Formation and resolution of double-strand break intermediates in V(D)J rearrangement

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A recently described pre-B cell line can be induced at high temperature to actively rearrange its immunoglobulin light-chain loci. We used this cell line to determine the fate of double-strand breaks generated by V(D)J rearrangement. After induction, 30%-40% of λ loci had broken λ1 signal ends. λ1-coding ends were detectable, but 10- to 100-fold less frequent. Both covalently closed [hairpin] and open, blunt, processed coding ends were observed. Coding junctions involving λ1 accumulated with similar kinetics as λ1 signal ends, arguing that coding ends can be resolved quickly and efficiently to coding junctions, whereas signal ends remain mostly unjoined. Signal ends are then joined rapidly when cells are returned to the low temperature. These results support the model that broken signal ends and hairpin coding ends are authentic intermediates in V(D)J recombination. It appears that hairpin coding ends are rapidly opened, processed, and resolved to coding junctions, whereas joining of signal ends is clearly uncoupled from the joining of coding ends and can be much slower. Efficient formation of signal junctions may require cell cycle progression, or down-regulation of the recombination machinery.

[Key Words: V(D)J recombination; double-strand break repair; immunoglobulin light chain; ts-abl; DNA hairpins]

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The variable domains of immunoglobulins and T cell receptors are generated by site-specific recombination of separate V-, D-, and J-coding segments [V(D)J rearrangement; for review, see Tonegawa 1983, Gellert 1992, Lewis 1994]. Recombination is triggered by conserved recombination signals that are located directly adjacent to each coding segment. The recombination signals consist of a conserved 7-bp DNA sequence (heptamer), separated by a relatively nonconserved spacer from a conserved 9-bp DNA sequence [nonamer]. The spacer is either 12 or 23 bp long, recombination occurs almost exclusively with one recombination signal of each type. Recombinations usually result in a precise head-to-head joining of the two signals (signal junction) and an imprecise joining of the two coding segments (coding junction). Coding junctions may contain short deletions or additions of terminal sequence, or both. When no DNA is lost from the end of a coding segment, a short sequence addition that is complementary to the terminal sequence of the coding segment may be observed in the coding joint [P nucleotide addition] [Lafaille et al. 1989, McCormack et al. 1989].

Our laboratory [Roth et al. 1992a,b] and others [Schlissel et al. 1993] have identified possible intermediates in V(D)J rearrangement in both thymus and bone marrow. Double-strand breaks are observed at the border of recombination signals, the DNA end that retains the recombination signal [the signal end] is almost always blunt, 5' phosphorylated, and has no sequence deletion or addition [Roth et al. 1993, Schlissel et al. 1993], as might be predicted from the precision of signal junctions. The presence of signal ends is confined to the G1 phase of the cell cycle [Schlissel et al. 1993].

Coding ends were first observed in thymocytes of mice with the scid mutation but were not detectable under similar conditions in normal mice [Roth et al. 1992a,b]. scid mice do not possess mature B and T cells because they do not form coding junctions efficiently and therefore cannot assemble immunoglobulins and T cell receptor genes. A recent report estimates that coding ends are at least 1000-fold less frequent than signal ends at the T cell receptor β locus in normal mouse thymocytes [Zhu and Roth 1995].

In scid mice the two strands of each coding end are covalently sealed together, forming a hairpin structure [Roth et al. 1992b]. The accumulation of hairpins in scid mice could be the result either of an inability to resolve a normal, hairpin intermediate or abnormal diversion of coding ends to an aberrant hairpin product. However, several lines of evidence suggest that hairpins are a normal, obligatory intermediate in V(D)J rearrangement. The utility of hairpins in explaining the occurrence of P nucleotides suggests that hairpins are an intermediate in at least some rearrangements, and the observation that...
hairpin ends from scid mice include all of the germ-line-encoded coding sequence, with no additions (Zhu and Roth 1995), argues further that hairpin formation may be linked directly to cleavage at the recombination signal. Finally, it was demonstrated recently that hairpins are the major coding end species made in a cell-free reconstitution of the cleavage step of V(D)J rearrangement with mouse cell extracts (van Gent et al. 1995). As these cells did not contain the scid mutation, recombination in a scid background is not necessary for hairpin production.

If hairpins are an obligatory, direct product of cleavage at the recombination signal/coding segment border, hairpins must then be opened to allow deletion and addition (processing) prior to formation of the coding junction. This suggests that open, and possibly processed coding ends might exist as an intermediate in the resolution of hairpins to coding junctions.

The source of DNA in all previous examinations of double-strand breaks generated by V(D)J rearrangement has been mouse tissue. Therefore, it has not been possible to determine whether the observed double-strand breaks are an authentic intermediate in recombination, for example, these broken molecules may represent long-lived end products of failed recombinations. Furthermore, actively rearranging cells in tissue populations are typically in the minority, thus making the detection of such intermediate species difficult. Here we use a recently described cell line that can be induced to rearrange its IgK loci at a very high frequency and then be directly observed via Southern blots (Roth et al. 1995). As described by Chen et al. (1994), cell lines transformed by Abelson murine leukemia virus with a temperature-sensitive variant of the abl oncogene demonstrate a high frequency of V(D)J rearrangement when cultured at the nonpermissive temperature. The induction of rearrangement is presumably attributable, at least in part, to the observed large increases in RAG-1 and RAG-2 mRNA levels. High-frequency rearrangement in ts-abl cell lines (as well as appropriate size-matched controls; see Materials and methods) indicates that after induction of both the K1 signal end species also contained a significant portion of this probe by an arrest in the G1 phase of the cell cycle, followed by cell death. Cell survival is prolonged in cell lines that express high levels of an introduced BCL-2 gene.

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Here we identify double-strand breaks at the Igk locus in DNA from a ts-abl/BCL-2 cell line grown at the nonpermissive temperature. Signal ends at /k elements are observed in great abundance, but coding ends, although detectable, are 10- to 100-fold less frequent. We are able to show that this difference is attributable to efficient formation of coding junctions and inefficient formation of signal junctions at the nonpermissive temperature. Return of a culture to the permissive temperature results in more efficient formation of signal junctions, indicating that the signal ends that accumulate at the nonpermissive temperature retain the ability to form signal junctions and therefore represent an authentic intermediate in V(D)J rearrangement. The ability to detect coding ends allows us to determine their structure in the absence of the scid mutation. We observe both covalently closed (hairpin) coding ends, as well as blunt, open coding ends. Open coding ends possess short deletions and insertions in a pattern that is mostly consistent with the processing observed at coding segment termini in coding junctions.

### Results

**/k1 signal ends accumulate when V(D)J rearrangement is induced**

The immunoglobulin / light-chain locus contains a single exon coding for the constant domain (Cx). Upstream of the Cx exon are five / segments in the same transcriptional orientation as Cx. The central / segment (/k3) is mutated in the recombinogenic signal and does not participate in V(D)J rearrangement. Approximately 160 functional / segments are located farther upstream and exist in both orientations, thus allowing V(D)J rearrangement to occur either by deletion or inversion (see Fig. 1A, for review, see Koller et al. 1992). Rearrangement at the / locus is rapidly induced in the ts-abl-transformed cell line 103/BCL-2 following a shift in the growing conditions, from 34°C to 39.6°C (Chen et al. 1994).

DNA from 103/BCL-2 cells grown at 34°C (samples labeled L in all figures), as well as DNA from cells grown for 48 hr at 39.6°C (samples labeled H in all figures) was digested with the restriction endonucleases EcoRI and SacI, Southern transferred, and probed with a DNA fragment located immediately 5' of /k1 (probe I, see Fig. 1A). In addition to the 2.95-kb EcoRI–SacI fragment corresponding to an intact germ-line / locus, we observed an abundant 11-kb species in DNA from the cells grown at 39.6°C (Fig. 1B). This latter species is the appropriate size for a /k1 signal end, and was sensitive to digestion with ATP-dependent exonuclease (data not shown), confirming its identity as an open DNA end. Comparison of the intensity of the /k1 signal end species to the germ-line fragment (as well as appropriate size-matched controls, see Materials and methods) indicated that after induction between 30% and 40% of / loci possess a /k1 signal end. No significant further accumulation of signal ends was observed in DNA samples from cells grown for >48 hr at this temperature. Unless stated otherwise, all further DNA samples were recovered from 103/BCL-2 cells grown for 48 hr at the nonpermissive temperature.

The blot displayed in Figure 1B was stripped and reprobed with a fragment spanning the /k1- and /k2-coding regions (probe II, Fig. 1A). A minor species (1%–5% of total DNA) of ~1.85 kb suggested the presence of /k1 coding ends (Fig. 1C). This blot was then stripped and reprobed with a fragment of the / locus immediately 3' of /k5 (probe III, Fig. 1A), revealing that the 1.85-kb species also contained a significant portion of this probe sequence, further supporting its identification as a /k1-coding end (Fig. 1D). Moreover, it was also possible to detect species of sizes appropriate for a /k1-coding end, at similar relative intensity, in independent digests using...
The restriction enzymes BgIII, PstI, or XbaI (data not shown). Direct identification and structural characterization of κ1-coding ends is discussed later in this paper. Two other species, of size appropriate for κ2- and κ4-coding ends (1.51 and 0.88 kb, respectively), were also observed using probe III [Fig. 1D]. Potential κ5-coding ends were also observed in some DNA samples, but detection of both κ4- and κ5-coding ends was inconsistent.

**κ2 signal ends occur largely in the context of κ1 rearrangements**

As described above, 30%–40% of κ loci were shown to possess κ1 signal ends, using a probe located 5' of κ1 [Fig. 1B]. No other major species were observed with this probe, even after prolonged exposure to X-ray film (data not shown). One possible reason why κ2, κ4, and κ5 signal ends were not observed in this experiment might be that rearrangements were not initiated at these signals. This does not seem likely, as we observed similar levels of coding ends at κ2 and κ4 as were observed at κ1 [Fig. 1D], and coding junctions involving κ2, κ4, and κ5 accumulate at a rate similar to coding junctions involving κ1 (data not shown).

Alternatively, the majority of κ2, κ4, and κ5 signal ends may exist largely within molecules that have completed a rearrangement at κ1 and thus would not be detected with a probe 5' of κ1. We therefore digested a sample of DNA from induced 103/BCL-2 cells with a restriction enzyme possessing a site just 3' of the κ1 signal/coding boundary and used a fragment spanning the sequence between κ1 and κ2 as a probe (probe II, Fig. 1A). Digestion with BanI, which has a site 16 bp 3' of the κ1 signal/coding segment border, will generate a 336-bp species if κ2 signal ends are present, regardless of the presence of a rearrangement at κ1. We compared this restriction digest to a restriction digest of the same DNA sample with Scal, which has a site 12 bp 3' of the κ1 signal/coding segment border. Digestion with Scal will generate a 366-bp species if κ2 signal ends are present, but only if a rearrangement at κ1 has not occurred, as rearrangement would remove the Scal site (see Fig. 2A).

Restriction digests were electrophoresed, Southern transferred, and probed with a fragment spanning the κ1- and κ2-coding segments [probe II, Fig. 1A]. Rsal digests of DNA from kidney, uninduced, and induced 103/BCL-2 cells are included as size/quantification controls, and produce a 366-bp germ-line fragment [Fig. 2B, lanes 1, 2, and 3, respectively]. DNA from induced 103/BCL-2 cells was digested with BanI [lane 4] or Scal [lane 5]. After correcting for the amount of DNA in each sample, we determined that while a species of size appropriate for a κ2 signal end was observed in <2% of κ loci following Scal digestion [Fig. 2B, lane 5], it was found in 15%–20% of κ loci after BanI digestion [Fig. 2B, lane 4]. The species produced by BanI digestion was removed by pretreatment of the sample with ATP-dependent exonuclease, confirming its identity as an open DNA end (data not shown).

Detection of the majority of κ2 signal ends following BanI digestion, but not Scal digestion, indicates that κ2 signal ends occur principally in the context of molecules that possess an interruption in the germ-line sequence within the 28 bp between these two sites or near the end of the κ1-coding segment. The most probable explana-
Formation of coding junctions

As described above, 48 hr after induction of V(D)/J rearrangement, we observed that ~50% of κ loci remained in the germ-line configuration, 30%–40% of κ loci terminated in a κ1 signal end, and only 1%–5% of κ loci remained in a κ1-coding end. These results suggest that κ1-coding ends are rapidly and efficiently resolved to junctions, whereas κ1 signal ends remain mostly unresolved. We therefore analyzed coding junction formation over several time points following induction using a quantitative PCR assay for Vκ1 rearrangements, and compared this to a ligation-mediated PCR (LMPCR) assay (Mueller and Wold 1989) for κ1 signal ends. LMPCR detects open, blunt, double-strand breaks by ligation of a double-stranded DNA linker to a DNA sample (see Fig. 3). The ligated material is amplified using a primer derived from one strand of the linker and another primer specific to the locus of interest. A culture of 103/BCL-2 cells was shifted to the nonpermissive temperature, and aliquots harvested at 12-hr intervals until 48 hr following the shift. We amplified Vκ1-coding junctions from DNA extracted from these samples using a degenerate Vκ primer described previously (Schlissel and Baltimore 1989) and a primer be-

Figure 3. Time course of the appearance of signal ends and coding ends. [A–C] PCR amplification of 0.5 μg of DNA samples from aliquots of a culture of 103/BCL-2 cells. (Lane 1) A sample of DNA from 103/BCL-2 cells grown at the permissive temperature; (lanes 2–5) samples from the same culture, taken at 12-hr intervals, after shift to the nonpermissive temperature; (lanes 6–9) samples from the same culture, taken at 12-hr intervals, after return to the permissive temperature. Electrophoresis and transfer to nylon membranes as described in Materials and methods. (Right) Graphic descriptions of each PCR product. The locations of primers and probes are indicated by arrows and bars, respectively. [A] Control PCR amplification (germ-line sequence J' of κ1). [B] PCR amplification of Vκ1-coding junctions. [C] LMPCR of κ1 signal ends. [D] Quantification of Vκ1-coding junctions. (Lanes 1–3) Replicate amplifications of 0.5 μg of DNA samples from the culture described above. (Lane 1) DNA from cells grown at the permissive temperature; (lane 2) DNA from cells grown at the nonpermissive temperature for 48 hr; (lane 3) DNA from cells returned to the permissive temperature for 48 hr; (lane 4) 0.5 μg of DNA from the cell line 70Z/3. (Lanes 5–7) Mixtures of DNA from 70Z/3 and DNA from the T cell line B6Mo3, to a total of 0.5 μg DNA for each amplification. (Lane 5) 50% of DNA from each cell line; (lane 6) 25% of DNA from 70Z/3, and 75% of DNA from B6Mo3; (lane 7) 12.5% of DNA from 70Z/3, and 87.5% of DNA from B6Mo3; (lane 8) 0.5 μg of DNA from the T cell line B6Mo3.
between Jk1 and Jk2, and determined that coding junctions are formed most actively in the first 24 hr [Fig. 3B, lanes 1–5]. The appearance of Jk1 signal ends, as measured by LMPCR [Fig. 3C, lanes 1–5], followed a time course similar to the appearance of V/k1-coding junctions, supporting the argument that resolution of Jk1-coding ends occurs very shortly after the introduction of the double-strand break.

To determine the frequency of V/k1-coding junctions, we compared the amount of PCR product from V/k1 amplifications of DNA samples from induced 103/BCL-2 cells to the amount of PCR product from serially diluted DNA samples from 70Z/3 cells [this cell line has two copies of a V/k1 rearrangement; D.A. Ramsden, unpubl.]. We estimated that after 48 hr of induction, 103/BCL-2 cells have V/k1 rearrangements in 20%–40% of k loci [Fig. 3D, cf. lane 2 and lanes 4–8]. This estimate is supported by the observation of the 520-bp species resulting from Rsal digestion, observed in Fig. 2, lane 3. This 520-bp species is probably indicative of V/k1 rearrangements. Slightly over half of a pool of 109 Vk segments possess an Rsal site ~165 bp 5' of the Vk recombination signal, because of conservation of invariant amino acids at codons 35 and 36 [Kofler et al. 1989]. The distance between the Jk1-coding/signal boundary and the Rsal site in Jk2 is an additional 355 bp; thus Rsal digestion will produce a 520-bp species when an appropriate Vk is rearranged to Jk1. The 520-bp species represents ~10%–15% of total DNA, suggesting that 20%–30% of total DNA has V/k1 rearrangements. The frequency of V/k1 rearrangements after 48 hr of induction is therefore similar to the frequency of Jk1 signal ends, as estimated by Southern analysis, at this time point.

Signal junctions are formed efficiently at the permissive temperature

One possible explanation for the accumulation of signal ends would be that they are incapable of resolution into signal junctions and would therefore not represent an intermediate in V(D)J rearrangement. Alternatively, their joining may depend on a return to conditions where levels of V(D)J rearrangement are much lower, and/or where normal cell growth has resumed. After 48 hr at the nonpermissive temperature we therefore returned this culture to the permissive temperature or they might have been resolved to signal junctions.

Intermediates in V(D)J rearrangement

To aid in the interpretation of this experiment, each DNA sample was first digested with EcoRI and then split evenly into two aliquots. One aliquot was digested with ApaLI, whereas the other aliquot was mock digested. Hybridization of this blot with a control probe (RAG-1) confirmed that DNA content for each pair of samples was equivalent and allowed rough comparison between different sample pairs.

Inclusion of ApaLI in a digest of DNA from 103/BCL-2 cells grown at the nonpermissive temperature for 48 hr increased the intensity of the 1.1-kb species slightly or not at all, demonstrating that signal junctions involving Jk1 are infrequent at this time point [Fig. 4B, cf. lanes 3 and 4]. This culture was returned to the permissive temperature, and aliquots were harvested every 12 hr. Comparison of paired digests indicative of Jk1 signal ends [Fig. 1B]. Digestion with EcoRI and ApaLI produces a 1.1-kb species that will result from signal junctions involving Jk1, in addition to Jk1 signal ends, as long as the signal junction possesses an ApaLI site [see Fig. 4A]. Signal junction formation can therefore be estimated by subtracting the amount of 1.1-kb species in a sample digested only with EcoRI from the amount of 1.1-kb species in a duplicate sample digested with both EcoRI and ApaLI. To aid in the interpretation of this experiment, each DNA sample was first digested with EcoRI and then split evenly into two aliquots. One aliquot was digested with ApaLI, whereas the other aliquot was mock digested. Hybridization of this blot with a control probe (RAG-1) confirmed that DNA content for each pair of samples was equivalent and allowed rough comparison between different sample pairs.

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from these later samples reveals that *ApoAI* digestion now significantly increases the abundance of the 1.1-kb species [Fig. 4B, lanes 5–10]. *K1* signal ends are therefore being resolved to *ApoAI*-sensitive signal junctions. Moreover, the ratio of *K1* signal junctions and signal ends to total DNA [cf. even-numbered lanes in Fig. 4B to the corresponding lanes in Fig. 4C] remains roughly constant throughout the time course, in contrast to the decreasing ratio of *K1* signal ends to total DNA. This indicates that resolution of *K1* signal ends to signal junctions is, for the most part, efficient and accurate.

We extended our analysis to the *λ* locus, which has a much simpler organization, to determine whether formation of a specific signal junction is more efficient following return of the culture to the permissive temperature. Only four rearrangements are typically observed; *Vα1* can rearrange to either *Jα3* or *Jα1*, and *Vα2* or *Vαx* can rearrange to *Jα2* (Carson and Wu 1989; Storb et al. 1989). We chose to analyze both products arising from *Vα1*–*Jα1* rearrangement, coding and signal junctions, following shift to nonpermissive growing conditions. Coding junctions increased at an approximately linear rate following shift to the nonpermissive temperature and continued to increase, although more slowly, following return to the permissive temperature [Fig. 5A, lanes 1–9]. In contrast, signal junctions are first detectable 36 hr after shift to the nonpermissive temperature but remain at low levels until the culture is returned to the permissive temperature [Fig. 5B, lanes 1–9]. At the first time point following return to the permissive temperature there is a fourfold increase in signal junctions; subsequent time points following the return to the permissive temperature show only modest increases in signal junction formation.

**Coding end structure**

We report above the first observation of coding ends in cells that are not from *scid* mice. As discussed above, coding ends observed in *scid* mouse DNA are covalently closed [hairpins] (Roth et al. 1992b). We therefore first examined the structure of *K1* coding ends by two-dimensional electrophoresis to determine whether hairpins are a major species of coding ends under these conditions. In this method, samples are first electrophoresed under native conditions. The lanes containing the samples are then cut out, equilibrated in alkali, and positioned perpendicular to the direction of applied voltage in an alkaline [denaturing] gel. Double-stranded DNA fragments with both ends open are observed on the diagonal. Species with a covalently closed [hairpin] end, however, run above this diagonal at a position consistent with a size in the denaturing dimension that is twice its size in the native [double-stranded] dimension.

DNA samples from 103/BCL-2 cells grown at the permissive temperature or the nonpermissive temperature were digested with *XbaI* and subjected to two-dimensional electrophoresis. The gel was then Southern transferred and hybridized with a 1.6-kb fragment including all five *K* segments.

The 3.5-kb germ-line fragment is observed as a spot on the diagonal in both DNA samples [Fig. 6]. In the sample from 103/BCL-2 cells grown at the nonpermissive temperature, two rare species were also observed above the diagonal, consistent with sizes of ~1.7 and 1.35 kb in the native dimension and approximately twice these sizes in the denaturing dimension. This is indicative of the presence of hairpin coding ends at *K1* and *K2*, respectively. We note that the probe location is such that it favors detection of the more 5' *Ks* and should not be taken as evidence that the distribution of hairpins is skewed toward the more 5' *Ks*. Prior hybridization with a probe derived from DNA between *K1* and *K2* detected only the *K1* hairpin, supporting our identification of these species [data not shown].

We investigated the possibility that open coding ends were present, in addition to the hairpin species. As described previously, LMPCR recovers blunt, 5' phosphorylated double-strand breaks; treatment of a sample with T4 DNA polymerase prior to LMPCR also permits the recovery of double-strand breaks that are not blunt.

DNA from 103/BCL-2 cells grown at the nonpermissive temperature contains a high frequency of random double-strand breaks, because of the presence of a significant portion of cells undergoing programmed cell death. As this poses difficulties in amplification of locus-specific double-strand breaks, particularly in samples that have been treated with T4 DNA polymerase, we first enriched DNA samples for double-strand breaks in the approximate region of *K1* by size fractionation.

DNA samples from BALB/c mouse kidney or induced 103/BCL-2 cells were treated with T4 DNA polymerase or mock treated. These samples were then digested with *XbaI* and subjected to gel electrophoresis. As the fragment with a *K1* coding end is ~1.7 kb using this enzyme, DNA molecules in the size range of 1.6-1.8 kb were purified from a gel slice and LMPCR was performed on this material. Although several bands were observed following LMPCR of DNA from induced 103/BCL-2 cells, the major product was 430 bp, the appropriate size for a *K1* coding end [Fig. 7A,B]. The 430-bp product was observed in all other repetitions of this experiment, but the other products were not reproducible, indicating that only the 430-bp product is the result of a site-specific double-strand break. No products were observed in a parallel experiment using kidney DNA [Fig. 7B] or in similar experiments with DNA from 103/BCL-2 cells grown at
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Figure 6. Detection of hairpin coding ends by two-dimensional electrophoresis. (A) A map of the \( \kappa \) locus, showing the location of the restriction enzyme sites and the probe used in B. (B) Southern analysis of DNA samples from a culture of 103/BCL-2 cells grown at the permissive temperature (L) or the nonpermissive temperature for 48 hr (H). The direction of native electrophoresis is along the horizontal axis, and the direction of denaturing electrophoresis is along the vertical axis. Ten micrograms of each DNA sample was digested with \( XbaI \), and electrophoresed and transferred as described. Hybridization was with a 1.6-kb probe including all \( K \) segments. Digestion, electrophoresis, transfer, and hybridization were performed in parallel for the two samples. The arrows show the expected sizes after native electrophoresis of the germ-line \( K \) locus (3.4 kb) and broken \( \kappa 1 \) (1.7 kb) and \( \kappa 2 \) (1.35 kb) coding ends, and were determined by comparison to molecular weight standards.

the permissive temperature (data not shown). The amount of amplified coding end did not increase significantly if the DNA was pretreated with T4 DNA polymerase, arguing that the majority of open coding ends are blunt.

We obtained 23 sequences of LMPCR products from the sample that was not pretreated with T4 DNA polymerase and 14 sequences of LMPCR products from the sample that was pretreated with T4 DNA polymerase (Fig. 7C). Consistent with the observation that T4 DNA polymerase pretreatment did not change the amount of amplified coding ends, the pattern of coding end sequences was also not significantly different between the two samples. The pooled sample of 37 sequences contained 34 that were consistent with coding segment termini, as observed in coding junctions. There were deletions ranging from 2–7 bp; in two examples, a non-germ-line-encoded addition of a single nucleotide was also observed. Three clones were not consistent with identification as potential coding ends; two of them retained part of the heptamer sequence, and one had a 28-bp deletion. The double-strand breaks recovered in the above described LMPCR reactions are therefore mostly consistent with identification as open, processed coding ends.

Discussion

Signal junction formation is not coupled to coding junction formation

The 103/BCL-2 cell line rapidly initiates rearrangement of its immunoglobulin light-chain loci in response to a shift in growing conditions. Following induction of V(D)J rearrangement, we observe accumulation of signal ends to levels as high as 30%–40% of total DNA after 48 hr of growth at the nonpermissive temperature. Coding ends are detectable, although 10- to 100-fold less frequent. Coding junctions involving \( \kappa 1 \) accumulate at a

Figure 7. Detection and characterization of open coding ends. (A) A description of the LMPCR product of \( \kappa 1 \)-coding ends. (B) DNA samples from kidney (K) or 103/BCL-2 cells induced for 2 days at the nonpermissive temperature (H) were treated with T4 DNA polymerase, or mock-treated, digested with \( XbaI \), and enriched for DNA species in the size range 1.6–1.8 kb by gel electrophoresis. LMPCR was performed on this size-fractionated material, electrophoresed, and transferred as described in Materials and methods. Products were detected using probe II; only the relevant lanes of this blot are shown. (C) Sequences of cloned inserts derived from PCR amplification of the linker-ligated material described above. Sequence derived from the recombination signal heptamer is italicized. Non-germ-line-encoded sequence additions are in boldface type.
similar rate and to similar levels as /k1 signal ends, indicating that coding ends are rapidly and efficiently resolved to coding junctions, whereas signal ends remain mostly unresolved. Coding ends, therefore, are short-lived intermediates in V(D)J rearrangement.

Signal ends have been observed previously in mouse tissue (Roth et al. 1992a, Schlossel et al. 1993) and in a cell-free reconstitution of the V(D)J cleavage step (van Gent et al. 1995). In this previous work, it was not possible to determine whether the signal ends observed could be joined, however, leaving open the possibility that signal ends are not an intermediate in V(D)J rearrangement. Here we show that the signal ends that accumulate at the nonpermissive temperature are resolved efficiently once the culture is returned to the permissive temperature. This result indicates that at least the majority of the signal ends that accumulate in 103/BCL-2 cells at the nonpermissive temperature are capable of forming signal junctions and are therefore bona fide intermediates in V(D)J rearrangement. We suggest two possible explanations for the accumulation of signal ends at the nonpermissive temperature. The formation of signal junctions may be slow, or signal junctions that are formed may be reopened due to repeated secondary rearrangements. These two possibilities are discussed in more detail below.

The possibility that the formation of signal junctions may be slow, or perhaps blocked, at the nonpermissive temperature seems most likely. Differences in the rate of signal junction formation between permissively and nonpermissively grown cells could have several causes. It has been shown that when ts-abl cells are returned from the nonpermissive temperature to permissive conditions, they re-enter cell cycle and their level of RAG mRNAs is rapidly reduced (Chen et al. 1994). Efficient resolution of signal ends could therefore be cell-cycle dependent, or dependent on the down-regulation of recombination activity. The demonstration that RAG-2 protein levels fluctuate in a cell-cycle-dependent manner, and are highest in G1 (Lin and Desiderio 1994) suggests the intriguing possibility that down-regulation of recombination activity and re-entry into the cell cycle may be linked. We note further that the correlation between the release of ts-abl cells from G1 arrest upon return to the permissive temperature and the more efficient resolution of signal ends is consistent with previous reports that signal ends are observed only in the G1 phase of the cell cycle (Schlossel et al. 1993). The large excess of signal ends over coding ends at the nonpermissive temperature may be related to the previously mentioned report that signal ends are at least 1000-fold more frequent than coding ends in wild-type thymocytes (Zhu and Roth 1995). Pursuing the analogy, it is possible that the signal ends that accumulate in mouse thymocytes are also capable of being resolved to signal junctions more efficiently when the cells have advanced to a later developmental stage.

Alternatively, the rarity of signal junctions at the nonpermissive temperature might be attributable to their ability to participate in further rounds of recombination (Lewis et al. 1985). The reopening of a signal junction could occur as an open and shut recombination, such that a second recombination signal would not be necessary (Lewis and Hesse 1991) or one of the recombination signals in the signal junction could rearrange with a second signal located elsewhere on the chromosome. Neither pathway is a likely explanation for the inability to form signal junctions efficiently at the nonpermissive temperature for the following reasons.

When a signal junction is reutilized, one-half is recognized as a signal sequence but the other half acts functionally as coding sequence (Lewis et al. 1985). Thus, open and shut recombination of a signal junction would often result in loss of terminal sequence from the side used as a coding segment (Lewis and Hesse 1991). This would leave only the other signal as a potential substrate for further rounds of recombination and would not generate an ApaLI site upon reclosure. This expectation is inconsistent with our observation that ApaLI digestion recovers the majority of /k1 signal ends that are incorporated into junctions following return to the permissive temperature. /k1 recombination signals in signal junctions could still frequently participate in rearrangements with one of the many V k recombination signals located farther upstream. At the /3 locus, however, there are far fewer possible rearrangements, allowing us to determine directly that a specific signal junction, the VA1/ /3 signal junction, also forms much more rapidly following return of the culture to the permissive temperature. As the context of the VA1/ /3 signal junction is such that only the /3 recombination signal is available for secondary recombination (Carson and Wu 1989), participation of recombination signals in signal junctions in secondary recombinations is consequently unlikely to be the major reason for inefficient formation of signal junctions at the nonpermissive temperature.

The inability of the ts-abl cell line to resolve signal ends efficiently at the nonpermissive temperature suggests a possible role for V(D)J rearrangement in the induction of the extensive programmed cell death observed in cell lines of this type, in the absence of the exogenously introduced BCL-2 gene (Chen et al. 1994). Cells in the G1 phase of the cell cycle will not continue to S phase if sufficient DNA damage is present; rather, the presence of a large number of double-strand breaks can result in commitment to a programmed cell death pathway (Lane 1993). Previous investigators have suggested that accumulation of V(D)J-generated signal ends might be tolerated by cells if the signal ends were associated with V(D)J rearrangements that had occurred by deletion; the signal ends would therefore be termini of fragments that had been deleted from the chromosome, and failure to join these signal ends would have no effect on chromosomal integrity (Zhu and Roth 1995). At the / locus, however, as many as one-half of all V k segments rearrange by inversion (Shapiro and Weigert 1987), and rearrangement by inversion requires joining of both signal ends and coding ends to maintain an intact chromosome. The extensive programmed cell death observed in ts-abl cell lines in the absence of overexpressed BCL-2
may therefore be partly a consequence of the loss of chromosomal integrity, because of unresolved \( V(D)J \) rearrangements.

A subset of \( \kappa \) loci undergoes frequent rearrangement while the remainder undergoes no rearrangement

We did not observe significant accumulation of signal ends at \( \kappa \) elements located 3' of \( \kappa_1 \), when we used a probe 5' of \( \kappa_1 \). Using a probe located between \( \kappa_1 \) and \( \kappa_2 \), and restriction endonuclease sites flanking the site of the \( \kappa_1 \) double-strand break, we determined that \( \kappa_2 \) signal ends did accumulate to levels similar to the \( \kappa_1 \) signal end (15%–20% of total DNA), but \( \kappa_2 \) signal ends occurred almost exclusively in the context of molecules that had also initiated \( V(D)J \) rearrangement at \( \kappa_1 \). This was a surprising result, given that 50% of \( \kappa \) loci are still in the germ-line configuration at this time. We conclude that rearrangement is highly active on a subpopulation of loci, such that they frequently possess rearrangements at more than one \( \kappa \), whereas the remaining loci are in the germ-line configuration and, thus, apparently quiescent or resistant to rearrangement.

These observations could be explained if there was a highly ordered program of rearrangements, beginning at \( \kappa_1 \), and then progressing in the 3’ direction to other \( \kappa \)s. Alternatively, rearrangement may be limited to 50% of \( \kappa \) loci for unknown reasons, and saturation has occurred by accumulation of many rearrangements to randomly chosen \( \kappa \)s on those \( \kappa \) loci that are more permissive for rearrangement. For example, chromosomal accessibility may be a limiting factor, such that once the \( \kappa \) locus on a given chromosome becomes accessible, rearrangement is so much more efficient that it frequently occurs several times on the same chromosome. Rearrangement could also be much more active on the circular deletion products of primary rearrangements.

Coding end structure

We have demonstrated that a portion of the coding ends observed after induction of \( V(D)J \) rearrangement are covalently sealed [hairpins]. Hairpin coding ends were first detected in thymocytes from scid mice but not wild-type mice (Roth et al. 1992b; Zhu and Roth 1995). However, hairpins have also recently been detected in a cell-free reconstitution of the cleavage step of \( V(D)J \) rearrangement (van Gent et al. 1995). This latter observation, as well as the observation of P nucleotides in coding junctions from normal mice, suggests that hairpins are not attributable to an aberrant diversion of coding ends species specific to \( V(D)J \)-generated cleavages in the background of the scid mutation. We demonstrate here that hairpins are also detectable under conditions where formation of coding junctions is to all appearances normal and efficient, thus further supporting the argument that hairpin coding ends are a normal intermediate in \( V(D)J \) rearrangement.

When the sequence of hairpin ends has been examined, they have been found to contain the full coding sequence [Zhu and Roth 1995]. Thus it is likely that they are formed at the first step in \( V(D)J \) rearrangement, before any removal of nucleotides. A hairpin coding end intermediate must be opened next to permit the processing observed at coding segment termini in coding junctions. We were also able to demonstrate the existence of a new species of \( V(D)J \)-generated double-strand breaks; the majority of sequenced examples of this species were consistent with identification as open, processed \( \kappa_1 \)-coding ends. No examples with sequence terminating exactly at the end of the \( \kappa_1 \)-coding segment were observed, suggesting that all members of this species are the products, rather than the precursors, of hairpins. This is consistent with the argument that hairpins are the direct, obligatory product of a double-strand break at the recombination signal. Previous models for processing of coding ends have suggested that hairpins are opened by a nick in the DNA molecule near the terminus of the coding segment; the resulting single-stranded DNA extensions could generate a P nucleotide addition if fill-in DNA synthesis occurred or short deletions if blunted by a single-stranded DNA specific nuclease. Pretreatment with T4 DNA polymerase produced no increase in the recovery of open coding ends and did not significantly alter the pattern of recovered sequences, suggesting that few, if any, open coding ends possess single-stranded DNA extensions. If coding end species with single-stranded DNA extensions are frequent, they must be converted rapidly to the blunt coding ends described here.

Two distinct pathways for the resolution of \( V(D)J \)-generated double-strand breaks

Taken together, the evidence presented here suggests that \( V(D)J \) rearrangement is initiated by a double-strand break, after which coding ends and signal ends are processed very differently [Fig. 8]. Coding ends are short-lived and probably pass through both a hairpin and an open configuration prior to rapid and efficient joining. In contrast, the majority of signal ends remain unjoined for a longer time. Efficient signal end resolution may be dependent either on the down-regulation of \( V(D)J \) rearrangement, or reentry into the cell cycle.

It therefore seems likely that resolution of the signal end species into a signal junction is not coupled to resolution of the coding end species into a coding junction. Although the occasional observation of hybrid junctions indicates that the separation of joining pathways for coding ends and signal ends is not absolute (Lewis et al. 1988), uncoupling of the two joining pathways is also supported by the fact that the two different types of ends are processed differently, and by the different degree to which the scid mutation affects each pathway. The strong evidence that hairpins are the normal, direct product of the cleavage reaction suggests the possibility that the difference in structure between coding ends and signal ends might act to direct each species down the appropriate pathway. Alternatively, the proteins of a complex that initiated cleavage, and that remain associated
Materials and methods

Block signal ends from resolving at the same time, and with the recombination signal after cleavage, may act to block signal ends from resolving at the same time, and in the same manner, as coding ends.

Cell lines and preparation of DNA samples

103/BCL-2/4 was the gift of Dr. N. Rosenberg [Tufts University, Boston, MA], and was cultured as described previously (Chen et al. 1994). This cell line was maintained at 34°C, in 1 mg/ml of G418 (GIBCO), and induced at 39.6°C. The T cell line B6Mo3 was the gift of Rex Risser (University of Wisconsin, Madison). BALB/c mouse kidney DNA was obtained from Clontech. High molecular weight DNA used for direct Southern analysis was prepared by standard techniques (Sambrook et al. 1989). DNA samples used for PCR reactions were prepared either by standard techniques (Sambrook et al. 1989), or using the Nucleon genomic DNA extraction kit (Scotlab), as per the manufacturer's instructions. A sample enriched for small molecular weight DNA was prepared by first precipitating high molecular weight DNA. After high molecular weight DNA was removed by spooling, the supernatant was chilled to -20°C overnight, and small molecular weight DNA was recovered after centrifugation at 30,000g for 15 min. Gel electrophoresis of this sample indicated that the majority of recovered DNA was <1 kb in size.

Southern analysis

Restriction enzymes were purchased from New England Biolabs. Restriction digests were electrophoresed in 1.2% SeaKem LE agarose (FMC Bioproducts) gels, except the gel used in Figure 2, which was a 3% NuSieve GTG agarose (FMC Bioproducts) gel. Two-dimensional electrophoresis was performed as described previously (Roth et al. 1992b). Agarose gels were transferred as described previously (Roth et al. 1992a) to Genescreen Plus (New England Nuclear) nylon membranes.

The probes described in Figure 1A were obtained by PCR amplification, for 25 cycles, of 1–10 pg of pRBjCκ (the gift of S. Lewis, California Institute of Technology, Pasadena, CA), using our standard PCR conditions (see below). Probes I, II, and III were generated using the primer pairs DAR6 and DAR7, DAR19 and DAR25, and DAR15 and DAR16, respectively. Each product was purified by gel electrophoresis and labeled by a modified PCR reaction. In this modified PCR reaction, we used ~10 ng of gel purified product, 25 pmoles of each primer, a final concentration of 40 μM each of dATP, dGTP, and dTTP, and 100 μM of [α-32P]dCTP (6000 Ci/mmol), in a volume of 25 μl. Reactions were subjected to two cycles of PCR, with each cycle consisting of 30 sec at 95°C, 15 sec at 60°C, and 5 min at 72°C.

The RAG-1 probe used in Figure 4C was generated by gel purification of the 1.1-kb fragment produced by Sau3A digestion of a plasmid containing the RAG-1-coding sequence (the gift of M. Sadofsky, National Institutes of Health, Bethesda, MD). The probe including all five κ segments used in Figure 6 was generated by gel purification of the 1.6-kb fragment produced by digestion of pRBjCκ with Banl and XbaI. These latter two probes were labeled by extension of random hexamer primers [New England Biolabs], using standard techniques (Sambrook et al. 1989).

Southern blots were hybridized with ~1 × 10^6 cpm/ml of probe in a solution containing 1 mM NaCl, 1% SDS, and 10% dextran sulfate in overnight (14–18 hr) incubations at 65°C, with the exception of the those Southern blots hybridized with probe 1 (Figs. 1B and 4B), which were hybridized overnight at 48°C in a solution containing 2× SSC, 1% SDS, 10% dextran sulfate, and 50% deionized formamide. All blots were washed twice at room temperature in 2× SSC for 15 min each, followed by two washes at 65°C for 30 min each, in 2× SSC/1% SDS. The blots shown in Figures 1B, 4B and C, and 6 were washed further for 30 min at 65°C in 0.2× SSC/0.1% SDS. Southern blots were exposed first for 1–4 days to Phosphorlmager plates (Molecular Dynamics), followed by exposure for 1–3 weeks to X-ray film. The blot in Figure 1 was stripped by incubation in 0.4 N NaOH for 30 min, neutralized in 2× SSC, and stored for 3 months to allow further decay of any residual radioactivity prior to analysis with each subsequent probe.

A Molecular Dynamics Phosphorlmager was used for quantification. We generated a size-matched control for estimation of the amount of κ1 signal end and κ1-coding end species by digestion of kidney DNA with Banl, in addition to the same enzymes that were used for digestion of DNA from induced 103/BCL-2 cells. We corrected further for differences in the amount of DNA in each sample by comparison of the amount of signal observed with a RAG-1 probe. Estimates for the amount of signal ends and coding ends at κ1 were derived from such comparisons using DNA samples from three independent inductions.

PCR analysis

PCR reactions were performed using a Perkin-Elmer 9600 thermal cycler, in a volume of 50 μl, containing 2.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl at pH 8.3, 0.01% gelatin, 250 μM each of dATP, dGTP, and dTTP, 50 pmoles of each primer and 2.5 units of Taq polymerase [Perkin-Elmer]. Amplification was preceded by incubation at 95°C for 4 min, followed by a variable number of cycles, each consisting of a denaturation step for 30 sec at 94°C, an annealing step for 15 sec at 60°C, and a primer extension step for 30 sec at 72°C, with one exception. Annealing of primers for the amplification of κ signal junctions (Fig. 5B) was performed at 62°C. Each amplification was followed by a further incubation at 72°C for 10 min. One-third of each amplified sample was electrophoresed on 5% polyacrylamide gels and transferred electrophoretically to nylon membranes. Oligonucleotide probes were 5′ end-labeled using standard techniques.

To confirm the presence of approximately equivalent
amounts of target DNA in samples analyzed in Figures 3 and 5, we amplified a germ-line sequence 3' of \( \kappa \)1 with the primers DAR25 and DAR24 for 20 cycles, using 0.5 \( \mu \)g of DNA. This product was detected with \( \alpha \)-end-labeled DAR26. \( \alpha \)K1-coding junctions were amplified for 22 cycles, from 0.5 \( \mu \)g DNA samples, using the primers DAR14 and DAR24. This PCR product was detected using \( \alpha \)-end-labeled DAR25 as a probe. LP PCR of \( \kappa \)1 signal ends was performed by first ligating 0.5 \( \mu \)g of each DNA sample with the linker FM11/25, as described previously [Roth et al. 1993]. One-half (50%) of each ligation was amplified for 25 cycles with the primers FM25 and DAR10. This PCR product was detected using \( \alpha \)-end-labeled DAR3 as a probe. \( \alpha \)I1A1-coding junctions were amplified from 0.5 \( \mu \)g of DNA, using the primers DAR31 and DAR34, for 25 cycles, and the PCR product detected using \( \alpha \)-end-labeled DAR35. PCR amplification of \( \alpha \)I1A1 signal junctions were performed on 1 \( \mu \)g of DNA and amplified for 25 cycles, using DAR28 and DAR29, and detected using \( \alpha \)-end-labeled DAR30.

**Amplification and cloning of open \( \kappa \)1-coding ends**

Thirty micrograms of DNA from kidney or induced 103/BCL-2 cells was treated, in a buffer containing 10 mM Tris-HCl (pH 7.8), 50 mM NaCl, 10 mM MgCl\(_2\), 1 mM DTT, 50 \( \mu \)g/ml of BSA, and 125 \( \mu \)M each of dATP, dCTP, dGTP, and dTTP for 2 hr at 37°C. T4 DNA polymerase was subsequently heat inactivated by incubation at 75°C for 10 min. A reconstruction experiment with kidney DNA precut with BanI (BanI has a site 16 bp 3' of \( \kappa \)1 and leaves a 5' overhang) indicated that T4 DNA polymerase treatment was effective in blunting staggered cuts (data not shown). These samples were digested with Xhol and electrophoresed in a 1.2% SeaPlaque agarose (FMC Bioproducts) gel. A portion of the gel containing DNA of size 1.6–1.8 kb, as estimated by comparison to an adjacent lane containing molecular weight standards, was cut out. DNA from this gel slice was purified by agarase (New England Biolabs) digestion and ethanol precipitation. The gel-fractionated DNA (25%) was ligated to the linker FM11/25. One-half (50%) of each ligation was amplified for 25 cycles with the primers DAR19 and FM25, and analyzed by electrophoresis and Southern blotting, as described above.

Coding ends were cloned by amplification of 10% of the material from the ligations described in the preceding paragraph, for only 15 cycles, with the primer pair DAR19 and FM25. Thirty percent of this reaction was electrophoresed in a 3% NuSieve agarose (FMC Bioproducts) gel. DNA of size 400–500 bp was excised from this gel and subjected to a second round of PCR amplification, for 25 cycles, using the primer pair DAR26 and FM25. A species of ~130 bp was excised, purified by agarase digestion and ethanol precipitation, and cloned, using the TA cloning kit (Invitrogen). Inserts that possessed a DAR26-derived signal junction were performed on Ip-g of PCR product detected using \( \alpha \)-P-end-labeled DAR3 as a probe. LMPCR junctions were amplified for 22 cycles, from 0.5 \( \mu \)g DNA sample with the linker FM11/25, as described previously (Ramsden et al. 1994); DAR24, 5'-CTTTCGCTTGGAGAATCGTCAAACTACA-3' (Ramsden et al. 1994); DAR25, 5'-GACGTAGCTGAGACATGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
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