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Cell-free V(D)J recombination

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V(D)J recombination generates diversity in the immune system through the lymphoid-specific assembly of multiple gene segments into functional immunoglobulin and T-cell receptor genes (for reviews, see refs 1, 2). The first step in V(D)J recombination is cleavage of DNA at recombination signal sequences. Cleavage produces a blunt DNA end on each signal sequence and a hairpin end on adjacent coding gene segments^{3,4}, and can be reproduced *in vitro* by using purified RAG1 and RAG2 proteins^{5,6}. The later steps involve processing and joining of the cleaved DNA ends, and until now have been studied only in cells. Here we reconstitute the complete V(D)J recombination reaction in a cell-free system. We find that the RAG proteins are not only involved in cleavage, but are also needed in the later steps for efficient joining of coding ends. Joining is largely directed by short pieces of identical sequence in the coding flanks, but addition of human DNA ligase I results in greater diversity. Coding junctions contain short deletions as well as additions complementary to a coding flank (P nucleotides). Addition of non-templated nucleotides into coding junctions is mediated by terminal deoxynucleotidyl transferase. The cell-free reaction can therefore reproduce the complete set of processing events that occur in cells.

In establishing a cell-free reaction, we concentrated on the joining of coding segments because this process involves multiple steps that are highly characteristic of V(D)J recombination. The hairpin ends from the initial cleavage are opened and then nucleotides are often removed or added before joining. The frequent retention of self-complementary inserts is also characteristic of V(D)J coding junctions^{1,2}. These P nucleotides are derived from asymmetric opening of hairpin-coding ends. By comparison, signal-end joining could be accomplished by simple ligation of two blunt DNA ends⁷.

To reconstitute complete V(D)J recombination, we used a partially fractionated HeLa cell extract as a supplement to cleavage by RAG proteins, because the factors required for the later stages are not specific to any particular cell type⁸. Using the polymerase chain reaction (PCR) (Fig. 1a), we detected coding junctions when RAG proteins, the HeLa cell fraction and high-energy cofactors were included (Fig. 1b, lanes 1–5). No joining was observed if either the RAG proteins or the cell fraction were omitted. The HeLa cell fraction was inactivated by heating (Fig. 1b, lane 3), suggesting that its activity was due to protein factors. Joining was optimal when both ATP and all four deoxynucleoside triphosphates (dNTPs) were included (Fig. 1b, lane 4), although some activity was detectable

with either ATP or dNTPs alone. In the presence of ATP, addition of dideoxynTPs did not stimulate joining like dNTPs, suggesting that dNTPs are substrates for a DNA polymerase (data not shown). In the absence of any added nucleoside triphosphates, joining was minimal (Fig. 1b, lane 5). Coding junctions were detectable within 3 hours of addition of extract, and the amounts continued to increase for 20 h. Signal junctions were also formed under similar conditions, but did not require addition of the cell fraction if T4 DNA ligase and ATP were included (Fig. 2b).

These reactions ('standard') were done in two stages: the first included only factors required for cleavage, the second included the HeLa cell fraction and high-energy cofactors. When joining factors were included from the start, cleavage and joining were both reduced (data not shown).

To determine whether RAG proteins participate in joining as well as cleavage, we compared the standard reaction with one in which the RAG proteins were removed by deproteinization before addition of the HeLa cell fraction (Fig. 2). When RAG proteins were present in the joining phase, they stimulated formation of the coding junctions as much as 50-fold (Fig. 2a), indicating that they may direct hairpin opening and/or recruit other factors for joining coding ends.

Stimulation of coding-junction formation was not due to continued cleavage by the RAG proteins in the second stage of the reactions (confirmed by direct staining of cleaved DNA; Fig. 2c). Furthermore, a second DNA substrate (designed to give a different-sized PCR product) added only in the second stage was not recombined (with or without an additional aliquot of RAG proteins). This verifies that residual cleavage activity makes no significant contribution to the amount of joined product (data not shown).

In contrast, signal-end joining by the HeLa cell fraction was inhibited more than 5-fold in the presence of RAG proteins (Fig. 2b). This effect was specific to the HeLa cell fraction, as joining of signal ends by T4 ligase was unaffected (Fig. 2b). RAG proteins therefore promote joining of coding ends but inhibit signal joining, consistent with the observation that signal ends persist for much longer than coding ends in cells^{3,9}. Inhibition of signal joining could be due to a continued stable association of RAG proteins with signal ends¹⁰ which blocks access by mammalian joining factors.

As already noted, the processing event specific to V(D)J recombination is the frequent addition of P nucleotides, so we determined whether the junctions generated *in vitro* also have these insertions. We sequenced *in vitro* derived coding junctions and identified several examples of such P nucleotides (Table 1).

Figure 1 Requirements for cell-free V(D)J recombination. **a**, Plasmid assay. Black triangles, 12-bp spacer recombination signals; white triangles, 23-bp spacer recombination signals. PCR primers are indicated by arrows. PCR products and sizes are described above or below the primer pair used to generate them. CJ, coding joint PCR product; SJ, signal joint PCR product; S, substrate PCR product. **b**, Basic requirements for coding joint formation. RAG proteins, HeLa fraction, ATP and dNTP were included as indicated. Triangle indicates the cell fraction was heated at 68 °C for 10 min.

Coding junctions made by *V(D)J* recombination are usually highly diverse after gaining or losing nucleotides during the process². Under cell-free conditions, we observed five different junction sequences in a sample of 25 junctions (from two independent joining reactions; Table 1a). The most common junction (19/25) has one copy of a 5-base-pair (bp) direct repeat (CAGGT; direct repeats are underlined in Table 1) precisely deleted from the two coding flanks. The next most frequently observed junction (3/25) may also have a precise deletion of a copy of a 5-bp direct repeat; at this junction, the direct repeat would have been generated by extending the coding flank of the 12-spacer signal with five more P nucleotides (ACCTG). Joining directed by short homologous sequences of DNA is also a feature of *V(D)J* recombination in cells², although the length of the deletion or addition (12 bp) observed here is greater than usual. The extent to which short repeats direct joining was further characterized by insertion of 30 bp between the 5-bp identical sequence and the 12-spacer signal. In this modified substrate, most joining still deleted precisely one copy of this motif,

even though deletion of over 40 bp of coding flank sequence was now required (data not shown).

Processing of coding flanks that contain directly repeated sequences can generate complementary overhangs. The added stability provided by these overhangs may result in homology-directed joining. DNA ligases differ greatly in their ability to join ends without complementary overhangs: for example, T4 DNA ligase and mammalian DNA ligase I (ref. 11) can join blunt ends, whereas the other known mammalian DNA ligases (DNA ligases II, III and IV; refs 11–13, respectively) require complementary overhangs. (Ligase II appears to be a fragment of ligase III rather than a separate entity^{14,15}.) The preference for homology-directed joining might reflect a low ligase activity, reduced during partial fractionation of the extract or by inappropriate storage of the cell fraction (DNA ligase I, for example, loses activity after freezing¹⁶).

We therefore supplemented the HeLa cell fraction with different purified ligases. Joining under standard conditions produced a major PCR product of 283 bp, representing junctions with a 12-bp

Table 1 Structure of coding joints generated by cell-free *V(D)J* recombination

No.	12 flank CAGGTCGACTTA	(12 RSS) P	N	(23 RSS) P	23 flank CGGTGGA
(a) Extract (standard conditions)					
19	<u>CAGGT</u>				GGA
3	CAGGTCGACTTA	TAAGTCG		<u>ACCTG</u>	CAGGTGGA
1	CAGGTC				<u>GA</u>
1	CAGGTCG			<u>ACCTG</u>	CAGGTGGA
1	CAGGTCGACTTA	TAAGT			TGGA
(b) Extract + ligase I					
8	CAGGTCGACTTA				CAGGTGGA
3	<u>CAGGT</u>				GGA
2	CAGGTCGACTTA	TAAGTCG		<u>ACCTG</u>	CAGGTGGA
2	CAGGTCGACT			CCTG	CAGGTGGA
2	CAGGTCGACTTA	T		CCTG	CAGGTGGA
1	CAGGTCGACTTA	TAA		CCTG	CAGGTGGA
1	CAGGTCGA			CCTG	CAGGTGGA
1	CAGGTCGACTTA			CCTG	CAGGTGGA
1	CAGGTCGACTTA				GTGGA
1	CAGGTCGACTTA			G	AGGTGGA
1	CAGGTCGAC				CAGGTGGA
1	CAGGTCGACTTA				GGTGGA
1	CAGGTCGACT				CAGGTGGA
(c) Extract + ligase I + TdT					
5	CAGGTCGATTA				CAGGTGGA
1	CAG		N ₂₇		CAGGTGGA
1	CAGGTCGACTTA	TA	GCGAC	G	CAGGTGGA
1	CAGGTCGACTT				GA
1	CAGG				GGTGGA
1	CAGGTC		N ₅₄	CTG	CAGGTGGA
1	CAGGTCGACT		CGATACCGCTCTCCT		CAGGTGGA
1	CAGGTCGACT		ATCGGA	G	CAGGTGGA
1	CAGGTCGACTTA	TA			GGTGGA
1	CAGGTCG		TACGAXCGGTCTCTGA		AGGTGGA
1	CAGGTCGACTT		CGGAAC	G	CAGGTGGA
1	CAGGTCGACTTA	T	GAATTGAAXC		GGTGGA
1	CAGGTCGAC		CT		CAGGTGGA

Sequence flanking the 12 signal and 23 signal in the starting plasmid is shown at the top of the table. No., number of examples of a coding joint sequence. Joints at which a significant (greater than 1 bp) homology may have directed joining are underlined. P, added sequence palindromic to a coding flank; P additions are listed adjacent to their parental coding flank. N, addition of non-templated sequence (for two joints, we record only N addition length (27 and 54 bp) and not sequence). Ambiguous bases are denoted by an X. **a**, Standard conditions; **b**, reactions supplemented with DNA ligase I, as for Fig. 3a, lane 8; **c**, reactions supplemented with DNA ligase I and 1.5 U TdT (Gibco-BRL), as for Fig. 4, lane 6.

Figure 2 Effect of RAG proteins in joining reactions. In standard (std) reactions, RAG proteins were present throughout; in deproteinized (deprot.) reactions, RAG proteins were removed after cleavage; the HeLa cell fraction was included (xt) or not (-), or 20 U T4 DNA ligase was added instead of the HeLa cell fraction (T4). **a**, Coding junctions; **b**, signal junctions; **c**, SE (double-cut signal end), a DNA fragment produced by cleavage at both 12 and 23 type signals. Samples are SE DNA from (-) reactions (stained directly; see Methods).

deletion (marked "c" in Fig. 3a, b) and a minor product of 307 bp, representing junctions with 12 bp of P nucleotide addition (marked "a"). Joining of both these products is directed by 5-bp homologies (see above). Addition of recombinant human DNA ligase I to the standard reaction changed the size of the preferred ligation product. The major species now migrated between the two homology-directed joining products (marked "b", lane 8 in Fig. 3a, and lanes 3 and 4 in Fig. 3b) at a position consistent with joints that are no longer homology directed. This same species could also be generated by addition of ligase I which was partially purified from HeLa cells but not frozen during preparation (data not shown).

Sequencing confirmed that the structure of the junctions made in the presence ligase I was altered as expected. Only 20% of these sequenced junctions used the 5-bp homologies (Table 1b). Coding junction sequences now included several examples of joints without deletion, as well as junctions with short deletions and P additions, in a pattern highly consistent with coding junctions derived from cells. The effects of DNA ligase I addition were not observed in reactions supplemented instead with T4 DNA ligase (Fig. 3a, lanes 3 and 4), recombinant human DNA ligase III (Fig. 3a, lanes 5 and 6), or DNA ligase IV purified from HeLa cells (Fig. 3b, lanes 5 and 6). The specificity of ligase I in altering coding joint structure suggests that this enzyme may be important for joining of *V(D)J* recombination intermediates.

It has previously been reported that a cell line having only mutant DNA ligase I still supports *V(D)J* recombination^{17,18}. Residual DNA ligase I activity (estimated to be 5–10% of normal levels) in this cell line may be sufficient for normal *V(D)J* recombination, as this level is sufficient to support DNA replication¹⁹. Alternatively, homology-independent joining in the absence of DNA ligase I may use an accessory factor or an as-yet unidentified mammalian DNA ligase.

Another important source of diversity in *V(D)J* rearrangements is the addition of non-templated nucleotides (N addition), which is mediated by terminal deoxynucleotidyl transferase (TdT) (reviewed in ref. 2). When we added TdT and ligase I to the cell-free reaction, most coding junctions contained N additions (Table 1c). We found a much wider range of N additions (2–54 bp) than in

Figure 3 a, b, Effect of DNA ligases on formation of coding junctions. T4, T4 DNA ligase. L-III, recombinant human DNA ligase III. L-IV, human DNA ligase IV purified from HeLa cells. L-I, recombinant human DNA ligase I. CJ-a, 307-bp coding junction product. CJ-b, approximately 295-bp coding junction product. CJ-c, 283-bp coding junction product. CJ-a was visible only after long exposures (**b**). Each ligase was tested at two concentrations differing by a factor of 5. The activities of human DNA ligases added were similar, as determined by an oligonucleotide nick-sealing assay, whereas the activities of T4 DNA ligase (50 and 250 U) were at least 4-fold higher.

junctions derived from cells (typically 1–15 bp)².

Formation of signal junctions *in vivo* is rarely accompanied by base loss or addition, irrespective of the presence of TdT². To determine whether this is also true under cell-free conditions, we made use of the observation that precise formation of signal junctions generates a site for the restriction enzyme *Apa*LI. When PCR products of signal junctions were tested by *Apa*LI digestion, less than half of the signal junctions were precise (sensitive to *Apa*LI) in the absence of added ligase I, but this frequency was not affected by addition of TdT (Fig. 4b). Addition of ligase I increased both the efficiency (>10 fold; Fig. 4a) and accuracy of signal junction formation (Fig. 4b). Over 90% of junctions formed in the presence of ligase I were now precise, and again this frequency was unaffected by the presence of TdT (Fig. 4b). In contrast, the majority of the coding junctions from the same joining reaction had N additions (Table 1). We conclude that TdT activity is preferentially targeted to coding junctions in the cell-free reaction, as it is *in vivo*.

The absence of signal end modification by TdT may again be explained by their binding of RAG proteins. Otherwise, one would expect signal ends to be acceptable substrates for TdT addition. Blunt ends are known to be fully competent for TdT addition, comparable (within 2-fold) to substrates with 3' overhangs²⁰, as might be generated by the opening of hairpins.

The cell-free reaction described here reproduces the steps of *V(D)J* recombination quite accurately. When complete, the system carries out cleavage at two recombination signal sequence (RSS) sites, opens the resulting DNA hairpins, inserts or deletes nucleotides at these opened ends, and finally joins both coding and signal ends. The retention of P nucleotides at some coding junctions is also similar to the cellular process. We have shown that the RAG proteins not only perform cleavage, but have important effects on joining, providing evidence that the two stages of *V(D)J* recombination, cleavage and joining, are intimately linked. The fact that RAG proteins affect joining in a way specific to the type of end (signal or coding) has implications for *V(D)J* recombination. RAG proteins may confer directionality on the reaction (a signal end rarely joins to a coding end) and provide a basis for the different

Figure 4 Effect of ligase I (L-I) and TdT on signal junction formation. 0.3 U (+) of TdT or 1.5 U (++) of TdT was added to reactions as indicated. **a**, 0.5% of reactions without, and 0.1% of reactions with DNA ligase I were analysed for signal junctions. A, *Apa*L1 site; A, potential *Apa*L1 site. **b**, 60% of the PCR reactions in **a** were digested with *Apa*LI; the two digestion products of precise junctions co-migrate.

processing events at each type of end. Furthermore, persistence of a synaptic complex following cleavage could help to enforce joining of double-strand breaks originating from the same cleavage event, and reduce the potential for intermolecular (or interchromosomal) joining. Cell-free *V(D)J* recombination has identified a role for RAG proteins in the joining phase of the reaction and helped to distinguish the different effects of cellular DNA ligases. It may now also be useful in determining the role of other factors in processing hairpins and in the repair of double-strand breaks, including Ku (XRCC5 and XRCC6), DNAPK α (*scid*, XRCC7) and XRCC4 (see ref. 21 for example). If some of these double-strand-break repair factors are essential for cell growth, a cell-free system may be the best means for characterizing them. □

Methods

Reaction conditions. 50 ng plasmid pDVG54, which consists of the *Bam*HI/*Sal*I fragment of pDVG42 with recombination signals²² cloned into pUC18, was cleaved by ~200 ng RAG1 and RAG2 proteins⁶ in the presence of 40 ng HMG1 protein. Cleavage reactions were done in 10 μ l at 37 °C for 1 h in 25 mM MOPS, pH 7.0, 2 mM DTT, 30 mM potassium glutamate, 30 mM KCl, 1% glycerol, 100 μ g ml⁻¹ BSA, 4 mM MgCl₂ and 0.4 mM MnCl₂. About 70% of the DNA was cleaved under these conditions. In 'standard' reactions, joining factors were directly added to cleavage reactions; 'deproteinized' reactions were treated after the cleavage step with proteinase K (Gibco-BRL), extracted with phenol and chloroform, precipitated with ethanol, and reconstituted in cleavage buffer with HMG1 but without RAG proteins. Joining reactions were done overnight (16–20 h) at 25 °C with 500 ng HeLa cell extract, 0.5 mM ATP (Pharmacia) and 50 μ M of each dNTP (Pharmacia) in 20 μ l containing 10% w/v polyethylene glycol (average relative molecular mass 8K), and supplemented as necessary to maintain 25 mM MOPS, pH 7.0, 4 mM MgCl₂, 30 mM potassium glutamate. Reactions were stopped by addition of Tween-20 (Sigma) to 0.5%. Equivalent cleavage and recovery of substrate DNA in standard and deproteinized reactions was confirmed by electrophoresis and direct staining of DNA with SYBR green dye (FMC Bioproducts) from reactions without added extract or T4 ligase.

Analysis of joining reactions. Coding junctions were detected by PCR amplification of 20% of each joining reaction for 25 cycles using the primers DAR147 (5'-CATTCGCCATTCAGGCTGCGCAACT) and DAR5 (5'-TGCTTCCGGCTCGTATGTTGGTGAAT). Each cycle consisted of 15 s at 94 °C, 30 s at 65 °C, and 15 s at 72 °C. Signal junctions were detected by amplification of 0.1% (Fig. 4, lanes 4–6 only) or 0.5% (Fig. 2b and Fig. 4, lanes 1–3) of each joining reaction as described above, but with the primers DG88 (5'-GTAGAACGAGCGGCGT CGAAGCCTG) and DG89 (5'-CACAGCAGCGCCATATCGAAGGTCG). One third of each PCR reaction was analysed by non-denaturing polyacrylamide gel electrophoresis and visualized either by staining with ethidium bromide (Fig. 3a) or by Southern transfer and hybridization with an oligonucleotide probe for the 23 signal coding flank

(DAR153: 5'-CCCGGGTACCGAGCTC). Faint bands of the wrong size were not detected by Southern analysis and so were considered to be PCR artefacts. Serial dilutions of template over a 10-fold range produced proportional amounts of PCR product after amplification, indicating that the PCR reaction is in a linear range. PCR products were quantified in this range in at least two independent experiments, using ImageQuant software. For sequencing, coding joint PCR products were separated from amplified non-recombinant substrate by agarose gel electrophoresis, diluted 200-fold, and re-amplified as before, but for only 12–15 cycles and substituting the primer DAR149 (5'-CGCCAGCTGG CGAAAGGGGGATGT) for DAR147. Products from the secondary amplification were cloned using the TA cloning kit (Invitrogen) and sequenced using a plasmid Quick Denature Sequenase kit (Amersham).

HeLa extract. A cell extract was prepared by homogenization of 9.0×10^9 HeLa cells in buffer A (0.5 M KCl, 50 mM Tris, pH 8.0, 1 mM DTT, 10% glycerol, 1 mM PMSF and 1 μ g ml⁻¹ each of leupeptin and aprotinin), followed by centrifugation at 100,000g. The supernatant was made 1 M in KCl, and nucleic acids were precipitated with 0.05% polyethyleneimine (PEI). To remove excess PEI, the supernatant was incubated with phosphocellulose, which was then removed by filtration. Material recovered in a 20–40% ammonium sulphate cut was resuspended in buffer B (0.1 M KCl, 20 mM Tris, pH 8.0, 1 mM DTT, 10% glycerol, 1 mM PMSF), dialysed, and loaded onto a ~15 ml Q-Sepharose column (Pharmacia). Bound protein was eluted with a gradient of 0.1–0.5 M KCl; active fractions eluted between 0.25 and 0.35 M KCl and were stored at –70 °C.

DNA ligases. Clones of histidine-tagged versions of human DNA ligases I and III (gift from T. Lindahl) were expressed in *E. coli* and purified by metal affinity. DNA ligase IV, purified from HeLa cells and free of the other human DNA ligases¹⁴, was also a gift from T. Lindahl. The activity of T4 DNA ligase (New England Biolabs) is defined in cohesive end units. The relative activity of each ligase was assessed using an oligonucleotide nick-joining assay.

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