Dual role of RAG2 in V(D)J recombination: catalysis and regulation of ordered Ig gene assembly

Susan A. Kirch1,2, Gary A. Rathbun3 and Marjorie A. Oettinger1,4

1Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114, 2Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138 and 3Harvard Institutes of Medicine, Department of Medicine, Beth Israel Hospital, Boston, MA 02115, USA
4Corresponding author
e-mail: oettinger@molbio.mgh.harvard.edu

Immunoglobulin genes are assembled during lymphoid development by a series of site-specific rearrangements that are tightly regulated to ensure that functional antibodies are generated in B (but not T) cells and that a unique receptor is present on each cell. Because a common V(D)J recombinase comprising RAG1 and RAG2 proteins is used for both B- and T-cell antigen receptor assembly, lineage-specific rearrangement must be modulated through differential access to sites of recombination. We show here that the C-terminus of the RAG2 protein, although dispensable for the basic recombination reaction and for Ig heavy chain DJH to JH joining, is essential for efficient VH to DJH rearrangement at the IgH locus. Thus, the RAG2 protein plays a dual role in V(D)J recombination, acting both in catalysis of the reaction and in governing access to particular loci. Keywords: B-cell development/immunoglobulin/RAG2/V(D)J recombination

Introduction

Functional immunoglobulin and T-cell receptor (TCR) genes are assembled from non-contiguous gene segments by a series of site-specific recombination reactions collectively termed V(D)J recombination (reviewed in Lewis, 1994; Gellert, 1997). Seven different loci containing V, J and sometimes D segments may be joined by this reaction: the TCR α, β, γ, δ and immunoglobulin H, κ and λ loci. All rearrangements are mediated by conserved recombination signal sequences (RSSs) that mark the border of the coding segments. Recombination is initiated by two lymphoid-specific proteins, RAG1 and RAG2 (Schatz et al., 1989; Oettinger et al., 1990), that recognize the RSS and introduce a double-strand break at the signal–coding boundary (McBlane et al., 1995). The rearrangement is then completed by a process that involves a number of double-strand break repair factors (Weaver, 1995).

The restricted expression of the RAG proteins to developing B and T cells serves to limit V(D)J recombination to immature lymphoid cells. The apparent cell cycle regulation of RAG2 protein expression further restricts the initiation of V(D)J recombination to cells in G1 or G0 (Lin and Desiderio, 1995). While a common machinery is responsible for antigen receptor assembly in both developing B and T cells, rearrangement is lineage specific such that TCR loci are never rearranged fully in B cells and vice versa. Rearrangement also occurs frequently in a preferred temporal order. For example, at the Ig heavy chain locus, joining of DH to JH segments precedes VH to DJH joining, and heavy chain rearrangement generally precedes light chain assembly (Lewis, 1994).

It is generally assumed that regulation of antigen receptor recombination is established by modulating the accessibility of particular sites to the recombination machinery in a manner that is specific to the tissue, lineage and developmental stage (Blackwell and Alt, 1988). For example, non-lymphoid cells (e.g. fibroblasts) co-expressing the RAG1 and RAG2 proteins are fully capable of recombining a variety of extrachromosomal and stably integrated recombination substrates, but no rearrangements of the endogenous Ig and TCR loci are detectable (Schatz et al., 1992). Thus, although these fibroblasts express all the necessary factors for V(D)J recombination, some form of negative regulation renders the endogenous Ig and TCR loci inaccessible. While this phenomenon of differential accessibility is readily apparent, little is understood about the molecular mechanism responsible for it.

The initial cleavage at an RSS appears to be the primary point at which V(D)J recombination is regulated (Cuomo et al., 1996; Ramsden et al., 1996). Mutations in recombination signal sequences have very similar effects on V(D)J recombination in vivo and V(D)J cleavage in vitro, suggesting that once cleavage has been initiated the recombination reaction will proceed, provided all the requisite general DNA repair factors are present. As indicated above, the regulation of this cleavage event can take place at multiple levels; there are requirements for a pair of functional RSSs, an active recombinase capable of recognizing these RSSs and organizing them into a synaptic complex, and an accessible DNA architecture permitting cleavage at a locus. It has long been noted that transcription at a locus is tightly correlated with recombination, but transcriptional activity in the presence of an active V(D)J recombinase is not always sufficient to make a locus recombinationally active (Lewis, 1994). Recent work has demonstrated that the DNA architecture that renders a locus accessible during a particular stage of lymphoid development is stable (Stanhope-Baker et al., 1996); for example, DNA in nuclei isolated from cells actively undergoing VH to DJH joining remains capable of undergoing primarily this rearrangement and not others. All the functions necessary for carrying out V(D)J cleavage in vitro are contained within ‘core’ domains of RAG1 (amino acids 384–1008 out of 1040) and RAG2 (amino acids 1–383 out of 527) (McBlane et al., 1995). When expressed together with full-length RAG2, the
RAG1 core is sufficient to mediate the complete recombination event in vivo (Sadofsky et al., 1993; Silver et al., 1993; Kirch et al., 1996), albeit at a slightly diminished frequency (Sadofsky et al., 1993; Silver et al., 1993; Kirch et al., 1996; McMahen et al., 1997; Roman et al., 1997). Similarly, the core of RAG2 functions in vivo in conjunction with full-length RAG1 or an N-terminal RAG1 deletion (amino acids 331–1040) efficiently to promote the recombination of extrachromosomal and integrated recombination substrates (Cuomo and Oettinger, 1994; Sadofsky et al., 1994; Kirch et al., 1996). The formation of signal joints, coding joints, and deletional recombinants is also essentially unaffected by the C-terminal truncation of RAG2 (Cuomo and Oettinger, 1994; Sadofsky et al., 1994).

Thus, the RAG2 C-terminus is an integral part of the integration sites and expression levels could be magnified, given the high degree of conservation of this region through evolution. One previously unexplored possibility is that the C-terminus of RAG2 might play a role in the rearrangement of endogenous loci. Here we compare V(D)J recombination mediated in pre-B cells by full-length RAG2 or its truncated counterpart. We find that while the C-terminus of RAG2 is dispensable for D\(\gamma\) to J\(\gamma\) rearrangement at the Ig heavy chain locus, VH to DJH recombination is impaired. Because decreases in recombination efficiency could be masked in an animal by the selective pressures leading to the proliferation of cells with proper rearrangements, we chose to carry out these studies in cultured murine cell lines. We employed Abelson murine leukemia virus (Ab-MuLV)-transformed RAG2–/– pro-B cell lines derived from both mouse fetal liver (Shinkai et al., 1992) and adult (3-week-old) bone marrow which are poised to initiate Ig V(D)J assembly upon RAG2 expression (Taccioli et al., 1992; G.Rathbun and F.W.Alt, unpublished).

The RAG2-deficient pro-B cells were stably transfected with linearized plasmids expressing either full-length RAG2 or the truncated core. RAG2 expression was driven by a V\(\gamma\) chain promoter and E\(\gamma\) enhancer that we previously found to be active in these cell lines (see Materials and methods). Control cell lines carrying only the expression vector and co-selectable marker were generated in parallel. Rather than considering effects in individual clones where small differences resulting from integration sites and expression levels could be magnified, we chose to analyze populations of cells independently transfected with the expression constructs. In this way, we could examine the intrinsic effect of the RAG2 deletion on the recombination event itself.

Overall V(D)J recombination activities in the cell populations derived from the full-length and truncated RAG2 proteins were shown to be comparable by transiently transfecting cells with an artificial recombination substrate (pJH200) (Hesse et al., 1987) and measuring recombination frequency. Recombination levels in cells carrying the wild-type RAG2 were 2- to 3-fold higher than for those carrying the truncation, in keeping with our earlier results on episomal and integrated recombination substrates in NIH 3T3 cells (Cuomo and Oettinger, 1994; Kirch et al., 1996). No recombination was detected in the controls transfected with the vector alone (Table I). The comparable level of recombination for the wild-type and truncated RAG2 clones makes it possible to consider the role of the RAG2 C-terminus in promoting endogenous rearrangement. We studied three different rearrangement events, IgH DJ and V to DJ joining and Ig\(\kappa\) V to J recombination, in parallel in the same cell populations.

The extent of DJ\(\gamma\) joining in the transfected cell populations was measured using a standard semi-quantitative PCR strategy (Schlissel et al., 1991). Genomic DNA was prepared from cells that were passaged for 2–6 weeks post-transfection (~56–168 doublings) to permit endogenous rearrangement to occur. Representative data are shown in the figures, and the average normalized phosphorimager quantitations from at least five independent PCR amplifications and at least three independent DNA preparations for each cell population are shown in Table I. A specific J\(\gamma\) primer was used in combination with a pool of degenerate primers that recognize essentially all known D segments at the IgH locus (Schlissel et al., 1991). Because the sequences complementary to the primers are very far apart in their germline configuration, amplification products 1 kb or less in size are expected only following DJ\(\gamma\) rearrangement. Efficient DJ\(\gamma\) joining was observed for pools of cells expressing either the full-length or core RAG2 proteins. (Figure 1A, Table I). In keeping with the decreased recombination activity measured with the plasmid substrate, the amount of DJ\(\gamma\) rearrangement mediated by the truncated clone was consistently 2- to 3-fold lower than that for full-length RAG2. Thus, the truncated clone is fully capable of assembling D\(\gamma\) and J\(\gamma\) segments and there is no overall distinction between the relative recombination frequencies measured for plasmids or endogenous loci.

Similarly, there was also no apparent effect of the absence of the RAG2 C-terminus on rearrangement at the

| Table I. Levels of rearrangements in cells expressing the RAG2 core protein (relative to the full-length protein) |
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| Cell population | pJH200 rec. freq. | V\(\kappa\) to J\(\kappa\), % | DH to J\(\gamma\), % | VH to DJ\(\gamma\), % |
| BM-1B | 0.34 | 0.50 | 0.67 | 0.005 |
| FL-1B | 0.30 | 0.75 | 0.29 | >0.005 |
| FL-2B | 0.55 | 0.73 | 0.38 | >0.005 |
| FL-3B | 0.57 | 0.60 | 0.44 | >0.005 |

aBM-1B and FL-1B, 2B and 3B are derived from bone marrow (BM) and fetal liver (FL) cells.
bWild-type frequencies were between 1 and 3%.
cEndogenous rearrangements are reported as phosphorimager values normalized to rearrangement level measured for full-length RAG2 in each individual experiment. Each number represents the average of at least five independent PCR amplifications and at least three independent DNA preparations.
Ig κ locus (Figure 1B, Table I). PCR amplification of κ rearrangements showed comparable levels of VκJκ joints. Recombination in cells expressing the core RAG2 was again reduced 2- to 3-fold compared with the wild-type counterparts, in keeping with the overall decrease in recombination efficiency of this mutant.

By contrast, Vκ to DJκ rearrangement showed a striking dependence on the RAG2 C-terminal region. Separate PCR amplifications were carried out using degenerate oligonucleotides specific for three different Vκ family segments. Two of these families, Vκ7183 and VκQ52, are the most Jκ proximal and are utilized preferentially in endogenous VH-DJH rearrangements. The third, Vκ558, is a more distal family that is used less frequently (Lansford et al., 1996). By pairing these Vκ family primers with a primer downstream of Jκ4, we were able to measure 12 distinct rearrangement products, representing assembly of the three Vκ gene segments with one of four possible DJκ products. All 12 products were readily apparent in the DNA prepared from cells expressing wild-type RAG2 protein (Figure 2A, lanes 1–3). Figure 2A (lanes 10–12) shows the nine expected products from rearrangement detected using a Jκ3 primer. However, VH to DJκ rearrangements were decreased dramatically in cells expressing the core RAG2 protein (four independent populations) when compared with those expressing the full-length protein (Figure 2A, lanes 4–6 and 13–15, and data not shown). Indeed, no VH to DJκ rearrangement was detected in three of those cell populations (Figure 2A, Table I).

When each pair of cell populations expressing wild-type and core RAG2 was compared, the apparent decrease in VH to DJκ rearrangement with core RAG2 as determined by phosphorimager quantitation was at least 200-fold (Table I). Because a completed DJκ rearrangement is a prerequisite for detecting the VH-DJκ product, we took into consideration both the 2- to 3-fold decrease in DJκ starting material and the 2- to 3-fold decrease in recombination activity in the overall population. Even with the most conservative assumption (that only one-ninth as much VH to DJκ joining could be expected when the RAG2 core was present) in the one case where VH to DJκ joining was observed, the level of VH-DJκ product was reduced ~20-fold in the populations expressing core RAG2 compared with the corrected wild-type counterparts. This level of decrease was consistent for all 12 rearranged species. To determine the maximum level of rearrangement that could be present in the three cell populations in which rearrangement was not detected, cells expressing full-length RAG2 were diluted into cells expressing only the pSV2His vector, and the fraction of wild-type expressing cells required to detect a signal by PCR in the context of equivalent amounts of total genomic DNA was determined (Figure 2B and C and data not shown). In the three cell populations in which no VH to DJκ joining was detected, we estimate from these experiments that VH to DJκ recombination in these cells must also be decreased at least 20-fold (corrected in the same manner) to reduce the recombination to undetectable levels.

**Discussion**

We show here that VH to DJκ rearrangement is greatly depressed by the removal of the RAG2 C-terminus, and that this depression affects proximal and distal V families equivalently. The observed decrease in VH to DJκ joining is unlikely to reflect the idiosyncrasies of expression or rearrangement of an individual clone, as recombination was measured in bulk populations and the same results were also obtained from multiple independent cell populations. Furthermore, we ruled out the possibility that the decrease in VH to DJκ joining resulted from a loss of functional RAG2 expression over time, by confirming that the cell populations continued to recombine a transiently transfected plasmid recombination substrate at a constant level over time. Moreover, the consistency of the 2- to 3-fold decrease in plasmid DJκ to Jκ and κ rearrangement provides an internal control demonstrating that recombination could occur in these cells and that all transfected populations have equivalent rearrangement potential. Because the only consistent difference between cell populations is the expressed RAG2 construct, the most probable explanation for the results presented here is that the RAG2 protein is involved in the regulation of V(DJ
rearrangement. Thus, the work presented here provides the first direct link between the recombination machinery itself and the locus-specific regulation of recombination activity.

Previous observations of the natural regulation of V(D)J recombination in vivo indicate that VH to DJH joining is more subject to regulation than the DH to JH step. Thus, our observation that the RAG2 C-terminus is important in the regulation of VH to DJH, but not DH to JH, joining is consistent with the known pattern of regulated Ig rearrangement. For example, IgH DH to JH joining is a very early event in developing B cells and not very tightly regulated. In contrast, VH to DJH joining is severely impaired in the absence of heavy chain assembly. Rather, the consistent 2- to 3-fold decrease in rearrangement observed here in the absence of the RAG2 C-terminus could be the result of some global regulation of V(D)J recombination, as the TCRβ locus in these cells remains in the germline configuration (data not shown). Because the k rearrangement measured here is likely to reflect the natural level of rearrangement that occurs independently of heavy chain joining, we cannot say whether the RAG2 C-terminus plays a role in the up-regulated k rearrangement that occurs following successful heavy chain assembly. Rather, the consistent 2- to 3-fold decrease in rearrangement observed at this locus in cells expressing truncated RAG2 should be viewed simply as an additional internal control for recombination potential in the cell populations.

The accessibility of an individual locus could be modulated in any of a number of ways, and it is possible that multiple forms of control are imposed. It is likely that the DNA architecture is regulated to facilitate excluding or attracting the RAG proteins. In this regard, the correlation of transcription and recombination may reflect not the need for transcription per se, but the need for an altered chromatin configuration. Recently, sterile transcription from unrearranged V segments (a marker of chromatin...
configuration) was shown to be silenced in mice lacking IL-7Rα, providing an insight into the cause of the diminished V to DJ joining observed in these animals. Loci may also include regions of DNA (e.g. the TCRβ enhancer region) that contain not only the binding sites for transcription factors, but also possibly binding sites for recombination enhancers (Villey et al., 1996). The accessibility of a locus to rearrangement may also be controlled on a more local level. For example, the apparent requirement for unpairing of the DNA at the RSS border to facilitate cleavage (Cuomo et al., 1996; Ramsden et al., 1996) suggests that factors that affect the state of the DNA could be used to control recombinational accessibility.

A number of models could explain the dependence of efficient Ig Vβ3 to DJH joining on the RAG2 C-terminus. This region of the RAG2 protein could be involved directly in gaining access to the site of rearrangement, perhaps by displacing bound proteins that block the site. The yeast Ty1 and Ty5 recombination proteins, for example, are known to be targeted specifically to open or silent chromatin respectively (Devine and Boeke, 1996; Zou et al., 1996), and may thus interact with proteins determining these regional specificities. Alternatively, the RAG2 C-terminal domain might act at a recombinational enhancer site, interacting with the DNA or cooperating with other proteins to open up the Vβ3 locus to the recombination machinery. The 59 amino acid acidic region (25 acidic residues) within the RAG2 C-terminus might, by analogy with transcription factors, be involved in protein–protein interactions that can regulate the state of the DNA or the recruitment of other required factors.

Here we have shown that the RAG2 protein appears to play a dual role in V(D)J recombination, acting both as a catalyst of the cleavage reaction itself and as a factor required for the regulation of recombination. These functions of RAG2 appear to be associated with two separable domains, the N-terminal three-quarters determining the catalytic activity and the C-terminal quarter involved in site accessibility. Other proteins with similar dual roles have also been described. MyoD, for example, contains a domain distinct from that involved in transcriptional activation which plays a role in reorganizing chromatin to facilitate the binding and activity of MyoD itself (Gerber et al., 1997). The identification of RAG2 as a factor involved in the regulation of V(D)J joining suggests that RAG2 should prove useful as a tool to identify further factors in the same process and for investigating their roles in the regulation of endogenous V(D)J recombination.

Materials and methods

Constructs

Standard subcloning procedures, Escherichia coli transformation and plasmid preparation procedures were as described in Ausubel et al. (1989). Full-length and core RAG2 (R2CC14, Cuomo and Oettinger, 1994) were subcloned into the 2C expression vector containing a fused E4–E1 promoter (Dildrop et al., 1989) and an SV40 poly(A) addition site. The RAG2 full-length construct contains the XhoI–Smal fragment from LG4.3 (Cuomo and Oettinger, 1994) subcloned into matching sites in the 2C vector. Core RAG2 was generated from R2CC14 (Cuomo and Oettinger, 1994) digested with EcoRI, treated with Klenow fragment and followed by digestion with XhoI; the resultant RAG2-containing fragment was subcloned into XhoI–Smal sites in 2C. For stable transfection, the RAG2 expression constructs were prepared by two rounds of CsCl purification to ensure no bacterial genomic DNA or RNA contamination was present. Finally, RAG2 plasmids were linearized with NotI and the co-selectable marker pSV2His was linearized with EcoRI, purified by organic extraction and ethanol precipitated prior to transfection.

Cell culture and transfection

The fetal liver-derived, RAG-2-deficient cell line R2FL63-12 has been described previously (Shinkai et al., 1992). The bone marrow-derived RAG2-deficient cell line R2BM3-7 used for these studies is described here. Bone marrow cells were obtained from a 3-week-old RAG2–/– mouse (generous gift of F.W. Alt) and transformed with Ab-MuLV as described previously (Rosenberg and Baltimore, 1976) and under the guidance of N.Rosenberg and K.Parmar. The cell populations BM1A, 1B, 1C and FL2A, 2B and 3B were generated by co-transfection of the appropriate expression vector and pSV2His which served as a co-selectable marker. BM-1A, 1B and 1C are derived from bone marrow and contain wild-type, core and vector alone, respectively. FL-1A, 1B, 2B and 3B were derived similarly from fetal liver (Shinkai et al., 1992); wild-type = full-length RAG2, core = RAG2 (1–383), vector = the 2C expression vector. All of these cell lines were cultured in RPMI (Gibco-BRL) containing 0.2 mM glutamine, 0.05 mM 2-mercaptoethanol, 10–20% fetal calf serum (FCS; Sigma) and 50 mg/ml gentamicin sulfate, and adjusted for a final pH 7.2 at 5% CO2 and 37°C.

For stable transfection, cells were harvested at 5–8 x 106 cells/ml by centrifugation at 1000 r.p.m. (Beckman GH-3.7 rotor), 5 min at room temperature, washed once in 1 x phosphate-buffered saline (PBS) and resuspended at 6 x 106 cells/ml in RPMI (as described above) supplemented with 20% FCS. A total of 1.5 x 106 cells were mixed with the appropriate combination of linearized DNA at a 2C expression vector: pSV2His ratio of 10:1, transferred to electroporation cuvettes (Bio-Rad 0.45 cm gap) and incubated on ice for 10 min. A Bio-Rad Gene-Pulser was used to deliver 250 V at 960 μ. The cells were transferred to 37°C immediately and allowed to recover for 10 min before transfer to 10 ml of RPMI media containing 20% FCS. Cell death was ~30–50% under these conditions as tested with methylene blue. Cells were cultured in non-selective media for 48 h, at which time they were centrifuged, washed with 1 x PBS and transferred to media containing 0.06 mM histidinol. Cells containing pSV2His are histidinol resistant. The concentration of histidinol was increased gradually to 0.25 mM over a period of 2 weeks or until the mock-transfected population died. Selection was maintained by cultivating these cells in 0.125 mM histidinol.

PCR and Southern blot analysis

Endogenous recombination in the stable cell lines was monitored by standard PCR and Southern blot methods with a few modifications to protocols previously described (Ausubel et al., 1989; Schlissel et al., 1991; Marshall et al., 1996). Briefly, genomic DNA was prepared using a Genomic Prep Kit (Qiagen, Cat. 10245) or by lysing 106 cells in 200 μl of PCR lysis buffer (Schlissel et al., 1991). PCRs were performed on 4 μl of lysate or 1 μg of genomic DNA in a final volume of 50 μl containing 1 x Boehringer-Mannheim amplification buffer with 0.15 mM MgCl2, 2.5 mM each of dATP, dCTP, dGTP and dTTP (Pharmacia), 100 ng of each primer and 1 U of Taq polymerase (Boehringer-Mannheim). For detecting DJH, Vβ1DJH and VκJκ rearrangements, 30 cycles of amplification were performed in a DNA thermal cycle (MJ Research, Inc.) at 94°C for 30 s, 58°C for 1 min, 72°C for 2 min with 5 s/cycle increase in extension time. A 20 μl aliquot of the PCRs were run on 1.2% agarose gels in Tris–borate, transferred in 0.5 M NaOH/0.1 M EDTA to Zetabind (American Bioanalytical) and probed with 32P-labeled DNA. IgH chain gene rearrangements were probed with a fragment generated from PCR amplification of the JH3–1–JH4 gene segments from murine 1–8 cells with the Mu0/JH4 primer pair (Schlissel et al., 1991). PCRs were performed on 4 μl of PCR lysis buffer (Schlissel et al., 1991). Igκ rearrangements were probed with the 2.5 kb HindIII fragment from pRBJCe which contains the mouse Jκ1–Jκ8 gene segments (Lewis et al., 1982). β-actin was detected by 24 cycles of 1 min at 94°C, 1 min at 60°C and 1.75 min at 72°C, under the conditions described above with the 5′ACT (5′-CCAAGGCCCAACCGTGAAAAG-3′) and 3′ACT (5′-TTTCATGTCTGCTAGGACCA-3′) primers. Ten μl of these PCRs were run on 1% agarose gels in Tris–borate and the products visualized by ethidium bromide. Band intensities were quantitated using a phosphorimagery and ImageQuant software (Molecular Dynamics).

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