris–HCl [pH 8.0], 150 mM KCl, 2 mM DTT, 10% [v/v] glycerol) for 3 hr. Aliquots were frozen in liquid nitrogen and stored at −80°C. This procedure yielded approximately 500 μg of more than 90% pure RAG1 protein.

The RAG2 fusion protein was expressed in E. coli strain TB1 (New England Biolabs). A freshly transformed colony was grown in Super Broth (Advanced Biotechnologies, Incorporated, Columbia, MD) at 37°C to an OD_{600} of 1. Then 0.3 mM IPTG was added, and cells were harvested after 2 hr of induction. The fusion protein was purified on an amylose resin column as described by the manufacturer (New England Biolabs). Protein-containing fractions were pooled and dialyzed against buffer R for 3 hr. Aliquots were frozen in liquid nitrogen and stored at −80°C. This procedure yielded approximately 1 mg of fusion protein per liter of induced E. coli culture.

Preparation and Fractionation of Nuclear Extracts

For a typical extract preparation, 1 liter of 103/BCL-2 cells was grown at 34°C to a density of approximately 5×10^5 cells per ml. The temperature was then shifted to 39.5°C for 20–24 hr. Nuclear extracts were prepared as described (Parker and Topol, 1984). Other cell types were grown at 37°C to a density of 5×10^5 cells per ml, and nuclear extracts were prepared in the same way. Extracts could be stored for at least several months at −80°C without loss of activity.

Extracts were fractionated by cation-exchange chromatography. Nuclear extract from 1 liter of induced 103/BCL-2 cells in buffer C (25 mM HEPES–KOH [pH 7.5], 40 mM KCl, 0.1 mM EDTA, 10% [v/v] glycerol, 1 mM DTT) was loaded onto a Mono S HR 5/5 column (Pharmacia) and washed with 5 ml of buffer C. Proteins were eluted with a linear gradient from 40 mM to 1 M KCl in buffer C; 20 fractions of 0.5 ml each were collected. Of each fraction, 100 μl was precipitated

and used for Western blotting. The rest of each fraction was concentrated 10-fold by using Microcon-10 concentrators (Amicon) before activity assays.

Preparation of RAG2 Antiserum and Western Blotting

The coding region of a *RAG2* cDNA was cloned into the T7 expression vector pAR2113 (Rosenberg et al., 1987). The protein was expressed in E. coli strain BL21(DE3). The resulting insoluble protein was purified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the most prominent protein band was electroeluted. Protein was injected into rabbits by standard methods. After several injections antiserum was collected, and antibodies against RAG2 were purified on a column on which RAG2 fusion protein had been immobilized as described (Harlow and Lane, 1988); 5 ml of antiserum was purified on a 0.5 ml antigen column, and antibodies were eluted in approximately 10 ml. Antiserum was used at a 1:100 dilution on Western blots as described (Harlow and Lane, 1988).

Cleavage Reactions

The standard cleavage reaction contained 37.5 mM HEPES-KOH (pH 7.5), 2.5 mM Tris-HCl, 95 mM KCl, 1 mM $MnCl_2$, 0.05 mM EDTA, 2.7 mM DTT, 6% (v/v) glycerol (including components contributed by extract and protein preparations), 2 μg of nuclear extract proteins, 20 ng of RAG1 protein, and 20 ng of DNA per 10 μl reaction volume. Reaction mixtures were preincubated at 0°C for 30 min, followed by a 1 hr incubation at 30°C. The reaction was stopped by addition of 40 μl of proteinase buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.1% SDS) and 10 μg of proteinase K. After a 2 hr incubation at 50°C, the mixtures were extracted successively with buffer-saturated phenol, phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1). Then 2 μg of tRNA was added, and the DNA was alcohol precipitated and resuspended in 10 μl of H_2O. Reaction products were detected by LMPCR (signal ends) or two-dimensional agarose gel electrophoresis, followed by Southern blotting (coding ends).

Extract fractions from the Mono S column were assayed similarly, but less extract protein could be added because of the high KCl concentration in the fractions. KCl concentration was normalized to 95 mM final concentration in the reaction, and bovine serum albumin (New England Biolabs) was added to 0.1 mg/ml.

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