**In vitro V(D)J recombination: Signal joint formation**

(recombination activating protein RAG1/recombination activating protein RAG2)

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**ABSTRACT** The first step of V(D)J recombination, specific cleavage at the recombination signal sequence (RSS), can be carried out by the recombination activating proteins RAG1 and RAG2. In vivo, the cleaved coding and signal ends must be rejoined to generate functional antigen receptors and maintain chromosomal integrity. We have investigated signal joint formation using deletion and inversion substrates in a cell free system. RAG1 and RAG2 alone or in combination were unable to generate signal joints. However, RAG1 and RAG2 complemented with nuclear extracts were able to recombine an extrachromosomal substrate and form precise signal joints. The in vitro reaction resembled authentic V(D)J recombination in being Ku-antigen-dependent.

Lymphocyte antigen receptors are encoded by multiple copies of gene segments in germ-line DNA. During B- and T-lymphocyte development, these gene segments are assembled into functional transcription units by the mechanism of V(D)J recombination. The DNA sequence requirements for V(D)J recombination consist of highly conserved heptamer and nonamer DNA motifs separated by a spacer of 12 or 23 base pairs (12RSS and 23RSS, where RSS is recombination signal sequence). During the V(D)J recombination reaction, two types of DNA joints are formed: signal joints, generally involving precise head-to-head ligation of two heptamers, and coding joints, usually containing deletions or additions of a few nucleotides (1).

Several lymphoid-specific factors are known to be involved in V(D)J recombination. These include terminal deoxynucleotidyltransferase (2–4) and the recombination activating proteins RAG1 and RAG2 (5–7). RAG1 and RAG2 are sufficient for the formation of specific double-strand DNA breaks at RSSs (8, 9). Efficient recombination occurs almost exclusively between RSSs with different spacers. This restriction is known as the 12/23 rule and is evident at the initial cleavage event (10–12). The cleavage reaction involves the formation of hairpin loops at the coding ends and precise double-strand breaks at the signal ends (8, 9, 13–16). RAG1 and RAG2 can be communoprecipitated from cells, suggesting that they function as part of a complex during V(D)J recombination (17, 18).

Other proteins that are not restricted in their pattern of expression to lymphoid cells are also required for V(D)J recombination. These include two components of the DNA-dependent protein kinase, the p86 subunit of the p70/p86 Ku antigen (product of XRCC5), and the large catalytic subunit (product of SCID), as well as the XRCC4 protein. These proteins are involved in DNA repair as well as in V(D)J recombination (19, 20). In addition other as yet unidentified factors may be required for antigen receptor assembly.

Herein we report an *in vitro* system in which RAG1 and RAG2 complemented with a nuclear extract are able to catalyze signal joint formation. This system depends on Ku proteins and may provide the means for identification of the range of factors involved in generation of the signal joint.

**METHODS**

PCR Analysis. Recombined DNA was amplified and labeled with α-32P by using 30 cycles of 94°C for 0.5 min, 65°C for 1 min, and 75°C for 1 min, followed by incubation at 72°C for 10 min. The PCR amplification mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 5.5 mM MgCl2, BSA (0.1 mg/ml), 100 ng of each appropriate primer (R5, 5′-CCAGCTGTAACGCACTGTGCAC-3′; R14, 5′-TCCACCGAAACGTCCTGCT-3′), all four dNTPs (each at 20 μM), 1 μCi of [α-32P]dCTP (300 Ci/mmol; 1 Ci = 37 GBq; Amersham), and 1 unit of Taq DNA polymerase (Boehringer Mannheim). When primer R14 was used in combination with primer R3 (5′-TGGTCCAGTGTCAGCCTTGAC-3′), PCR was for 30 cycles of 95°C for 10 sec, 55°C for 0.5 min, 72°C for 1 min, followed by incubation at 72°C for 10 min.

**Purification of Truncated Glutathione S-Transferase Fusion RAG1 and RAG2 Proteins.** Truncated versions of RAG1 (amino acids 330–1040) and RAG2 (amino acids 1–383) were expressed as glutathione S-transferase-fusion proteins under the transcriptional control of the elongation factor 1α promoter (21). 293T cells [293 cells expressing the simian virus 40 large tumor (T) antigen] were transiently transfected with the RAG1 and RAG2 constructs by calcium phosphate precipitation (22). Two days after transfection, the cells were harvested and lysed for 5 min in RSB buffer (10 mM Tris-HCl, pH 7.4/10 mM NaCl/5 mM MgCl2/0.5% Nonidet P-40/protease inhibitors). Lyses were brought to 0.6 M NaCl with buffer LSB (20 mM Tris-HCl, pH 7.4/1.0 M NaCl/0.2 mM MgCl2/0.1% Nonidet P-40/protease inhibitors) and incubated for 30 min on ice. Extracts were centrifuged and the supernatant was incubated with glutathione-agarose beads. RAG proteins were eluted (50 mM Tris-HCl, pH 8.3/20 mM glutathione/1 M NaCl/10% glycerol/protease inhibitors) and dialyzed against buffer D (20 mM HEPES-NaOH, pH 7.5/1 mM DTT/10% glycerol/0.3 M NaCl/0.1 mM EDTA/protease inhibitors). Both proteins were expressed at levels corresponding to approximately 1 to 2 μg of each protein from one 100-mm tissue culture dish. Although RAG1 and RAG2 were highly purified (as determined by Coomassie blue staining, data not shown), we cannot rule out the possibility that our preparations contained additional factors that contributed to the biochemical activities described. All of the experiments described were done at least three times with two different preparations of extracts and proteins.

**Preparation of Nuclear Extracts.** 293 and CHO cells stably transfected with full-length RAG1 and RAG2 are indicated as 293-S and CHO-S, respectively. BASC6C2 is a pro-B-cell line and 22D6 is an Abelson virus-transformed pre-B-cell line.

Abbreviations: RAG, recombination activating protein; RSS, recombination signal sequence.
Nuclear extracts were prepared using a modification of the Dignam protocol (23). Briefly, cells were harvested and resuspended in 5 vol of buffer A (10 mM Hepes-KOH, pH 7.9/1.5 mM MgCl₂/10 mM KCl/0.1% Nonidet P-40/protease inhibitors). After 10 min of incubation on ice, samples were centrifuged at 1000 x g for 10 min. The pellet was resuspended in 1.5 vol of buffer C (20 mM Hepes-KOH, pH 7.9/20% glycerol/0.6 M NaCl/1.5 mM MgCl₂/0.2 mM EDTA/0.5 mM DTT/0.1% Nonidet P-40/protease inhibitors) and incubated on ice for 30 min. Cellular debris was removed by high-speed centrifugation, and the supernatant was dialyzed against low salt buffer C (100 mM NaCl) as described by Dignam et al. (23).

In Vivo Recombination. Fifty nanograms of pJH200 or 10 ng of pJH288 was incubated with RAG proteins and nuclear extracts in 20 μl in the presence of 12.5 mM Hepes-KOH, pH 7.5/100 mM KCl/1 mM MnCl₂/0.05 mM EDTA/5% glycerol/0.5 mM ATP/all four dNTPs (each at 50 mM), RAG1 (200 ng), RAG2 (200 ng), or nuclear extracts from 293-S (8 mg/ml), BASC6C2 (11 mg/ml), HeLa (10 mg/ml), CHO-S (9 mg/ml), or 22D6 (3 mg/ml) were added to the reaction mixtures. After a 5-hr incubation at 30°C, the samples were treated with proteinase K for 2 hr. The samples were then extracted once with phenol/chloroform and twice with chloroform. The DNA was recovered by ethanol precipitation, using 3 μg of poly(dI-dC) as carrier, and resuspended in 20 μl of 1 mM Tris-HCl, pH 7.5/0.1 mM EDTA. Five percent of the recovered DNA was used as template in the PCR detection assay.

Southern Blot Analysis. PCR products were resolved by electrophoresis through a 2% agarose gel, denatured, renatured within the gel, and then transferred onto Biotrans membrane (ICN). The DNA was UV-crosslinked to the membrane with a Stratalinker (Stratagene) and probed with an oligonucleotide which encompasses a correct signal joint (CT-GTGCACAGTGGTA) according to the protocol described by Oettinger et al. (7).

RESULTS AND DISCUSSION
As a further step toward defining the biochemical requirements for the V(D)J recombination reaction, we have develop-
opened an in vitro system to study signal joint formation. The system uses the active core regions of RAG1 (amino acid 330-1040) and RAG2 (amino acid 1–383), which were purified as fusion proteins with glutathione S-transferase (24–27). Truncated RAG1 and RAG2 fusion proteins mediated V(D)J recombination in vivo and specific cleavage of the RSSs in vitro (data not shown). The truncated fusion proteins were used for all of the experiments shown herein and will be referred to as RAG1 and RAG2.

A sensitive PCR assay was used to test for signal joint formation in a cell-free system (Fig. 1). The substrate used for the joining reaction was pJH200, a plasmid that undergoes deletional V(D)J recombination (28). Recombined signal joints were detected by amplification with primers R5 and R14 to yield a 252-bp PCR fragment (Fig. 1A). Hybridization of the R5 primer to recombined DNA is precise, whereas hybridization to the unrecropped substrate results in a mismatch at the 3’ end of R5 (Fig. 1B). Despite the mismatch, some amplification of a 456-bp PCR fragment was observed with R5 and R14 on the unrecropped substrate (Fig. 1C, lane 5). However, there was a preference for the recombined product even when the unrecropped plasmid was present in vast excess (Fig. 1C). Furthermore, the presence of the unrecropped substrate did not significantly inhibit detection of the recombined product (Fig. 1C). As a further control for the in vitro signal joining reaction, pJH200 was cotransfected with or without RAG1 and RAG2 into 293T cells. The 252-bp product was only amplified from DNA recovered from cells transfected with the combination of pJH200, RAG1, and RAG2 (Fig. 2A).

Using this PCR assay, we found that RAG1 and RAG2 or a combination of both were not sufficient to carry out signal joint formation in vivo. However, when RAG1 and RAG2 proteins were complemented with nuclear extracts, signal joints were generated as evidenced by the presence of the 252-bp PCR-amplified fragment (Fig. 2A, B, and D). Different nuclear extracts can complement RAG proteins in signal joint formation. RAG1 (R1) and RAG2 (R2) were supplemented with extracts prepared from BASC62 (BASC), 22D6, HeLa, CHO-S, or 293-S cells, as indicated. Each sample was processed as in A and the bands were visualized by autoradiography.

To estimate the number of signal joints formed in vitro, we constructed a standard curve with different amounts of pre-recombined molecules diluted in 2.5 ng of the unrecropped substrate (Fig. 1C). We found that a typical in vitro reaction resulted in formation of approximately 10^7 recombined molecules, corresponding to an average of 0.2% of the pJH200 substrate being transformed into recombined product.
Nuclear extracts from BASC6C2, 22D6, HeLa, CHO-S, or 293-S cells complemented the RAG proteins in signal joint formation (Fig. 2D). However, nuclear extracts prepared from 293 cells stably expressing RAG1 and RAG2 (293-S) were at least 5 times more active than all other extracts tested for signal joint formation. Low levels of recombination were seen in unsupplemented extracts both from BASC6C2 pro-B cells, which constitutively express RAG1 and RAG2 and actively recombine their immunoglobulin genes, and CHO-S cells, which stably express low levels of RAG1 and RAG2. This recombination activity was enhanced by the addition of recombining RAG1 and RAG2 (Fig. 2D).

To determine whether the amplified products from in vivo and in vitro reactions were molecularly identical, we purified the 252-bp fragment from both reaction mixtures and performed restriction digests with RsaI and HindIII. We found that the digestion pattern of the PCR products of the in vitro V(D)J joining reaction was identical to that displayed by authentic in vivo recombination reactions (Fig. 2C).

Because the R5 primer crosses the recombination border and could theoretically give an artificial result, we used an alternative pair of flanking primers, R14 and R3 (Fig. 1A), to confirm in vitro signal joint formation. The recombinant product was detected by Southern blot analysis with a probe that covered the recombination border. The results obtained with the flanking primers were identical to those obtained with the R5 and R14 primers. Using these primers, we found in vitro signal joint formation only when the RAG proteins and nuclear extract were present (Fig. 3A). Since the heptamer–heptamer junction generates an ApuLI site, digestion with this enzyme was used to define the precision of the signals joints amplified with the flanking primers. PCR was performed with R3 and R14 flanking primers, on in vitro and in vivo recombination reactions, in the presence of [32P]dCTP (Fig. 3B) and the 256-bp recombinant product was analyzed by restriction digestion (Fig. 3C). Almost 100% of the amplified products from the in vitro and in vivo reaction were ApuLI-sensitive (Fig. 3C), indicating that the majority of the signal joints produced in vitro were precise.

Inversion V(D)J reactions are characteristic of antigen receptor gene rearrangement in lymphoid cells. To examine signal joint formation in an inversion substrate, we used pJH288 (30) and the PCR assay with oligonucleotides R5 and R14. In this assay, the recombinated plasmid should generate a PCR product of 231 bp. Neither RAG1 nor RAG2 alone nor the combination was sufficient to generate an inversion product. However, extracts from 293-S cells were able to complement the purified RAG proteins and produce signal joints by inversion (Fig. 4A). To verify the accuracy of the inversion-mediated signal joints, the products of in vivo and in vitro reactions were compared by restriction enzyme digestion (Fig. 4B). Just as with deletional joining, the joints mediated by inversion in vitro were indistinguishable from their counterparts produced in vivo. Signal joints in this inversion substrate were detected after approximately 2 hr (Fig. 4C), whereas cleavage could be observed after a few minutes (data not shown).

During in vivo V(D)J recombination, a DNA sequence with a 12 RSS recombines with a sequence containing a 23 RSS. This restriction is known as the 12/23 rule and has been shown in vivo to regulate cleavage when Mg2+ is included in the reaction (10, 12). Under our reaction conditions, we were unable to observe the 12/23 rule in cell extracts. Also, signal joint formation could not obey the 12/23 rule. We are pursuing further studies to attempt reconstitution of signal joint formation under conditions that allow observation of the 12/23 rule. Reproduction of this restriction is key to producing an in vitro system that completely mimics the in vivo reaction.

Cleavage of the RSS at the heptamer results in blunt-ended 5'-phosphorylated linear DNA molecules (8, 9, 13–16). These ends could potentially be joined to one another nonspecifically.

**Fig. 3.** Signal joints generated in vitro and detected by PCR are precise. (A) Southern blot analysis of pH200 recombinant in vitro and PCR-amplified with flanking oligonucleotides R3 and R14. Because primers R3 and R14 hybridize equally well to the recombined and uncombined pH200, the DNA recovered from in vitro recombination reactions was first digested with HincII before PCR amplification, to enrich for the recombined DNA. The in vitro recombination assay was performed with the indicated combinations of RAGs, 293-S nuclear extracts, and 50 ng of pH200 (lanes 1–5). The HincII-digested DNA from these recombination reactions, as well as a titration (0–100 pg) of pH200+R (lanes 6–10) or with (lanes 11–15) 2.5 ng of pH200, was then subjected to PCR with the following modifications: no [32P]dCTP was added to the mixture and the primer pair used was R3/R14 (which generates a 256-bp fragment). Unlike R5, which overlaps the signal joint, the 3' end of R3 stops 3 bp before the signal joint and is, therefore, considered a flanking primer. The PCR products were separated on an agarose gel, transferred to nylon membrane, subjected to Southern blot analysis using a [32P]labeled oligonucleotide that overlaps the signal joint and visualized by autoradiography. (B) pH200 recombinant in vitro and PCR-amplified with flanking oligonucleotides R3 and R14, in the presence of [32P]dCTP. pH200 was subjected to the in vitro recombination assay in the absence (lane 1) or presence of RAGs and 293-S nuclear extract (lane 2). As a control, pH200 was transiently transfected into 293T cells alone (lane 3) or along with RAGs (lane 4) to generate in vivo-recombined plasmid. DNA recovered from recombination reactions was digested with HincII and PCR-amplified using flanking oligonucleotides R3 and R14. [32P]dCTP was used during PCR amplification. The [32P]-labeled PCR products were fractionated on an 8% native polyacrylamide/1× TBE gel and detected by autoradiography. (C) Restriction enzyme digestion of signal joints formed in vivo and in vitro. The 256-bp product from in vivo and in vitro reactions was extracted from a gel and submitted to restriction enzyme digestion with ApeLI (A1), RsaI (R1), and HindIII (HIII). Undigested and digested aliquots were separated on an 12% native polyacrylamide gel and the [32P]-labeled PCR products were detected by autoradiography. ApeLI cuts at the correct signal joint, located 20 bp from the end of the PCR product.
by DNA ligases to complete the signal joining reaction. In fact, complementation of RAG1 and RAG2 with purified T4 ligase will cause signal joint formation (data not shown). However, genetic experiments indicate that several factors other than RAG1 and RAG2 are required for efficient V(D)J recombination in vitro including the p86 subunit of Ku (19). To determine whether in vitro signal joint formation has similar requirements, we performed immunodepletion experiments with antibodies against the Ku antigen. We found that polyclonal antibodies to p70, one of the Ku subunits, and monoclonal antibodies that recognize the p70/p86 Ku heterodimer (31) specifically inhibited signal joint formation (Fig. 5). Preimmune serum and monoclonal isotype controls had no effect on the reaction. Thus, the in vitro reaction resembles authentic V(D)J recombination in that it involves genetically defined factors other than RAG1, RAG2, and ligase.

In summary, we have developed a cell-free system that mediates signal joint formation. This in vitro reaction recapitulates key aspects of signal joint formation as observed during in vivo V(D)J recombination. The joints are precise head to head heptamer fusions and their formation involves factors beyond RAG1 and RAG2. Because RAG1 and RAG2 are known to be sufficient for cleavage at RSSs (ref. 8 and data not shown), the other cellular proteins must be needed for the joining reaction. Our antibodies identify Ku-70 as one component that we assume acts in complex with Ku-86 and the DNA protein kinase. Other factors could well be necessary. The availability of this system to study signal joint formation should allow for rapid progress identifying all of the factors required to mediate this reaction.

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