Immunity, Vol. 3, 715–726, December, 1995, Copyright © 1995 by Cell Press

# Localization, Interaction, and RNA Binding Properties of the V(D)J Recombination-Activating Proteins RAG1 and RAG2

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## Summary

**The RAG1 and RAG2 gene products are indispensable for activating somatic rearrangement of antigen receptor gene segments. The two proteins form a stable complex in primary thymocytes as well as when expressed in adherent cells. In both cell types, most cells localize RAG proteins at the periphery of the nucleus. However, when overexpressed in fibroblast cells, RAG1 is found largely in the nucleolus. Nucleolar localization of RAG1 is mediated by several domains containing stretches of basic amino acids, indicating that RAG1 has affinity for RNA or ssDNA. The RAG1 interacting proteins SRP1 and Rch1 directly bind to the nuclear localization signals of RAG1, which mediate the nuclear and nucleolar translocation of the protein. RAG1 appears to have a binary structure, each half containing multiple regions that can act as NLSs, binding sites for the SRP1/Rch1 family, and RNA binding domains.**

## Introduction

In the developing immune system, diversity of the antigen receptors, immunoglobulin, and T cell receptor (TCR) is generated by somatic rearrangement of gene segments in a site-specific process termed variable(diverse)joining (V[D]J) recombination (Tonegawa, 1983; Alt et al., 1987). This process is directed by highly conserved DNA recombination signal sequences (RSS) and executed by both ubiquitous and lymphoid-specific proteins (for review see Lewis and Gellert, 1989; Lieber, 1991; Schatz et al., 1992). Current models of V(D)J recombination are based mainly on the identification of V(D)J recombination intermediates (Roth et al., 1992; van Gent et al., 1995). Evidence for the involvement of ubiquitous activities stems from the analysis of the mutant nonlymphoid cell lines deficient in both V(D)J recombination and DNA repair (Kemp et al., 1984; Whitmore et al., 1989; Fulop and Philips, 1990; Hendrickson et al., 1991; Pergola et al., 1993; Taccioli et al., 1993). The ubiquitous DNA repair proteins, Ku70 and 80, along with the DNA-dependent protein kinase defective in mouse *scid* cells are three proteins essential for resolution of the hairpin loop formed during the V(D)J recombination process (Bosma and Carroll, 1991; Gottlieb and Jackson, 1994; Taccioli et al., 1994; Rathmell and Chu, 1994; Kirchgessner et al., 1995; Lees-Miller et al., 1995; Blunt et al., 1995).

The three lymphoid-specific components identified so far are the terminal deoxynucleotidyl transferase (TdT) enzyme, which contributes to junctional diversity (Landau et al., 1987; Gilfillan et al., 1993; Komori et al., 1993) and the V(D)J recombination-activating gene products, RAG1 and RAG2. These two genes reside adjacent to each other and were cloned by virtue of their ability to activate V(D)J recombination of an artificial recombination substrate in fibroblasts (Schatz et al., 1989; Oettinger et al., 1990). In mice carrying homozygous deletions of either RAG1 ($RAG1^{-/-}$) or RAG2 ($RAG2^{-/-}$), lymphocyte development is arrested at the point at which V(D)J recombination of the TCR or immunoglobulin loci would have normally occurred. As a result, the mutant mice are immunodeficient due to the lack of any functional B or T cells (Mombaerts et al., 1992; Shinkai et al., 1992; Spanopoulou et al., 1994). The total absence of antigen receptor rearrangements in the $RAG1^{-/-}$ or $RAG2^{-/-}$ mice emphasizes the indispensable role of these two proteins in the V(D)J recombination process. This notion is reinforced by the recent findings that in an in vitro system, the RAG1 protein is essential for specific cleavage and the formation of hairpin ends (van Gent et al., 1995).

Deletional analysis has defined the minimal regions of RAG1 and RAG2 required for their catalytic function. This analysis indicated that amino acids 330–1008 of RAG1 and 1–388 of RAG2 constitute the active core of the two proteins that mediate V(D)J recombination in fibroblastic cell lines (Silver et al., 1993; Sadofsky et al., 1993, 1994; Cuomo and Oettinger, 1994). Two proteins, Rch1 and SRP1, were found to associate with RAG1 (Cortes et al., 1994; Cuomo et al., 1994). SRP1 interacts with the N terminus of the protein (amino acids 1–288), outside of the RAG1 active core. To gain further insight into the precise function of RAG1 and RAG2 in V(D)J recombination, we analyzed their interaction and intracellular localization.

## Results

### RAG1 and RAG2 Protein Distribution

To examine the intracellular localization of the RAG proteins, both monoclonal and polyclonal antisera were raised against RAG1 and RAG2. The specificity of the sera was validated by comparing proteins in RAG-expressing normal cells versus cells derived from either $RAG1^{-/-}$ or $RAG2^{-/-}$ mice (see data in individual experiments below). Western blots were prepared from extracts of either primary thymocytes or cell lines expressing high levels of recombinant RAG protein and were probed with the spe-

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cific antisera. Both in thymocytes and overproducers, RAG2 was readily detectable (Figure 1a, lanes 5 to 7). By contrast, low levels of RAG1 protein were present in primary thymocytes (Figure 1a, lanes 2, 3), in spite of the relatively high levels of *RAG1* mRNA found in the thymus (Schatz et al., 1989; Oettinger et al., 1990). The majority of both proteins was not soluble in 0.2M NaCl but was solubilized in 0.7M NaCl (Figure 1a, compare lanes 2 with 3, and 6 with 7). No RAG proteins could be detected in the thymus of $RAG^{-/-}$ mice (Figure 1a, lanes 4, 8). Subsequently, the distribution and stability of the RAG proteins was followed by immunoprecipitation assays in the presence of high concentration of NaCl to include the insoluble nuclear fraction. Using extracts from cells metabolically labeled with [$^{32}$P]orthophosphate, both RAG1 and RAG2 were found to be phosphorylated (Figure 1b, lanes 1–4).

The stability of the two proteins was followed by pulse–chase radiolabeling of fibroblasts expressing the RAG proteins, or of primary thymocytes. In these assays, RAG1 exhibited a half-life of about 15 min in both cell types (Figure 2a, lanes 5–7 and 8–12). In contrast, RAG2 had a half-life of about 1 hr (Figure 2a, lanes 1–4). The relative stability of RAG2 was also indicated by the weak intensity of the labeled immunoprecipitated protein from thymocytes even after 3 hr of labeling (Figure 2a, lane 14).

### Physical Interaction between RAG1 and RAG2

In extracts from primary thymocytes immunoprecipitated with an anti-RAG2 serum (pAb2), RAG2 coprecipitated a protein of the same molecular weight as RAG1 (Figure 2a, lanes 12 and 14), which was absent from $RAG1^{-/-}$ thymic extracts (data not shown). In the same experiment, the anti-RAG1 serum (pAb1) precipitated RAG1 but no obvious RAG2 protein could be observed (Figure 2a, lanes 13 and 15). This is probably due to the slow turnover rate of RAG2 protein.

To examine the specificity of the polyclonal anti-RAG1 (pAb1) and anti-RAG2 (pAb2) antisera used in these experiments, they were tested with lymphoid cell extracts derived from $RAG1^{-/-}$- or $RAG2^{-/-}$-deficient mice, respectively (Shinkai et al., 1992; Spanopoulou et al., 1994; Corbella et al., 1994). pAb1 and pAb2 detected RAG1 and RAG2, respectively, in extracts from wild-type lymphoid cells but not in lines derived from RAG-deficient mice, demonstrating their specificity (Figures 2b, 2c).

To confirm the RAG1–RAG2 interaction, the proteins were transiently expressed either separately or simultaneously in 293T cells, and extracts were immunoprecipitated using anti-RAG sera, subjected to Western blot analysis and analyzed with the same sera. In this experiment, the anti-RAG1 serum clearly immunoprecipitated RAG2 and vice versa (Figure 2d).

Interaction of the two RAG proteins could also be demonstrated with $^{35}$S-metabolically labeled extracts, independently of the anti-RAG sera. To accomplish this, RAG2 was tagged with glutathione S–transferase (GST–RAG2) and RAG1 with an influenza peptide (HA–RAG1). The expression constructs were transiently transfected into 293T cells and the cell lysates were subjected to immunoprecipitation using antisera against GST or HA. The anti-GST serum precipitated RAG2 along with a protein of the same molecular weight as RAG1 (Figure 3, lane 5). Conversely, the anti-HA serum precipitated RAG1 plus a protein of the same molecular weight as RAG2 (Figure 3, lane 6). The identity of the two coprecipitated bands was confirmed by the observation that they were absent from cells that

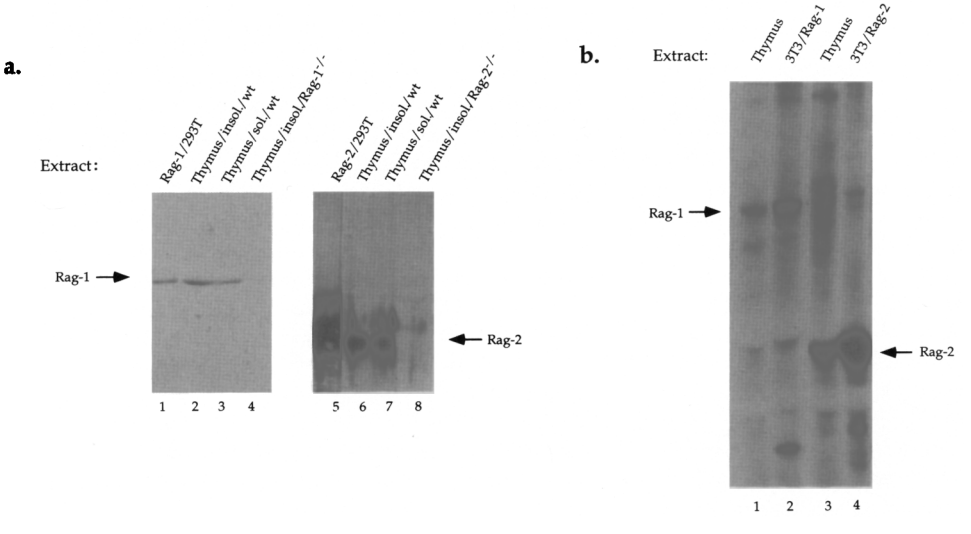


Figure 1. Analysis of RAG Proteins by Immunoprecipitation and Western Blotting

(a) RAG1 and RAG2 distribution analyzed by Western blotting. The material soluble in 0.2 M NaCl (lanes 3 and 7) and 0.7 M NaCl (lanes 1, 2, 4, 5, 6, and 8) of cellular extracts from murine thymocytes (lanes 2–4 and 6–8) or 293T cells expressing either recombinant RAG1 or RAG2 (lanes 1 and 5, respectively), were submitted to Western blot analysis using a mouse MAb against RAG1 (lanes 1–4) (MAb-1, Pharmingen) or rabbit polyclonal antisera against RAG2 (lanes 5–8) (pAb2).

(b) Both RAG1 and RAG2 proteins are phosphorylated. $^{32}$P-labeled lysates from primary thymocytes or 3T3 cells expressing RAGs (Silver et al., 1993) were analyzed by immunoprecipitation using the rabbit polyclonal antisera pAb-1 (lanes 1 and 2) and pAb-2 (lanes 3 and 4).

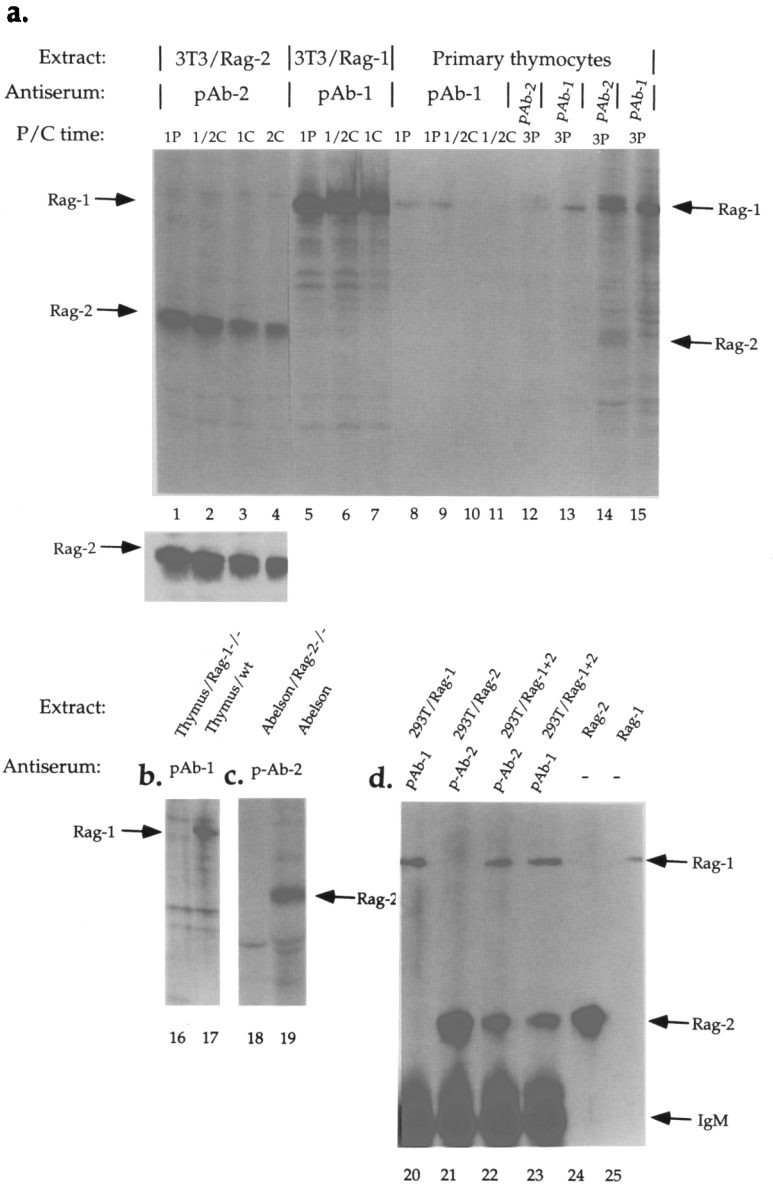


Figure 2. Stability and Interaction of the RAG Proteins

(a) Immunoprecipitation analysis of $^{35}$S-labeled extracts from 3T3 cells expressing either recombinant RAG2 or RAG1 (lanes 1–4 and 5–7, respectively) (Silver et al., 1993). Cells were pulsed for 3 hr and aliquots of equal numbers of cells were chased for the indicated times. The pulse–chase assay was also performed with $^{35}$S-labeled extracts from primary thymocytes (lanes 8–15) using the indicated antibodies. Abbreviations signify the following: pAb-2, rabbit polyclonal antisera against RAG2; pAb-1, rabbit polyclonal against RAG1; 1P, 1 hr pulse; 1/2,1,2C, chase time. Bottom represents a scanned image of lanes 1–4.

(b) Specificity of polyclonal rabbit anti-RAG1 serum, pAb1. Immunoprecipitate of RAG1$^{-/-}$/TCR$\alpha\beta^{+}$ (Corbella et al., 1994) (lane 16) and wild-type thymocyte extracts (lane 17).

(c) Specificity of polyclonal rabbit anti-RAG2 serum. Immunoprecipitate of extracts from Abelson virus transformed preB cell lines from RAG2$^{-/-}$ (Shinkai et al., 1992) (lane 18) and wild-type mice (lane 19).

(d) The RAG1 and RAG2 proteins interact when coexpressed in 293T cells. Extracts from 293T cells expressing either RAG1 (lane 20) or RAG2 (lane 21) or both proteins (lanes 22 and 23), were subjected to immunoprecipitation using the indicated polyclonal antisera. The immunoprecipitated products were subsequently analyzed by Western blotting using the same polyclonal antisera (pAb-1 and pAb-2).

expressed either RAG1 or RAG2 alone (Figure 3, lanes 1 and 4). To test whether the observed RAG1–RAG2 protein interaction is an artificial effect of the overexpression of the two proteins, RAG1 or RAG2 were coexpressed with the NF-κB components p65 or p50, respectively (Ghosh et al., 1990). Despite the high levels of expression of p65 or p50 in these cells, no obvious RAG1–p65 or RAG2–p50 interaction was observed (Figure 3, lanes 7–10), thus confirming the genuine nature of the RAG1–RAG2 interaction. Interestingly, the ratio of RAG1–RAG2 in the coprecipitated complex was about 1/4 as judged by $^{35}$S-labeled extracts once the $^{35}$S content of the two proteins was taken into account (Figure 3, lane 6). It should be noted that pulse–chase experiments with cells stably expressing either wild-type RAG1 or RAG2, individually or both together, showed that the RAG1–RAG2 interaction does not appear to influence the stability of either protein (data not shown).

The RAG1–RAG2 interaction was also studied in the yeast two-hybrid system. RAG1 and RAG2 were cloned and expressed in the appropriate yeast expression vectors that would allow transactivation of a reporter construct upon specific interaction of the two proteins (Zervos et al., 1993; Cortes et al., 1994). However, coexpression of the two proteins in yeast cells failed to reveal any direct interaction between the RAG1 and RAG2 proteins. Similarly, the two proteins failed to interact when coexpressed in reticulocyte lysates (data not shown). Thus, there may be a third protein, as yet unidentified, mediating the RAG1–RAG2 interaction or the interacting species may be molecules that have been posttranslationally modified in mammalian cells.

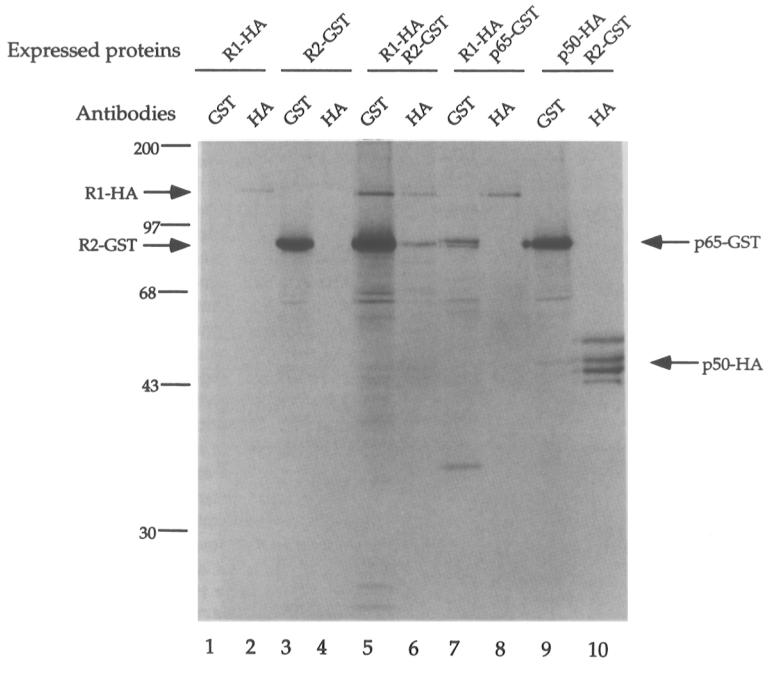


Figure 3. Coimmunoprecipitation of Hybrid RAG Proteins

RAG1 was tagged at the N terminus with an influenza epitope (R1–HA) and RAG2 was fused to the glutathione S–transferase protein (R2–GST). The two proteins were expressed either together (lanes 5 and 6) or separately (lanes 1–4) in 293T cells. After transient transfection (45 hr), the cells were subjected to $^{35}$S-labeling for 3 hr and the nuclear extracts were analyzed by immunoprecipitation using antibodies against either the influenza peptide (lanes 2, 4, and 6) or the GST protein (lanes 1, 3, and 5). As a control, RAG proteins were also coexpressed with modified versions of the NF-κB proteins, p50-HA and p65-GST (Liou et al., 1994). R1–HA was coexpressed with p65-GST (lanes 7 and 8), while R2–GST was coexpressed with p50-HA (lanes 9 and 10).

**Subcellular Localization**

To obtain further insight into the properties of the RAG proteins, the specific antisera were used in immunofluorescence studies. In primary mouse thymocytes, RAG1 was difficult to detect because of its low level, but there was a consistent specific signal at the periphery of most nuclei (Figure 4A). In addition, in some cells RAG1 was found in nuclear speckles, often coincident with nucleoli (Figure 4A). These signals were absent in thymocytes from RAG1$^{-/-}$ cells. RAG2 also localized to the periphery of the positive nuclei but at much higher levels than RAG1 (Figure 4B). This signal was absent in RAG2$^{-/-}$ thymocytes. Although not all cells give a uniform picture, it appears that RAG1 and RAG2 are in the nuclear periphery in the majority of thymocytes.

In fibroblast lines expressing relatively low levels of RAG1 and RAG2 from the retroviral vector pGD, both proteins were found near the periphery of the nucleus (Figure 4C, similar data for RAG1), in agreement with the distribution observed in primary thymocytes. However, a very different pattern of distribution was observed when the RAG proteins were expressed at high levels in the same cells (Silver et al., 1993). RAG2 was then evenly distributed throughout the nucleus but spared the nucleolus (Figure 4D). However, RAG1 showed a very different distribution, with the majority of the protein being localized as speckles within the nucleus (Figure 4E). Speckled distribution was also evident in fibroblasts coexpressing both RAG1 and RAG2, but in this condition much of the RAG1 was more evenly distributed in the nucleus, presumably by virtue of its interaction with RAG2 (Figure 4F).

The speckles of RAG1 coincided with the position of nucleoli evident by phase contrast microscopy. The nucleolar localization of RAG1 was confirmed by costaining with rhodamine-labeled anti-RAG1 and fluorescein-labeled anti-Nopp 140, a nucleolar chaperone protein (Meier and Blobel, 1992). RAG1 colocalized with Nopp 140 in the nucleolus (Figure 4G). However, as previously reported (Meier and Blobel, 1992), Nopp140 localized only in the fibrillar compartment of the nucleoli, while RAG1 was also present in the granular compartment.

To examine whether overexpressed RAG1 was in nucleoli because of an affinity for RNA, the nucleolar RNA was selectively depleted by treatment of the cells with low levels of actinomycin D (0.2 μg/ml), which specifically inhibits RNA polymerase I transcription (Perry, 1962; Goldberg and Friedman, 1971). The majority of the RAG1 protein 2 hr after treatment was no longer in the nucleoli but rather was distributed throughout in the nucleus in tiny speckles (Figure 4H), perhaps at places with a high concentration of RNA (Li and Bingham, 1991; Kalland et al., 1994) or in origins of replication (Adachi and Laemmli, 1994). By contrast, the localization of Nopp 140 remained unaffected by the actinomycin D treatment (data not shown). Thus, RAG1 appears to have a high affinity for RNA and when overexpressed without RAG2 it binds to sites of high RNA concentration.

**Role of RAG1 Basic Motifs: Binding Sites for the SRP1/Rch1 Family**

Several amino acid motifs have been described that mediate the binding of proteins to RNA (Bandziulis et al., 1989; Burd and Dreyfuss, 1994; LaCasse and Lefebvre, 1995). The RAG1 protein (Schatz et al., 1989) does not appear to contain within its primary sequence any of the motifs described for the RNA-associating nucleoproteins (Bandziulis et al., 1989), but it does contain multiple motifs of consecutive basic amino acids that could potentially confer RNA affinity on the protein, as demonstrated for the RNA binding proteins Tat, Rev, and Rex (Siomi et al.,

1988; Ruben et al., 1989; Cochrane et al., 1990; Calnan et al., 1991; Kjems et al., 1992). To define the domains of RAG1 that mediate nuclear and nucleolar localization, we generated several internal deletions (Δ) or amino acid alterations (M) or both within the basic domains of the RAG1 protein. The mutant RAG1 proteins were tested for their nucleolar localization when overexpressed in 293T cells and in functional assays for their ability to activate V(D)J recombination (Table 1). In addition, recent studies have indicated that the RAG1-interacting proteins SRP1 (Cortes et al., 1994) and Rch1 (Cuomo et al., 1994) mediate nuclear translocation of proteins containing a nuclear localization signal (NLS) (Gorlich et al., 1994; Weis et al., 1995). Our previous studies had shown that SRP1 interacts with an N-terminal segment of RAG1 (amino acids 1–288; Cortes et al., 1994) whereas a fragment of Rch1 (amino acids 432–529) interacts with the C-terminal region of the protein (330–1040; P. C. and D. B., unpublished data). Therefore, we used the yeast two-hybrid system and immunoprecipitation assays to test in parallel the ability of the different RAG1 mutants to interact with either SRP1 or Rch1.

Deletion of amino acids 13–330 in RAG1, which includes the ring finger motif, did not impair nuclear localization but decreased the amount of nucleolar RAG1, suggesting that sequences towards the N terminus of the protein may partly contribute to nucleolar binding (Silver et al., 1993). The Δ13-330 mutant protein retained 50% of wild-type functional activity (Table 1; Silver et al., 1993). This mutant failed to interact with SRP1 but it retained interaction with Rch1 (Table 1; Table 2). The deleted amino acid 13–330 region contains three basic regions: amino acids 141–146 (region BI), amino acids 222–225 (BII), and amino acids 243–249 (BIII). Mutation within the BI region (142,143 KK to II; Table 1) partly relocalized RAG1 to the periphery of the nucleus but had no effect on its activity (see Table 1; Silver et al., 1993). Mutation of BII (KRKR to IVKL; see Table 1) did not affect nucleolar localization or activity (see Table 1). Mutation of amino acids 233–236 (KKLK to LGLA) left the protein nucleolar and fully active (see Table 1). A deletion that encompasses the third basic domain BIII (amino acids 243–254, see Table 1) produced a fully functional protein that was partly in the nucleolus and partly in the nucleus (see Table 1). The latter suggests that RAG1 is partly driven in the nucleolus by regions BI and BIII and that additional sequences must contribute to this localization because of the behavior of the 13–330 deletion. Since additional basic motifs are present at the C terminus of RAG1 (amino acids 826–840 [motif BIV] and 969–973 [motif BV]), individual point mutations or internal deletion of these motifs were generated.

Deletion of amino acids 814–836 removing half of the BIV region (see Table 1) had an interesting effect. In some cells, the mutant protein was exclusively cytoplasmic, while in others it was present in both nucleus and cytoplasm but was not nucleolar. Mutation of the basic amino acids in this region (amino acids 823–828, KEERKR to IEELMI) resulted in only part of the protein residing in the nucleolus, while the rest was evenly spread through the nucleus. Both mutants were compromised in the V(D)J recombination assay (see Table 1). Mutation within the adjacent basic motif (amino acids 835–844) yielded a mutant RAG1 protein distributed between the nucleus, the nucleolus, and the cytoplasm but still 70% active (see Table 1). Finally, mutations within the BV region (amino acids 969–973, RRFRK to IKFLI) produced a totally cytoplasmic and inactive protein. Mutation of adjacent amino acids (amino acids 964–967, GNKL to AQGI) has no effect on localization or activity of RAG1.

The ability of the identified RAG1 basic motifs to drive localization of a heterologous protein to the nucleolus was tested by producing fusion proteins between the individual motifs and GST (Table 2). When a GST–RAG1 fusion protein containing the middle part of RAG1 (amino acids 278–477) was expressed in 293T cells as a negative control, the protein was cytoplasmic (data not shown). However, the GST fusion of amino acids 120–277 yielded a protein that localized to the nucleolus in most cells and was more diffusely nuclear in others (Figure 4I).

The C-terminal 330–1040 RAG1 sequence could also drive nuclear and nucleolar localization of GST. Fusion of amino acids 758–877 to GST gave a cytoplasmic protein (Table 2). Expression of amino acids 918–997, however, drove the GST fusion protein into the nucleus (Table 2).

To map more precisely the relevant sequences, we introduced mutations of the individual basic motifs into the RAG1-derived portions of the fusion proteins (Table 2) and studied their subcellular distribution by immunofluorescence. In parallel, we tested the ability of the wild-type and mutant fusion proteins to interact with SRP1 (Cortes et al., 1994) and Rch1 (Cuomo et al., 1994) (Table 2) using immunoprecipitation assays and the yeast two-hybrid system. This analysis revealed that all three basic motifs within the N terminus (BI, BII, and BIII) (Figure 5) of RAG1 were binding sites for SRP1, because mutations in any of these domains drastically reduced the RAG1–SRP1 interaction as judged by immunoprecipitation (Table 2). This reduction also affected the nucleolar localization of RAG1 (Table 2). SRP1 did not interact with the C terminus of RAG1 despite the presence of similar basic motifs. Similarly, Rch-1 only interacted with the C terminus of the protein with motifs BIV and BV (Table 2; Figure 5). Motif BIV was not sufficient to drive nuclear localization, but BV provided a strong signal for nuclear transportation (Table 2).

## Discussion

The stability, interaction, and localization of RAG1 and RAG2 were probed using specific antisera. In the nucleus of thymocytes, the two proteins are complexed together and concentrated in the periphery. When overexpressed individually, however, RAG2 is more evenly distributed and RAG1 shows a predominant nucleolar localization. Even though there is no correlation of activity with the nucleolar concentration of RAG1, we examined the basis of the nucleolar binding for the insight it might give to RAG1 function. The localization is due to not one but multiple signals that serve both to catalyze transport of the

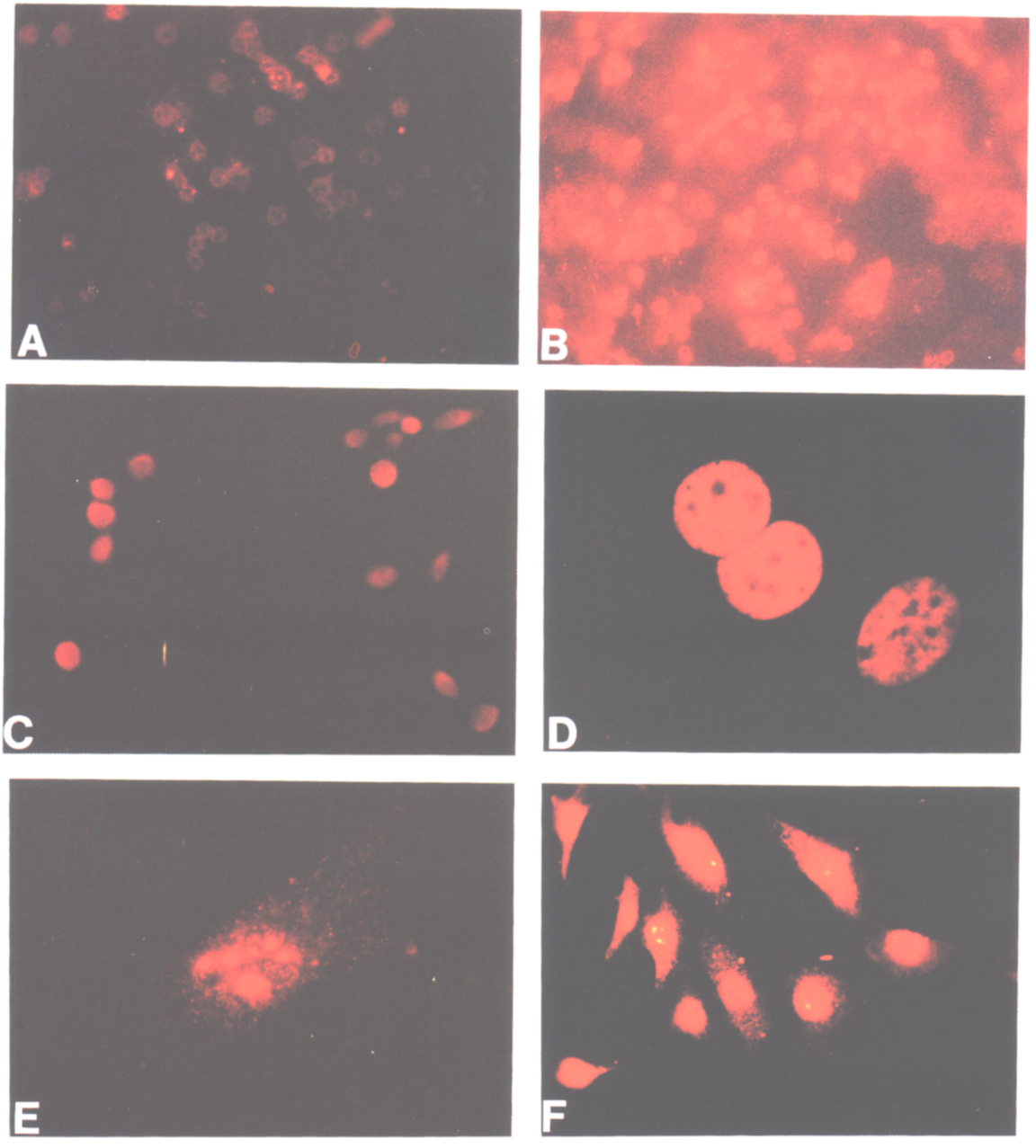


Figure 4. Immunofluorescence Analysis of Endogenous and Exogenously Expressed RAG1 and RAG2

In transfected 3T3 cells, only RAG1 or only RAG2 was expressed unless otherwise stated. (A) Endogenous RAG1 protein in primary thymocytes detected by the MAb-1. (B) Endogenous RAG2 protein in primary thymocytes detected by pAb-2. (C) RAG2 recombinant protein expressed at moderate levels in NIH 3T3 cells (pAb-2). (D) High level expression of recombinant RAG2 in NIH 3T3 cells (pAB-2). (E) High level recombinant RAG1 expressed in NIH 3T3 cells (MAb-1). (F) Detection of RAG1 in cells expressing RAG1 plus RAG2 (MAb-1). (G) Costaining of RAG1 (rhodaminated, anti-mouse IgM) (MAb-1) and Nopp140 (fluorescein-conjugated donkey anti-rabbit) (pAb-Nopp140) in NIH 3T3 cells overexpressing RAG1. (H) Detection of RAG1 protein after treatment of RAG1 overexpressing NIH 3T3 cells with 0.2 μg/ml of actinomycin D (MAb-1) for 2 hr. (I) Expression of the RAG1:120–277/GST fusion peptide in 293T cells (pAb-GST).

protein into the nucleus and to bind to RNA concentrated in the nucleoli. These signals serve as binding sites for two proteins known to interact with RAG1 and also known to be involved in transport of proteins to the nucleus.

### Stability of the RAG Proteins

RAG1 and RAG2 partition in the soluble as well as the insoluble part of the nucleus and their relative expression is inversely proportional to their steady-state mRNA levels (Oettinger et al., 1990). This suggests that the RAG proteins are under translational or posttranslational control. Indeed, both in primary thymocytes and fibroblast lines, RAG1 is a very labile protein (independently of the presence or absence of RAG2), in contrast with RAG2, which appears to be more stable. Because both proteins are needed for recombinational activity, RAG1 instability is

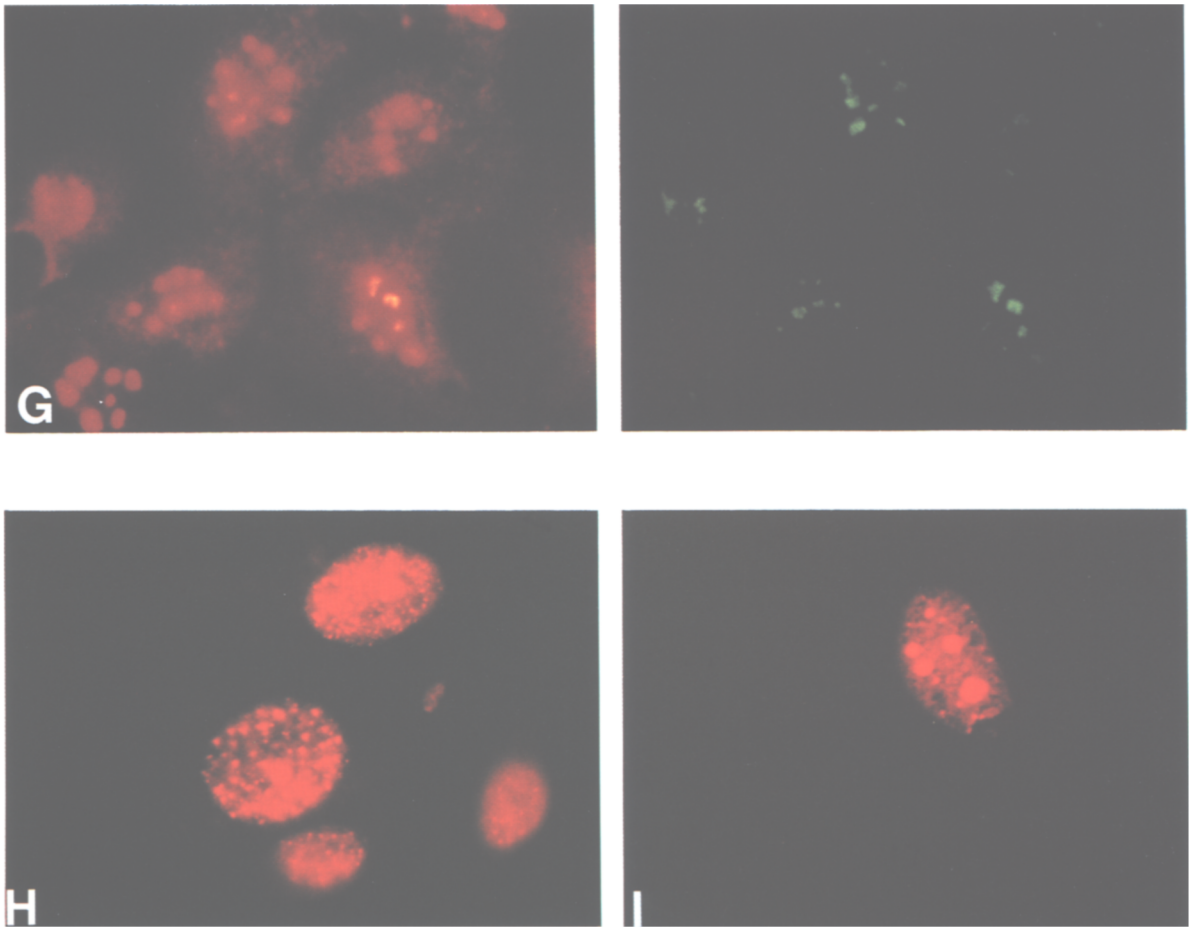


*Figure 4 continued.*

sufficient to ensure that once the synthesis of the proteins is terminated, their activity will not linger. Given the low efficiency of the V(D)J recombination process that ensures clonality of lymphoid cells, perhaps the labile nature of RAG1 reflects a rate-limiting step in the recombination reaction.

Our estimations of the stability of RAG2 differ from previous observations where RAG2 was found to be particularly unstable (Lin and Desiderio, 1992, 1994). We find that both in primary thymocytes and overexpressing fibroblasts RAG2 appears to be rather stable; it is RAG1 that exhibits instability. One explanation for this discrepancy is that we did not examine rapidly cycling cells because the previous work suggested a particular instability in S

Table 1. Effect of Mutations on the Localization and Activity of RAG1

| Mutant protein | Subcellular localization | Activity |
|---|---|---|
| Δ13–330[a] | Nuclear/Nucleolar | 40–50% |
| M 142, 143 KK to II[a] | Nucleolar | WT |
| Δ 141–146 (BI) | Nucleolar | WT |
| M 222–225 KRKR to IVKL (BII) | Nucleolar | WT |
| Δ 243–254 (BIII) | Nuclear/Less Nucleolar | WT |
| M 233–236 KKLK to LGLA | Nucleolar | WT |
| Δ 814–836 (BIV) | Cytoplasm, Nucleus/Cytoplasm | 10% |
| M 823–828 KEERKR to IEELMI (1/2BIV) | Nuclear/Low Nucleolar | 0 |
| M 835–844 KHLRKRMNLK to IHLLILMNLI (1/2BIV) | Cytoplasm, Nucleus/Cytoplasm/Low Nucleolar | 70% |
| M 969–973 RRFRK to ILFLI (BV) | Cytoplasmic | 0 |
| M 964–967 GNKL to AQGI | Nucleolar | WT |

Internal deletions (Δ) and multiple point mutations (M) were analyzed in the context of the full-length RAG1 protein. The basic motifs that were mutagenized are indicated in parenthesis (see Figure 5). Localization was determined in 3T3 (first two mutants) and 293T cells (rest of the mutants) expressing only the wild-type or mutant RAG1 forms by immunofluorescence using the MAb-1 antibody. Activity was determined both in 3T3 and 293T cells, by coexpression of wild-type RAG2 with the different RAG1 forms.

[a] See Silver et al., 1993.

Table 2. Properties of RAG1/GST Chimeric Proteins

| RAG1amino acids/GST | Mutation | Localization | Interaction with SRP1 | Interaction with Rch1 |
|---|---|---|---|---|
| N terminus | | | | |
| amino acids 13–330 | – | Nucleolar | ++++ | – |
| amino acids 120–277 | – | Nucleolar | ++++ | – |
| amino acids 120–277 | M 142/143 (BI) | Nuclear Periphery | + | |
| amino acids 120–277 | Δ 141–146 (BI) | Nuclear Periphery | + | |
| amino acids 120–277 | M 222–225 (BII) | Nuclear/Weak Nucleolar | + | |
| amino acids 120–277 | M 234–237 | Nucleolar | ++++ | |
| amino acids 120–277 | Δ243–254 (BIII) | Nuclear Speckles/Weak Nucleolar | + | |
| amino acids 120–277 | M 246–250 (BIII) | Nuclear Speckles/Weak Nucleolar | + | |
| Control | | | | |
| amino acids 278–477 | – | Cytoplasmic | – | |
| C terminus | | | | |
| amino acids 330–1040 | – | Nuclear/Nucleolar | – | +++ |
| amino acids 758–877 | – | Cytoplasmic | – | +++ |
| amino acids 758–877 | M 823–828 (BIV) | Cytoplasmic | | – |
| amino acids 918–997 | – | Nuclear/Low Cytoplasmic | – | +++ |
| amino acids 918–997 | M 969–973 (BV) | Cytoplasmic | | – |
| amino acids 918–997 | M 964–967 | Nuclear/Low Cytoplasmic | | +++ |

The analyzed proteins were fusions of the indicated amino acids of RAG1 with GST. The indicated mutations were put into these fusion proteins (see Table 1 for the precise changes in the M mutants, which all involved replacing basic regions with acidic or neutral ones). The SRP1 interactions were determined both in the yeast two-hybrid system and by coexpression of GST fusion polypeptides and HA–SRP1 into 293T cells and subsequent immunoprecipitation using antibodies against GST and the anti-HA (influenza) antibody to detect the coprecipitated SRP1 protein. The Rch-1 interactions were only determined in yeast since we were unable to coprecipitate Rch-1 and Rag-1 from extracts from 293T cells coexpressing the two proteins. Localization was determined by immunofluorescence in 293T cells using antibodies against the GST moiety. The basic motifs that were mutagenized are indicated in parenthesis (see Figure 5).

phase. Furthermore, we made an effort to include both the soluble and insoluble fractions. Perhaps the most soluble form of RAG2 protein shows a different behavior.

### RAG1-RAG2 Interaction

RAG1 and RAG2 could be coprecipitated from extracts derived from primary thymocytes as well as overexpressing fibroblasts, indicating that they are maintained together in a physical complex and might act in coordination during the recombination reaction. Interestingly, in fibroblasts coexpressing RAG1 and RAG2 we were unable to detect any RAG2 in the nucleolar compartment, suggesting that the form of RAG1 localizing in the nucleolus fails to interact with RAG2. In contrast, coexpression of RAG2 relocalizes part of RAG1 into the nucleus, underlining their propensity to interact. The RAG1–RAG2 interaction, although readily detectable in a number of ways in mammalian cells, could not be reproduced in the yeast two-hybrid system or in reticulocyte lysates (data not shown), suggesting that the interaction is generated in a complex with one or additional protein(s), or requires a specific posttranslational modification. The unequal ratio of the interacting proteins (RAG1–RAG2 is ~1/4) suggests that another protein might determine the stoichiometry. This interaction is of particular significance in understanding the precise functions of RAG1 and RAG2 in the V(D)J recombination reaction in view of the recent findings that RAG1 is directly involved in the recombination process. In an in vitro reaction, RAG1 participates at the very first step of V(D)J recombination, the introduction of a double-stranded break (van Gent et al., 1995). Given that RAG1–RAG2 are in the same complex, it is expected that RAG2 would also have a direct role in the catalysis of V(D)J recombination.

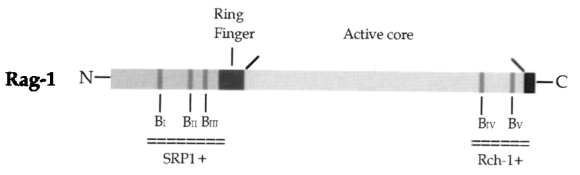


Figure 5. Motifs of Basic Amino Acids (BI to BV, Shaded in Red) within the RAG1 Sequence that Direct Nuclear and Nucleolar Localization

BIII constitutes the dominant nucleolar signal, while BV is the dominant nuclear localization signal, in the context of the full-length protein. Motifs BI, BII, and BIV are also involved in nucleolar organization auxiliary to the BIII function. Amino acids 141–146 (BI), 221–224 (BII), and 243–249 (BIII) participate in the SRP1 interaction, whereas amino acids 826–828/838–840 (BIV), and 969–973 (BV) interact with Rch1. The ring finger domain is indicated (Schatz et al., 1989; Freemont et al., 1991; Silver, 1992). The light grey area represents the minimal part of RAG1 that is required for V(D)J recombination of extrachromosomal substrates expressed in fibroblast cells (Silver et al., 1993; Sadofsky et al., 1993).

### Nuclear Localization of RAG1 and RAG2

In thymocytes, both proteins localize in the periphery of the nucleus, while RAG1 is also seen as speckles in a percentage of cells, presumably those where its concentration is particularly high. The peripheral localization of RAGs can be reproduced when the proteins are individually expressed in 3T3 cells at low levels, suggesting that the cell has a limited number of sites for nuclear RAG1

binding after which the excess protein goes to the nucleolus. It could be that a nonlymphoid protein serves as an anchor to these sites for both RAG1 and RAG2. Taking into account the propensity of the two proteins to precipitate, it is tempting to speculate that V(D)J recombination occurs in certain compartments of the nuclear periphery where RAGs are constrained and attached to the nuclear envelope, matrix, or both. Such a mechanism might help to overcome the topological constraints imposed by the long distances at which the recombining RSSs are located on the DNA.

The subcellular localization of RAG proteins in overexpressing fibroblasts showed a number of interesting features. RAG2 localizes throughout the nucleus but spares the nucleolus. Although clearly nuclear, RAG2 does not contain any sequence of basic amino acids homologous to the already described NLSs (Dingwall and Laskey, 1991; Friedberg, 1992; Burd and Dreyfuss, 1994; LaCasse and Lefebvre, 1995). Thus, RAG2 must be driven into the nucleus by an inapparent type of NLS. Alternative NLSs have been described for other proteins (LaCasse and Lefebvre, 1995). The nuclear distribution of RAG1 is partly due to the dominant NLS present at the C terminus of the protein (region BV) (see Table 1; in the context of the full-length protein only the BV mutations render the protein totally cytoplasmic). Nuclear localization of RAG1 is also mediated by the BI, BII, and BIII basic motifs at the N terminus of the protein (Figure 5; see Table 1; deletion of the N terminus leaves 50% of the protein nuclear) and to a lesser extent by the BIV basic motif (Tables 1 and 2; Figure 5). Our studies confirmed that the N-terminal sequences constitute binding sites for SRP1 (Cortes et al., 1994), while C-terminal sequences have affinity for Rch1 (Cuomo et al., 1994). However, mutation of individual basic segments revealed that SRP1 and Rch1 each interact with several basic motifs of the RAG1 sequence. Perhaps this multifinger anchoring of NLS receptors onto RAG1 is mediated by the multiple repeats present within the SRP1/Rch1 family (Peifer et al., 1994; Adam, 1995).

### Nucleolar Localization of RAG1 and Interaction with the SRP1/Rch1 Family

High expression of RAG1 drives most of the protein to both the granular and fibrillar compartments of the nucleolus. Nucleolar localization of overexpressed proteins can occur for several reasons: first, affinity for RNA as in the RNA binding proteins Tat, Rev, and Rex, all of which show nucleolar distribution because of their ability to bind RNA (Siomi et al., 1988; Ruben et al., 1989; Cochrane et al., 1990; Kalland et al., 1994); second, affinity for topoisomerase sites that are present in multiple copies in the ribosomal RNA (rRNA) genes (Bonven et al., 1985); third, interaction with other nucleolar proteins (Fankhauser et al., 1991); fourth, serving a role as a nucleolar chaperone protein (Meier and Blobel, 1992); fifth, affinity for single-stranded DNA (ssDNA) as described for the yeast protein SSB1 (Jong et al., 1987). Taking into account the observations that, first, mutations within the basic motifs of RAG1 partly abolish nucleolar localization; second, low actinomycin D treatment moves part of the protein out of the nucleolus; and third, peptides encompassing the N terminus motif can cause another protein, GST, to localize in the nucleolus (as was observed in the case of Tat, Rev, a Rex; Siomi et al., 1988; Ruben et al., 1989; Cochrane et al., 1990), the data suggest that RAG1, like Tat and Rev, localizes in the nucleolus by virtue of its affinity for structured RNA (Calnan et al., 1991; Kjems et al., 1992) or ssDNA (Jong et al., 1987). It should be noted that, in preliminary electrophoretic mobility shift assays for RNA binding, we were unable to observe any binding of RAG1 to in vitro transcribed RNAs such as sterile transcripts from the immunoglobulin-κ (Igκ) locus or a 400 nt transcript containing the RSSs (data not shown). Thus, it appears that the nucleolar binding of RAG1 is not due to a general nonspecific affinity of the protein for RNA.

Two of the RAG1 basic domains, BI and BIII, mediate both nuclear and nucleolar localization of RAG1. These motifs also interact with SRP1 and mutations within either the BI, BII, or BIII motifs reduce the SRP1–RAG1 interaction and abolish nucleolar localization, suggesting that the SRP1 family of proteins could be implicated both in nuclear and nucleolar translocation. In fact, it has been shown that NLSs may also display RNA binding properties (for review see LaCasse and Lefebvre, 1995). These data, along with the Tat/Rev paradigm, suggest a separation of the nucleolar targeting of RAG1 into two phases. First, active nucleolar transportation mediated by SRP-1/Rch1 and second, retention in the nucleolus by virtue of its affinity for RNA/ssDNA.

The functional significance of the RAG1 RNA affinity remains to be determined. The N-terminal third of the protein, which has one of the clusters of basic regions, is dispensable for the V(D)J recombination activity of RAG1 in fibroblast cells (Silver et al., 1993; Sadofsky et al., 1993). However, it is indispensable for the V(D)J activity of RAG1 in Abelson virus–transformed preB cell lines (C. Roman and D. B., personal communication), suggesting that in lymphoid cells both of the clusters participate in recombination. Although it is conceivable that RNA binding plays some role in recombination, no such role has been directly implicated and it seems more profitable to consider the possibility that there are multiple single-stranded DNA binding regions. Perhaps RAG1 uses its basic motifs for specific binding to nonconventional DNA structures (e.g., a bubble of "melted" DNA) similar to the fashion by which the C terminus of p53 (which contains motifs of basic amino acids) binds to mismatched DNA (Lee et al., 1995; Jayaraman and Prives, 1995). Interestingly, two other proteins, T160 (Shirakata et al., 1993) and the DNA repair protein Ku80 (Taccioli et al., 1994), both of which have been implicated in the V(D)J recombination process, also localize to the nucleolus when overproduced (Hsu et al., 1993; Higashiura et al., 1992). In fact, the subcellular distribution of the Ku protein remarkably resembles that of the RAG1 protein, suggesting that like Ku, RAG1 may bind to ends or short single-stranded regions in DNA. It is likely that in the process of recombining DNA RAG1 must have affinity for cleaved DNA ends or short single-stranded segments.

Finally, it should be noted that the presence of NLS,

nucleolar attachment motifs and SRP1/Rch1 binding sites in both the N and C terminus of the protein suggest that RAG1 has a binary structure (Figure 5). In this regard, it may be significant that a recombination protein has to bind to two DNA elements so as to appose them for recombination.

## Experimental Procedures

### Recombinant DNA Clones

For bacterial expression, the *RAG* fragments containing the coding nucleotides, 1890–3120 of *RAG1* and 360–1581 of *RAG2*, were generated by polymerase chain reaction (PCR) and cloned as 5′ BamHI–XhoI 3′ fragments into the pT7 vector (Andino et al., 1993).

For expression in mammalian cells, wild-type *RAG1* and *RAG2* plasmids were expressed in the CDM-8 vector as previously described (Oettinger et al., 1990). Also, the wild-type full-length RAG1 and RAG2 proteins and all of their truncated forms (Tables 1 and 2) were expressed as glutathione transferase fusion proteins in the peBG expression vector (5′ BamHI–NotI 3′ fragments). peBG (a gift of Dr. B. Mayer) is a modified version of peFOS (Mizushima and Nagata, 1990) that allows production of fusion proteins to the glutathione transferase protein.

For cloning into the yeast transactivation vector peg202 (Zervos et al., 1993), parts of *RAG1* were amplified by PCR, sequenced, and cloned as 5′ BamHI–NotI 3′ fragments. The full-length SRP1 and region 432–529 of Rch-1 were cloned into the yeast library vector pJG4–5 (Zervos et al., 1993) as 5′ EcoRI–XhoI 3′ fragments.

### Antibodies

Purified polyclonal antisera against the glutathione transferase protein were a gift from Dr. B. Mayer. Polyclonal antisera against the nucleolar chaperone Nopp140 protein were provided by Dr. T. Meier. Anti-influenza haemmaglutinin (HA) monoclonal antibody (MAb) (12CA-5) was purchased from Berkeley Antibody.

### Generation of Anti-RAG Antibodies

Fragments of the *RAG1* and *RAG2* genes encoding amino acids 630–1040 for *RAG1* and amino acids 120–527 for *RAG2* were cloned into the bacterial expression vector pT7 and expressed in the T7 polymerase–expressing bacterial cells BL21 (DE3) after IPTG induction (Andino et al., 1993). Bacterial lysates were separated on polyacrylamide gels, stained with Coomasie Blue, fixed with methanol/acetone, and the RAG1 and RAG2 polypeptides were excised, dried, and injected subcutaneously into rabbits as described (Antibodies, CSH). The same polypeptides were separated by electrophoresis, electroeluted from the gel, and injected into mice to generate MAbs (Antibodies, CSH). A MAb against RAG1 (PharMingen, catalog number G109–256) was successfully used in immunofluorescence, immunoprecipitation, and Western blot analyses.

### Protein Preparation

cDNAs encoding full-length *RAG1* or truncated GST fusion proteins were transfected into 293T cells by calcium phosphate precipitation, expressed transiently for 48 hr, and purified by a one-step purification procedure using glutathione–agarose beads (Molecular Probes) (see below).

### Western Blot Analysis

Cellular extracts were lysed in 10 mM Tris (pH 7.5), 10 mM NaCl, 5 mM $MgCl_2$, 0.5% NP-40, 0.2 mM PMSF to separate cytoplasmic from nuclear fractions by centrifugation. Nuclear fractions were subsequently solubilized in lysis buffer containing 20 mM HEPES (pH 7.9), 0.7 M NaCl, 10 mM EDTA, 1% NP-40, 0.2 mM PMSF, 10 μg/ml aprotinin (Sigma), leupeptin, and pepstatin A (Boehringer). Extracts were subsequently sonicated and resuspended in 2× Laemmli loading buffer (1× final) and loaded directly. For the phosphorylation studies, 1 mM orthovanadate and 50 mM NaF were also included. Western blot analysis of the extracts was performed as described elsewhere (antibodies, CSH). Specific antisera against RAG1 and RAG2 were incubated overnight, in 1× 10 mM Tris (pH 8.0), 150 mM NaCl, 0.05% Tween (TBST), at 4°C and after three washings, antimouse or antirabbit IgM horseradish peroxidase (HRP)–conjugated antibodies (Amersham) were applied for 30 min at room temperature. Blots were washed three times and developed by enhanced chemiluminescence (ECL) (Amersham).

### Immunoprecipitation Assays

Nuclear proteins were solubilized as described for Western blot, specific monoclonal or polyclonal antisera were added, and reactions were incubated for 4 hr at 4°C. Subsequently, reactions were incubated with protein A/G–agarose beads (Oncogene Science) for 30 min at 4°C, and after six washings in lysis buffer, beads were resuspended in Laemmli loading buffer (antibodies, CSH). Immunoprecipitated products were separated on 8% polyacrylamide gels. The GST fusion proteins were collected on glutathione–agarose beads (Molecular Probes); after 1 hr incubation at 4°C, beads were washed three times and resuspended in Laemmli loading buffer.

### In Vivo Protein Labeling

293T cells were transiently transfected with RAG1, RAG2, or both expressed in CDM8 and cells were subjected to labeling 46 hr after transfection. 3T3 cells were infected with the MFG retrovirus expressing full-length RAG1, RAG2, or both as described (Pear et al., 1993; Silver et al., 1993). After 30 min of starvation in methionine-free DMEM, 5% dialysed fetal calf serum (FCS) (GIBCO), cells were incubated with fresh DMEM medium containing 200 μCi/ml [$^{35}$S]methionine (ICN) for 3–4 hr. At the end of the labeling, medium was replaced with DMEM, 10% FCS and cell lysates from different timepoints and equal number of cells were prepared as above. Primary thymocytes were treated similarly except they were grown in RPMI. [$^{32}$P]orthophosphate labeling was also performed in the presence of dialysed FCS in DMEM- or RPMI-free of sodium pyruvate (GIBCO). Cells were also labeled for 3 hr but cell lysates were prepared in the presence of 50 μg/ml DNaseI and 100 μg/ml RNaseA. Immunoprecipitations were performed as described above. Radioactivity was quantitated by Phosphoimager (Molecular Dynamics) analysis as well as image scanning of autoradiograms (National Institutes of Health, Image Program 1.52).

### Immunofluorescence

Mouse primary thymocytes from postnatal day 7 were teased out of the thymus, immobilized on glass slides by cytospin, fixed in 50% methanol, 50% acetone, rehydrated in phosphate-buffered saline, preblocked with TBST, 2% donkey serum, 2% bovine serum albumin for 1 hr (1 × TBST = 10 mM Tris [pH 8.0], 150 mM NaCl, 0.05% Tween), incubated with the primary antibody (for RAG1, mouse MAb1, for RAG2 affinity-purified rabbit polyclonal, pAb2) in TBST, 1% donkey serum, 1% bovine serum albumin for 2 hr at room temperature. Slides were then washed three times in TBST, preblocked for 10 min, and incubated with rhodaminated donkey anti-mouse or anti-rabbit $F(ab)_2$ (Jackson Immunoresearch) for 30 min. After three washes with TBST, samples were air dried very briefly before mounting with fluorescent G and coverslip protection. Cells were visualized with Nikon fluorescence. 3T3 fibroblasts stably expressing full-length RAG1 or RAG2 were infected by pGD retroviral-RAG DNA as described (Pear et al., 1993). The GST fusion proteins were expressed in 293T cells. 293T cells growing in DMEM, 10% FCS were transfected by calcium phosphate precipitation in 35 mm tissue culture plastic dishes. The medium was removed 48 hr later and cells were washed gently, twice with phosphate-buffered saline. Subsequently, the cells were fixed on the plastic by 50% methanol, 50% acetone treatment for 3 min. Cells were then rehydrated in phosphate-buffered saline and treated and visualized similarly to the thymocyte preparations (see above).

### Yeast Two-Hybrid Screening

Screening was performed as described (Zervos et al., 1993; Cortes et al., 1994).

### Acknowledgments

Both E. S. and P. C. made key independent contributions. We thank Drs. T. Meier for the anti-Nopp140 serum; M. Scott for guiding E. S. in immunofluorescence techniques; B. Mayer for donating anti-GST

sera and pERG vector; Y. W. Choi for hospitality; and M. Konarska, R. Andino, A. Hodtsev, and D. Alexandropoulos for several valuable suggestions. E. S. was initially supported by a European Molecular Biology Organization long-term fellowship and later by a Cancer Research Institute fellowship. P. C. was supported by a fellowship from the Irvington Institute and is currently supported by the Lymphoma Research Foundation of America. This work was supported by National Institutes of Health grant CA54162 to D. B.

Received September 11, 1995; revised October 11, 1995.

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