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A Basic Motif in the N-Terminal Region of RAG1 Enhances V(D)J Recombination Activity

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The variable portions of antigen receptor genes are assembled from component gene segments by a site-specific recombination reaction known as V(D)J recombination. The RAG1 and RAG2 proteins are the critical lymphoid cell-specific components of the recombination enzymatic machinery and are responsible for site-specific DNA recognition and cleavage. Previous studies had defined a minimal, recombinationally active core region of murine RAG1 consisting of amino acids 384 to 1008 of the 1,040-residue RAG1 protein. No recombination function has heretofore been ascribed to any portion of the 383-amino-acid N-terminal region that is missing from the core, but it seems likely to be of functional significance, based on its evolutionary conservation. Using extrachromosomal recombination substrates, we demonstrate here that the N-terminal region enhances the recombination activity of RAG1 by up to an order of magnitude in a variety of cell lines. Deletion analysis localized a region of the N terminus critical for this effect to amino acids 216 to 238, and further mutagenesis demonstrated that a small basic amino acid motif (BIIa) in this region is essential for enhancing the activity of RAG1. Despite the fact that BIIa is important for the interaction of RAG1 with the nuclear localization factor Srp-1, it does not appear to enhance recombination by facilitating nuclear transport of RAG1. A variety of models for how this region stimulates the recombination activity of RAG1 are considered.

The genes that encode the variable portions of immunoglobulin and T-cell receptor polypeptides are assembled from component V (variable), J (joining), and in some cases D (diversity) gene segments by a site-specific recombination reaction known as V(D)J recombination. This reaction is essential for lymphocyte development and in many species is critical for generating a diverse repertoire of antigen receptors.

The *cis*-acting recombination signals that specify the sites of recombination have been well defined and consist of conserved heptamer and nonamer elements separated by a spacer of variable sequence whose length is 12 ± 1 or 23 ± 1 bp. The enzymatic machinery that performs V(D)J recombination is less well understood but is known to consist of both lymphoid cell-specific and ubiquitously expressed factors (for a review, see reference 15). The first phase of the reaction (DNA recognition, synapsis, and double-strand cleavage) is mediated largely by the lymphoid cell-specific proteins encoded by the recombination activating genes, *RAG1* and *RAG2* (5, 19, 33). Signal recognition is mediated in part by an interaction between RAG1 and the nonamer (4, 31), and formation of a stable, cleavage-competent complex requires the heptamer, the nonamer, and both RAG proteins (11). The second phase of the reaction (DNA end processing and joining) is dependent on a number of ubiquitous factors that play important roles in the repair of DNA double-strand breaks (for reviews, see references 2 and 33). A role for the RAG proteins in the second phase of the reaction has been hypothesized based on their stable association with the recombination signals after DNA cleavage (1).

It has proven difficult to perform the complete V(D)J recombination reaction in vitro, and it is only recently that the Gellert laboratory established an in vitro system for performing the cleavage phase of the reaction (32). Development of this in vitro cleavage system was aided by the observation that while the full-length RAG1 protein is insoluble under physiological conditions (14, 30), a truncated form of this protein is both biologically active (25, 29) and substantially more soluble (32). This truncated form, referred to as the RAG1 core, consists of amino acids 384 to 1008 of the 1,040-amino-acid murine RAG1 protein (25) and has become a vital reagent for in vitro studies of the mechanism of V(D)J recombination. Experiments relying on the RAG1 core, however, would not detect possible contributions made by the N-terminal 383 amino acids of the protein.

The RAG1 core corresponds to the most highly conserved region of the protein (Fig. 1A) and together with RAG2 is sufficient for recognition and cleavage at recombination signals (19). Little is known about functional domains of the core other than that the first approximately 60 amino acids are sufficient to mediate sequence-specific recognition of the nonamer (31). The N-terminal domain of RAG1 also exhibits substantial sequence conservation (Fig. 1A), suggesting that it may play an important role in V(D)J recombination. To date, however, no discrete region of the N-terminal domain has been shown to make any contribution to the recombination reaction.

Previous studies have identified several motifs of particular interest in the N-terminal region of RAG1. Among them are three basic regions (BI, BII, and BIII) that are important for RAG1 to interact with the nuclear transport protein Srp-1 (30). Srp-1 is a member of a family of proteins that recognize nuclear localization signals and are thought to facilitate binding to the nuclear pore and subsequent nuclear transport (9, 35). Mutation of any one of these three N-terminal basic regions of RAG1 substantially reduces its interaction with Srp-1 and subtly alters its localization within the nucleus but was

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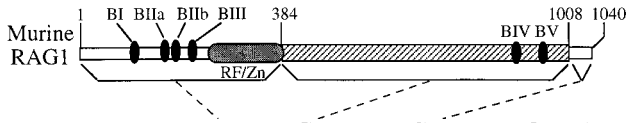

	N-term	Core	C-term	Overall
Human	81 (91)	96 (98)	84 (84)	90 (95)
Rabbit	79 (89)	96 (98)	78 (84)	90 (95)
Chicken	56 (73)	86 (95)	50 (69)	76 (86)
Xenopus	46 (63)	87 (92)	50 (69)	71 (81)
Trout	36 (59)	76 (88)	29 (45)	61 (77)
Shark	40 (59)	80 (90)	29 (45)	64 (77)

B Basic Regions in the N-terminus of the RAG1 Proteins

	BI (aa 141-146)	BIIa (aa 218-224)	BIIb (aa 233-236)	BIII (aa 243-254)
Mouse	RKKEKR	RG..LKRKR	KKLK	R..RDRKRRTQARV
Human	------	--..----S	----	-QA-Q----A---I
Rabbit	------	--..----N	----	-QA-Q----V---I
Chicken	------	--..V---S	--V-	-VN-GI-NQV...I
Xenopus	------	W-..K--SA	--R-	-KS-TSSGNSIQWK
Trout	--VGC-	--ERK---P	--T-	PQGPGL-PWVRSS-
Shark	--MGYG	.T..R---G	--A-	IRPGVI---D-KLS
Mutation	.....	--..-IVS-	ID-L	.....EL.....

FIG. 1. The RAG1 protein and its N-terminal domain basic regions. (A) Schematic diagram of the mouse RAG1 protein. Black ovals, conserved basic regions BI, BIIa, BIIb, BIII, BIV, and BV; stippled oval, the homodimerization domain including the ring finger and zinc finger (amino acids [aa] 265 to 389); hatched box, the RAG1 core (amino acids 384 to 1008). Brackets below the diagram demarcate the N-terminal (N-term), core, and C-terminal (C-term) regions of the protein. The RAG1 amino acid sequences from all species for which the complete sequence is known were aligned with the murine sequence by using the GCG program Bestfit. Using this alignment, the percent identical amino acids (percent similar amino acids) was calculated for each region for each species in comparison to the murine protein. The core regions for each species are as follows: mouse, 384 to 1008; human, 387 to 1008; rabbit, 386 to 1008; chicken, 385 to 1005; *Xenopus*, 391 to 1011; trout, 416 to 1040; and shark, 393 to 1015. (B) Amino acid sequences of the four N-terminal basic regions from the known RAG1 proteins. The analogous regions in each protein were determined by the GCG program Pileup. On the top line are indicated the relevant sequences of mouse RAG1. Other sequences are indicated below with a dash to indicate identity with the murine sequence, or the amino acid where different. Substitutions of lysine for arginine or vice versa are also marked with dashes to emphasize conservation of basic residues. A dot indicates a space introduced to maximize alignment. Indicated at the bottom are the murine RAG1 mutations of each motif used in this study. A dot and dash indicate deletion and retention, respectively, of the corresponding murine RAG1 amino acid.

reported not to affect recombination activity (30). None of these regions is required for nuclear transport of RAG1, due to the presence of additional nuclear localization signals in the RAG1 core (30). For the purposes of our study, the region referred to as BII previously (30) has been renamed BIIa to distinguish it from a nearby cluster of basic amino acids, which we refer to as BIIb. The locations and sequences of BI, BIIa, BIIb, and BIII are indicated in Fig. 1. Mutation of BIIb does not affect RAG1's interaction with Srp-1 or recombination activity (30).

Another well-conserved portion of the RAG1 N-terminal region is a cysteine-rich domain with sequence similarity to zinc binding motifs in a large family of proteins (7, 26). This C_3HC_4 ring finger domain of RAG1 is flanked by a classical C_2H_2 zinc finger, and together these two zinc binding fingers function as a homodimerization domain (24). The function of the ring finger domain in other proteins is not known, nor is it known how the dimerization domain contributes to RAG1 function.

Conflicting data exist concerning whether the RAG1 core performs recombination of plasmid substrates as efficiently as the full-length protein. Two studies found that truncation mutants similar to the core had lower activity than wild-type RAG1 (12, 29), while another found the core and wild-type RAG1 to be equally active (25). The reasons for this discrepancy remain unclear but may relate to subtle differences in assay conditions. The relative in vitro cleavage activities of the full-length and core RAG1 proteins have not yet been determined.

We reasoned that the RAG1 N-terminal region would not have been maintained through evolution if it did not play some role in V(D)J recombination. To test this idea, we performed extensive comparisons of the activities of RAG1 molecules containing or lacking the first 383 amino acids of the protein. We demonstrate that inclusion of the N-terminal region substantially and reproducibly enhances the ability of RAG1 to recombine extrachromosomal substrates. Using truncation and site-specific mutants, we localize the portion of the N terminus responsible for this effect to a small region between amino acids 216 and 238 and show that mutation of BIIa, but not the other N-terminal basic regions, eliminates enhanced recombination activity. Cell fractionation experiments indicate that BIIa does not increase recombination activity simply by enhancing accumulation of RAG1 in the nucleus. These results provide the first identification of a functionally important domain in the N-terminal region of RAG1 and indicate that this basic region performs a function at a step other than nuclear targeting.

MATERIALS AND METHODS

RAG expression plasmids. The positions of the human RAG1 core analogous to those of the mouse RAG1 core were determined by the Genetics Computer Group (GCG) program Bestfit. The human RAG1 expression plasmids have a *Sal*I site 5′ of the translation initiation codon. The human RAG1 A construct (phR1A) was created by PCR to position an *Mlu*I site at position 1008 of the human RAG1 coding sequence. This was then subcloned as an *Xho*I-*Mlu*I fragment along with the *Sal*I-*Xho*I fragment from pH36CD (28) into the *Sal*I-*Mlu*I sites of pMS127 (25), thereby placing three copies of the Myc epitope tag at the C terminus of the protein. The human RAG1 core (hR1c) construct was made by PCR to create an initiation codon immediately upstream of amino acid 387 and was cloned as a *Sal*I-*Sph*I fragment into phR1A to replace the wild-type N-terminal end of human RAG1. A Kozak translation initiation consensus sequence was created by PCR at the 5′ end of the coding region for both the R1A and R1c versions of human RAG1.

pCJM048 (mR1A) encodes amino acids 1 to 1008 of the murine RAG1 cDNA with three copies of the Myc epitope tag at the C terminus (20). pCJM116, pCJM117, pCJM118, pCJM119, and pCJM120 were made by PCR to place an initiation codon with the Kozak consensus sequence immediately upstream of amino acids 276, 264, 134, 216, and 238, respectively. *Xho*I-to-*Sph*I fragments were then used to replace the 5′ coding sequence of pCJM048.

The BI mutation is that described previously (30), while the BIIa/b, BIIb, and BIII mutations were made by the same method (30). The BI, BIIb, and BIII mutations were introduced into the context of R1A by subcloning the appropriate *Xho*I-to-*Sph*I fragment into pCJM048, generating plasmids pCJM219, pCJM220, and pCJM218, respectively. The BIIa/b, BIIb, and BIII mutations were introduced into the context of R1T by PCR to position an initiation codon immediately upstream of amino acid 216, followed by subcloning of *Xho*I-to-*Sph*I fragments into pCJM119, to generate pCJM226, pCJM224, and pCJM223, respectively.

The BIIa mutation was introduced into the context of R1T by PCR amplification using oligonucleotide MD-57 (5′-ACCTCGAGCATGGCCCATCGGGG ACTCATCGTCTCTAGACATCAGCCCAATG; the start codon is underlined) and a reverse primer located downstream of the *Sph*I site (CM-76; 5′-GGACT TTCTCGGCATTCCG). The resulting product was digested with *Xho*I and *Sph*I and inserted into pCJM048, thereby deleting amino acids 1 to 215 of R1A and creating pMJD242. Constructs pMJD243 and pMJD244 were cloned by a multistep PCR procedure. Primer MD-58 (5′-CATCGGGGACTCATCGTCTCTA GACATCAGCCC) was used with CM-76 for PCR amplification of pCJM048 (R1A) or pCJM206 (containing full-length RAG1 with the BIIb mutation [29]). The resulting products were purified from the reaction mix and used as PCR primers for the second round of amplification with CM-76 and a primer in the cytomegalovirus promoter (CM-37; 5′-GCTAACTAGAGAACCCACTG), using *Sac*I-digested CJM048 as the template. The products were then digested with *Xho*I and *Sph*I and cloned into pCJM048. Mutants were distinguished from wild-type R1A clones by use of an *Xba*I site introduced by the BIIa mutation. All constructs were then sequenced to confirm the mutations.

The construct pCJM228 (RFdel) was created by PCR in two steps. In the first

step, the upstream oligonucleotide (5′-CAGCAAGGAAGTCCTGAAGGTGCATATCAATAAAGGGGG, with codons for Lys 261 and Val 384 underlined) was used in conjunction with CM-76. The product of this reaction was used as the downstream primer in the second step with an oligonucleotide that creates an initiation codon immediately upstream of the amino acid at position 216. This product was subsequently subcloned as an *Xho*I-to-*Sph*I fragment into pCJM048, replacing the original N-terminal sequences. The deletion was confirmed by sequence analysis. The complete sequences of the expression plasmids used are available upon request.

Western blots. Gels were electrophoresed by using the Bio-Rad Mini Protean II gel system and electrotransferred to polyvinylidene difluoride membranes (Bio-Rad catalog no. 162-0182) by using a Mini Trans-Blot apparatus (Bio-Rad). Detection was performed by using either enhanced chemiluminescence or alkaline phosphatase. For the former, membranes were blocked in 5% nonfat dry milk in TBST (50 mM Tris [pH 7.5], 150 mM NaCl, 0.05% Tween 20) for more than 2 h. The primary antibody, anti-Myc (α-Myc) monoclonal antibody 9E10 (6), was affinity purified against the peptide EQKLISEEDL coupled to epoxy-activated agarose (Pierce catalog no. 20241X) and used at a concentration of 3 μg/ml. Membranes were sealed in plastic bags with 2 ml of antibody solution per filter and incubated on a rocker for more than 4 h. The membranes were then washed four times (15 min each) in TBST and incubated with rabbit anti-mouse horseradish peroxidase conjugate (1/20,000 dilution; Jackson ImmunoResearch, Inc. catalog no. 315-035-045) in 10 ml of TBST per filter for 1 h with rotation. The filters were washed as before, developed with an Amersham chemiluminescence kit (catalog no. RPN-2106) as instructed by the manufacturer, and exposed to Amersham Hyperfilm-MP (catalog no. RPN-1677).

For alkaline phosphatase detection, blocking for 1 h was followed by incubation with the primary antibody at the above-specified concentrations in 2 ml of TBST–3% nonfat dry milk with rocking overnight at room temperature. The remainder of the procedure was carried out as instructed by the manufacturer (Vector Laboratories), using AK-5001 (rabbit immunoglobulin G1) AK-5002 (mouse immunoglobulin G1), and SK-5400 (alkaline phosphatase substrate).

Recombination assay. COS cells, grown in Dulbecco modified Eagle medium (DMEM) plus 10% fetal calf serum, were seeded into 10-cm-diameter dishes at 1.5×10^6 cells per dish. Cells were transfected the following day by refeeding each plate with 7 ml of fresh medium containing 50.7 μM chloraquine and adding a DNA mixture containing 2 μg of the recombination substrate (pSJ [8]) and 6 μg of each RAG plasmid in a volume of 1 ml of medium containing chloraquine and 0.5 mg of DEAE-dextran per ml. After 5 h at 37°C, cells were shocked with 10% dimethyl sulfoxide in medium for 2 min and washed twice with 5 ml of medium, and 10 ml of medium was added. After 48 h, DNA was harvested from a portion of the cells by rapid alkaline lysis (10) and resuspended in 100 μl of 10 mM Tris–1 mM EDTA plus RNase A (10 μg/ml). The other portion of the cells was used for preparation of protein extracts for Western blotting (see Fig. 2 and 3) or for subcellular fractionation (see Fig. 6). One microliter of the DNA was mixed with 35 μl of electrocompetent *Escherichia coli* MC1061 and electroporated at 25 μF, 1.66 kV, and 200-Ω resistance with a Bio-Rad electroporator. The bacterial cells were resuspended in SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2 mM KCl, 20 mM glucose, 10 mM $MgCl_2$, 10 mM $MgSO_4$) medium and incubated at 37°C for 1 h with shaking. Portions of the cell suspension were plated on ampicillin (100 μg/ml) only or ampicillin (100 μg/ml) plus chloramphenicol (11 or 22 μg/ml). The percent recombination value (Rn value) was determined by dividing the number of chloramphenicol-resistant (Cam^r) plus ampicillin-resistant (Amp^r) colonies by the number of Amp^r colonies and multiplying by 100. RAG2 (amino acids 1 to 491) was expressed from pR2A (20). Control experiments demonstrated that it was not necessary to digest the DNA with *Dpn*I before plating (to eliminate unreplicated molecules), since this treatment had no measurable effect on the colony numbers or recombination frequency. The number of Amp^r colonies provides a measure of the transfection efficiency and was used to ensure that transfection efficiencies were uniform in experiments to analyze RAG1 protein levels and subcellular distribution. It is important to note that the RAG expression plasmids do not contain the *bla* gene and hence cannot confer resistance to Amp.

293 cells were grown in DMEM plus 10% fetal calf serum and seeded into dishes at 2×10^6 cells per 6-cm-diameter dish. 3T3 and HeLa cells were grown in DMEM plus 10% calf serum and seeded into dishes at 8×10^5 cells per 6-cm-diameter dish. All three were transfected by the calcium phosphate method (4, 27) with 6 μg of recombination substrate (pSJ [8] or pD243 [16]) and 6 μg of each RAG plasmid (pD243 replicates in rodent cells, while pSJ replicates in primate cells). After 5 h, 3T3 and HeLa transfections were shocked for 2 min in HEPES-buffered saline (6 mM dextrose, 137 mM NaCl, 5 mM KCl, 0.7 mM dibasic sodium phosphate, 21 mM HEPES [pH 7.05]) plus 15% glycerol and washed twice in 2 ml of medium, and 4 ml of medium was added. For 293 cell transfections, the medium was replaced with 4 ml of fresh medium after 10 h. Cells were harvested after 48 h; DNA was obtained by rapid alkaline lysis, resuspended in 30 μl of Tris-EDTA, and analyzed as described above. Samples transfected with pSJ and pD243 were added to plates containing 22 and 11 μg, respectively, of chloramphenicol per ml, the higher concentration being necessary to prevent background with pSJ (8).

Nuclear fractionation. Cells were transfected and harvested as described above and resuspended in 200 μl of buffer A (10 mM HEPES [pH 8.0], 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol) on ice for 10 min to allow swelling; 10 μl of a 10% Nonidet P-40 solution was then added to lyse the plasma membrane. The suspension was centrifuged for 1 min at 2,000 rpm at 4°C in a microcentrifuge. The supernatant was transferred to a Beckman TL100 rotor tube, 24 μl of 5 M NaCl was added, and the sample was centrifuged at 90,000 rpm for 30 min. The supernatant was transferred to a fresh Eppendorf tube, an equal volume of 2× protein sample buffer was added, and the cytoplasmic fraction was frozen at −20°C. The nuclear pellet was washed twice with 200 μl of buffer A and resuspended in 50 μl of 2× protein sample buffer. Samples were boiled for 5 min, and 10 and 20 μl of the nuclear and cytoplasmic fractions, respectively, were loaded on sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels. Expression of the control chloramphenicol acetyltransferase (CAT) protein from pCAT/E3 was performed as described previously (20), with fractionation and detection performed as described above.

Statistical analysis. The program DataDesk was used to analyze the statistical differences in recombination efficiencies. *P* values were obtained by using the two-tailed Student *t* test.

RESULTS

The RAG1 N-terminal region substantially increases its recombination activity. We first compared the recombination activity of the RAG1 core (mouse amino acids 384 to 1008; human amino acids 387 to 1008) to that of a nearly full length version of the protein (amino acids 1 to 1008 for both mouse and human [Fig. 2A]). The nearly full length versions of mouse and human origin are referred to as mR1A and hR1A, respectively. All of the proteins contained three copies of the Myc epitope tag at their C termini so that expression levels could be monitored by using the α-Myc epitope monoclonal antibody 9E10. The RAG2 partner for all functional assays was encoded by pR2A (20) and consisted of amino acids 1 to 491 of the 527-amino-acid murine RAG2 protein.

Transfection experiments were performed in monkey (COS), human (293 and HeLa), and mouse (NIH 3T3) cell lines to determine the recombination activity of each construct in each cell type. The recombination frequency was measured with replicating extrachromosomal recombination substrates which undergo deletion to retain a signal joint, either pD243 in 3T3 cells (16) or pSJ in COS, 293, and HeLa cells (8). In this assay, all substrate molecules confer ampicillin resistance in bacteria and confer chloramphenicol resistance only after recombination has occurred. The recombination frequency (expressed as percent recombination) is calculated by dividing the number of Cam^r plus Amp^r colonies by the total number of Amp^r colonies and multiplying by 100. As expected, cotransfection of a recombination substrate with pR2A only yielded low to undetectable levels of recombination (Fig. 2B, bars 1, 6, 11, and 16). The few Cam^r colonies that arise in the absence of RAG1 are the result of aberrant rearrangements of the substrate and do not represent correct V(D)J recombination events (data not shown). The no-RAG1 samples therefore provide a measure of the nonspecific background in the assay.

The mouse and human RAG1 core proteins (mR1c and hR1c, respectively) typically yielded levels of recombination that were clearly above background, the one exception being hR1c in 293 cells (Fig. 2B). In addition, mR1c and hR1c were not significantly different from one another in recombination activity, with the exception of the 293 cell transfections. We also tested the core version of *Xenopus* RAG1, which showed levels of recombination statistically similar to those of the mammalian RAG1 core constructs (data not shown). In all cell lines, mR1A and hR1A were significantly more active than their core RAG1 counterparts. The smallest difference, 1.5-fold between hR1A and hR1c in 3T3 cells (Fig. 2B, bars 19 and 20), represents a statistically significant difference. In all other cases, the nearly full length RAG1 proteins are 4- to 20-fold more active than their core counterparts (Fig. 2B and Table 1). These data demonstrate that while the core proteins are sim-

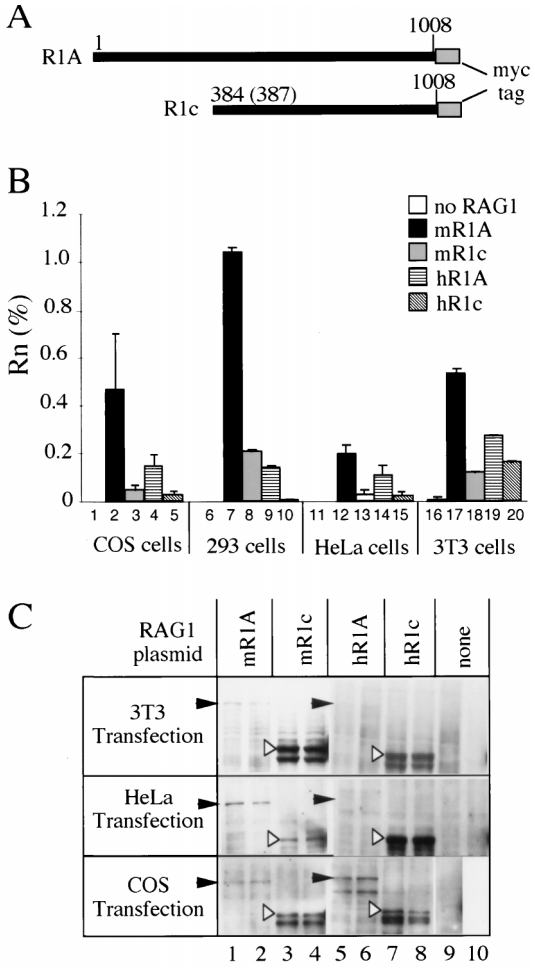

FIG. 2. Comparison of the recombination activities and expression of the R1A and R1c proteins. (A) Schematic diagram of the R1A and R1c proteins. The stippled boxes represent three copies of the Myc epitope tag fused to the C termini of the proteins. R1A consists of amino acids 1 to 1008 for both mouse and human RAG1. R1c consists of amino acids 384 to 1008 for mouse RAG1 and 387 to 1008 for human RAG1. (B) Recombination activities of R1A and R1c of mouse and human origin. The numbers of replicate samples were as follows: 2 for bars 6 to 10; 3 for bars 14 and 15; 4 for bars 4, 5, 11, 12, 13, 16, 19, and 20; 5 for bar 17; 6 for bar 18; 21 for bar 1; and 20 for bars 2 and 3. Only those experiments that yielded more than 100,000 Amp^r colonies were included, as the assay becomes less reliable if fewer than this number are obtained. Two-tailed Student *t* tests were performed to determine if differences between pairs of values were statistically significant. The recombination activity values obtained with no RAG1 (samples 1, 6, 11, and 16) were significantly different from those of the other samples in each cell line group ($P < 0.05$) except bars 10 and 15. In each group, mR1c was not statistically significantly different from hR1c ($P > 0.05$), except for bars 8 and 10, and the R1A protein was significantly different from its respective R1c protein in all cases ($P < 0.02$). All transfections included the RAG2 expression vector pR2A. Error bars represent 1 standard deviation. (C) Western blot of R1A and R1c proteins obtained by transient transfections of three different cell lines. The samples were transfected in duplicate, and a portion of the harvested cells was lysed and analyzed for total protein. Ten micrograms of total protein was electrophoresed per lane on SDS–7% polyacrylamide gels. Detection was with the α-Myc antibody. Filled arrowheads mark the position of the R1A protein; open arrowheads mark the position of the R1c protein.

ilar in recombination activity to each other, they have significantly lower activity than their R1A counterparts.

To determine if the lower recombination activity of the RAG1 core proteins was due to lower levels of expression, we measured steady-state protein levels by Western blotting with the α-Myc antibody. In contrast to what might have been expected based on measurements of activity, there was a consistently higher level of expression of the core proteins than in their R1A counterparts (Fig. 2C). This was true regardless of the cell line used (the one exception being murine RAG1 in 293 cells, where expression levels were comparable). In all other cases, the core proteins were expressed at levels 1.5- to 10-fold higher than those of their R1A counterparts (Table 1). Therefore, R1c is less active than R1A despite being expressed at higher levels.

TABLE 1. Relative activities and levels of expression of RAG1 core and R1A proteins

Cell line	Relative recombination activity[a]		Relative protein level[b]	
	Mouse RAG1	Human RAG1	Mouse RAG1	Human RAG1
COS	15	4	0.2	0.5
293	5	20	1	0.5
HeLa	7	5	0.5	0.1
NIH 3T3	5	1.5	0.2	0.1

[a] Determined by dividing the Rn value for R1A by the Rn value for R1c (Fig. 2B).

[b] Determined by dividing the protein level for R1A by the protein level for R1c. Protein levels were estimated from Western blots developed with the α-Myc antibody (Fig. 2C and data not shown). A portion of each harvested cell pellet was analyzed for recombination activity, and a separate portion was analyzed by Western blotting for protein levels. The number of Amp^r bacterial colonies recovered indicated that transfection efficiencies were approximately equal for mouse and human RAG1 plasmids in a given cell line.

Truncation mutants define a modified core RAG1 protein starting at amino acid 216. The recombination activities of truncation mutants of murine RAG1 were measured in COS cells to identify a region(s) of the N terminus important for activity. The first truncation proteins tested were encoded by pCJM116 and pCJM117 (beginning at amino acids 276 and 264, respectively), which begin N terminal to the dimerization domain. Neither of these proteins had the high level of recombination seen with mR1A (pCJM048) (Fig. 3A; compare bars 6 and 7 to bar 2). This result suggests that the dimerization domain alone is not responsible for the increased activity of the full-length RAG1 protein in the extrachromosomal assay.

We next tested the truncation mutants pCJM118, pCJM119, and pCJM120 (starting at amino acids 134, 216, and 238, respectively). These truncation mutants include all, three, and one, respectively, of the N-terminal basic regions depicted in Fig. 1. pCJM118 and pCJM119 had high levels of activity, while pCJM120 did not (Fig. 3A, bars 3 to 5). These data demonstrate that there are amino acids between positions 216 and 238 that are needed for high levels of recombination by the RAG1 protein. Together with data described below, this finding indicates that amino acids 216 to 1008 represent the minimal contiguous RAG1 region necessary for high activity and hence constitute a modified core for the RAG1 protein. This protein, which contains basic regions BIIa, BIIb, and BIII, is hereafter referred to as R1T.

Western blot analysis using the α-Myc antibody demonstrated that all of the truncated proteins accumulated to higher levels than R1A (Fig. 3B; compare lane 1 to other lanes). The proteins encoded by pCJM118 and pCJM116 accumulated to levels intermediate between those of mR1A and mR1c, while those encoded by pCJM119 (R1T), pCJM120, and pCJM117 were expressed at levels comparable to that of mR1c (Fig. 3B). Breakdown products are observed to various degrees with all forms of RAG1 tested, but because R1A is generally less well expressed, only longer exposures show its breakdown products.

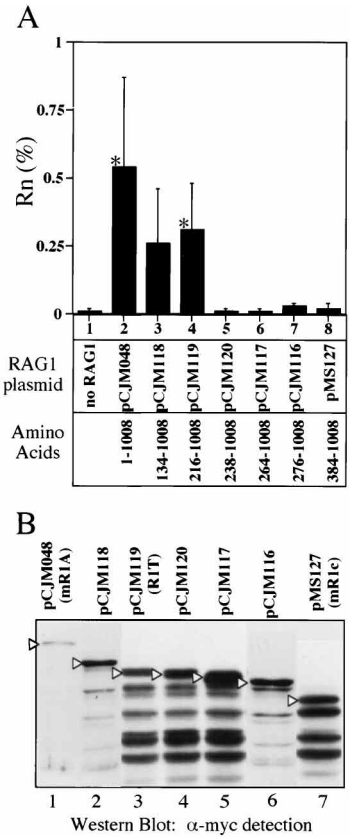

FIG. 3. Recombination activity and protein expression of RAG1 truncation constructs in COS cells. Construct names and encoded amino acids are indicated. (A) Recombination activity. The numbers of replicate transfections were as follows: 2 for bar 5; 4 for bars 3, 6, and 7; and 10 for bar 4. The data for bars 1, 2, and 8 are from Fig. 2B. The asterisks mark bars 2 and 4, which are statistically significantly different from bars 1 and 5 to 8 ($P < 0.001$). Bar 3 is not significantly different from bars 1 and 5 to 8 due to its relatively large standard deviation. Error bars represent 1 standard deviation. (B) Protein expression. Approximately 100,000 cell equivalents were run per lane on duplicate gels, one for detection with the α-Myc antibody and the second for Coomassie blue staining to ensure equivalent loading (not shown). Lanes 1, 2, and 6 were from one experiment; 3, 4, 5, and 7 were from a second experiment. The lanes were taken from films of equivalent exposure lengths (75 s). Arrowheads mark the expected positions of the RAG1 proteins.

These results demonstrate that the observed differences in activity are not explained by differential protein stability.

Mutation of the BIIa motif abrogates high recombination activity of the RAG1 protein. Both the BIIa and BIIb motifs are contained in the region between positions 216 and 238 (Fig. 1). BIIa is important for the interaction between RAG1 and Srp-1, while BIIb is not (30). To determine whether either of these motifs played a role in enhancing recombination by RAG1, we measured the recombination activities of proteins containing substantial mutations in one or both. For comparison, we also measured the recombination activities of proteins with deletions of, or mutations in, BI and BIII (both of which are important for the interaction with Srp-1 [29]).

In the context of R1A (amino acids 1 to 1008), the BI, BIIb, and BIII mutations had no effect on the recombination activity of the RAG1 protein. In sharp contrast, proteins containing mutations of BIIa, or both BIIa and BIIb, exhibited markedly lower levels of recombination (Fig. 4 and Table 2). Similar results were obtained when the mutations were assayed in the context of R1T (amino acids 216 to 1008). Mutation of BIIa, or BIIa and BIIb, again resulted in a significant decrease in recombination activity, to levels very close to that of R1c (Fig. 4 and Table 2). In the context of R1T, mutation of BIIb or BIII caused a decrease in activity, but this was not statistically significant (Table 2). We conclude that BIIa plays an essential role in enhancing RAG1 recombination activity, while BIIb does not. It is important to note that the BIIa/b mutation affects coding joint formation to the same extent as signal joint formation (data not shown) and that the signal joints formed by R1T and R1T-BIIa/b are precise, as are those formed by the RAG1 core (data not shown). Therefore, the BIIa region is not required for reaction fidelity or preferentially for one of the two joined products but rather has a more general effect on reaction efficiency. We also note that in the experiments represented by Fig. 4 and Table 2, R1A and R1T had essentially indistinguishable recombination activities (Rn values of 0.89 and 0.90%, respectively).

We next investigated whether the small region of amino acids containing BIIa, BIIb, and BIII was sufficient to enhance the recombination activity of the RAG1 core protein. We constructed a truncation/deletion mutant of RAG1 that encodes amino acids 216 to 261 fused to the RAG1 core, deleting all of the dimerization domain (Fig. 5A). In COS cells, the RFdel protein exhibited a level of recombination intermediate between those of R1T and R1c (Fig. 4 and Table 2). Due to the relatively large standard error of the measurement, the activity of RFdel was not statistically significantly different from that of R1A, R1T, or R1c. However, in an independent experiment, the activity of RFdel (Rn = 0.31% ± 0.13%) was significantly greater ($P < 0.02$) than that of R1c (0.03% ± 0.02%) but was not significantly different than that of R1A (0.45% ± 0.35%) or R1T (0.31% ± 0.2%). In 3T3 fibroblasts, RFdel was as active as R1A and R1T and was more active than R1c or than R1T containing mutations in BIIa and BIIb (Fig. 5B). Again, due to fluctuations between measurements, these differences are not statistically significant as measured by the two-tailed Student *t* test. Therefore, while the data suggest that amino acids 216 to 261 are sufficient to enhance the recombination activity of the RAG1 core, further experiments are necessary to confirm this and to determine whether the dimerization domain also contributes to the high activity seen with R1A and R1T.

Nuclear localization of RAG1 proteins. Because the BI, BIIa, and BIII motifs mediate the interaction of RAG1 with the nuclear transport protein Srp-1, it was important to determine if mutation of these regions (particularly of BIIa) altered the ability of RAG1 to localize to the nucleus. We determined nuclear localization by lysing cells in Nonidet P-40 followed by Western blotting of the nuclear and cytoplasmic extracts (see Materials and Methods). Control experiments with a Myc epitope-tagged CAT protein, which resides exclusively in the cytoplasm (25), demonstrated that this fractionation method avoided contamination of the nuclear fraction with cytoplasmic proteins (Fig. 6B).

We first examined nuclear localization of basic region mutants in the context of R1A. In all cases, the mutant proteins were detectable in the cytoplasmic fraction and accumulated in the nucleus to levels higher than that observed with R1A (Fig. 6, lanes 1 and 4 to 8). Mutation of BI, BIIb, or BIII increased nuclear levels substantially, while mutation of BIIa in the presence or absence of the BIIb mutation resulted in levels only slightly higher than those of R1A. The lower activity of the R1A-BIIa protein compared with R1A is therefore not attributable to reduced levels of the mutant protein in the nucleus.

In the context of R1T, the mutations do not affect overall

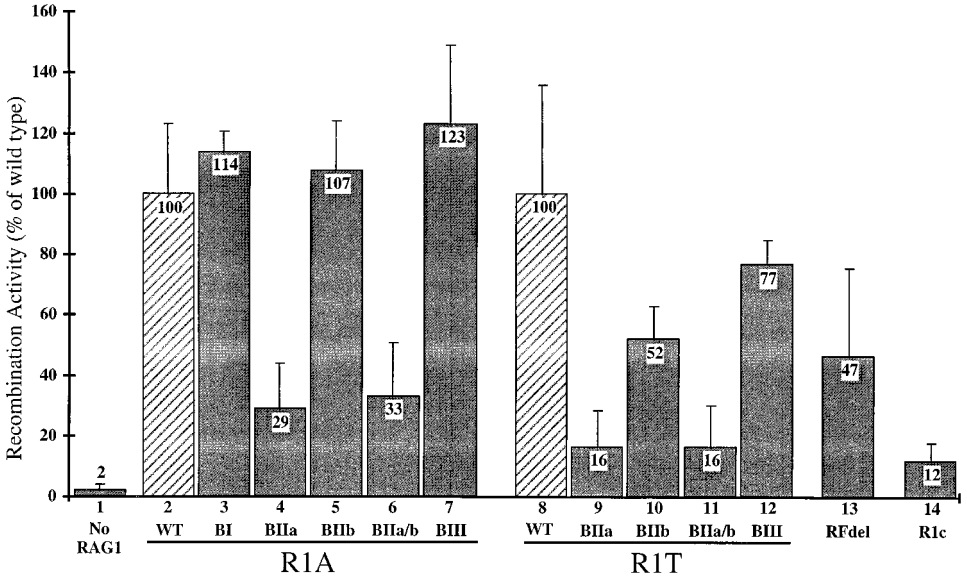

FIG. 4. Recombination activity of N-terminal mutants of RAG1 in COS cells. Mouse RAG2 (from pR2A) was expressed alone (bar 1) or together with wild-type (WT) or mutant forms of RAG1 in COS cells, and recombination activity was measured as described in Materials and Methods. Wild-type or mutant forms of R1A (bars 2 to 7), R1T (bars 8 to 12), or RFdel (bar 13) or wild-type R1c (bar 14) was expressed. The region mutated is indicated below each bar, and the bars for R1A and R1T are shaded differently for emphasis. The Rn values for samples 1 to 7 and samples 8 to 14 were then divided by the values for R1A and R1T, respectively, and multiplied by 100, to yield the relative recombination activity, expressed as a percentage of the relevant wild-type control. Note that the average Rn values for R1A (0.89%) and R1T (0.90%) are virtually identical in this experiment, and hence the results are not dependent on which value is used for normalization. The number of replicate transfections and a statistical analysis are provided in Table 2. In addition, note that the value for R1T-BIIb (bar 10) is statistically significantly greater than those for R1T-BIIa, R1T-BIIa/b, and R1c (bars 9, 11, and 14, respectively; $P < 0.002$). Error bars represent 1 standard deviation.

levels of RAG1 expression or disturb its nuclear accumulation (Fig. 6A, lanes 9 to 13). In particular, the BIIa, BIIa/b, and RFdel mutant proteins accumulate to levels comparable to those of R1T (lane 2) and to substantially higher levels than R1A (lane 1). Furthermore, R1c (lane 3) is found at higher levels in the nucleus than R1A, as would be expected from its higher overall levels of expression (Fig. 2 and Table 1). We conclude that neither levels of protein expression nor levels of

TABLE 2. Recombination activities and protein expression of N-terminal mutants of murine RAG1 in COS cells

Plasmid	Protein	Amino acids	Region mutated[a]	R (% of wt; mean ± SD)[b]	n[c]	P value (vs wt)[d]	Protein expression[e]
pCJM048	R1A	1–1008	None	100 ± 23	7		+
pCJM119	R1T	216–1008	None	100 ± 36	7	>0.05	+++
pMS127	R1 core	384–1008	None	12 ± 6	7	<0.0005	+++
RAG1 proteins in context of R1A							
pCJM048	R1A	1–1008	None	100 ± 23	7		+
pCJM219	R1A-BI	1–1008	BI	114 ± 7	3	>0.05	+++
pMJD243	R1A-BIIa	1–1008	BIIa	29 ± 15	5	<0.0005	++
pCJM220	R1A-BIIb	1–1008	BIIb	107 ± 17	5	>0.05	+++
pMJD244	R1A-BIIa/b	1–1008	BIIa and BIIb	33 ± 18	5	<0.0005	++
pCJM218	R1A-BIII	1–1008	BIII	123 ± 26	3	>0.05	+++
RAG1 proteins in context of R1T							
pCJM119	R1T	216–1008	None	100 ± 36	7		+++
pMJD242	R1T-BIIa	216–1008	BIIa	16 ± 12	6	<0.0005	+++
pCJM224	R1T-BIIb	216–1008	BIIb	52 ± 11	5	>0.05	+++
pCJM226	R1T-BIIa/b	216–1008	BIIa and BIIb	16 ± 14	5	<0.0005	+++
pCJM223	R1T-BIII	216–1008	BIII	77 ± 8	3	>0.05	+++
pCJM228	RFdel	216–1008	Deletion of 262 to 383	47 ± 30	3	>0.05	+++

[a] See Fig. 1B for amino acid mutations of BI, BIIa, BIIb, and BIII.

[b] The normalized recombination frequency (R) is the Rn value divided by the Rn value for the appropriate wild-type (wt) RAG protein (as described in the legend to Fig. 4). The average Rn values for R1A and R1T were 0.89 and 0.90%, respectively. These data (plotted in Fig. 4) were derived from separate experiments from those shown in Fig. 2B. Absolute recombination frequencies can vary between sets of experiments, but the relative values are reproducible.

[c] Number of independent transfections used to determine Rn.

[d] Statistical analysis of difference between R_{mutant} and R_{wt}. $P > 0.05$ indicates that the two values are not statistically significantly different, while $P < 0.0005$ indicates that the difference is highly significant.

[e] Estimated from Western blots. +, protein expression comparable to that of R1A; ++, expression ≥2-fold higher than that of R1A; +++, ≥, expression 10-fold higher than that of R1A.

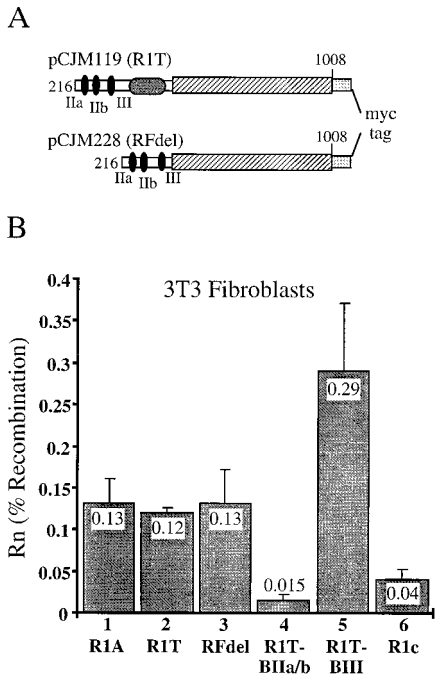

FIG. 5. Recombination activity of the RFdel protein. (A) Schematic diagram of R1T (pCJM119) and RFdel (pCJM228). The black ovals represent basic regions IIa, IIb, and III. The stippled oval and hatched box represent the dimerization domain and RAG1 core, respectively. The stippled box represents three copies of the Myc epitope tag. The number to the left represents the first amino acid of the RAG1 sequence after the initiation codon. In RFdel, amino acids 262 to 383 are deleted. (B) Recombination activities of various RAG1 proteins in 3T3 cells. The indicated forms of RAG1 were coexpressed with RAG2, and recombination frequency was measured as described in Materials and Methods. Error bars represent 1 standard deviation. Statistical analysis indicates that the value for RFdel is not significantly different ($P > 0.2$) from that for R1T-BIIa/b or R1c.

nuclear accumulation explain the decreased recombination activity observed when the BIIa motif is mutated.

DISCUSSION

Given the degree of evolutionary conservation of the RAG1 N-terminal region, it is not surprising that a portion of this region could enhance recombination activity. We have demonstrated that the R1T protein, spanning amino acids 216 to 1008 of murine RAG1, is substantially more active in vivo than the RAG1 core and is expressed at higher levels than R1A. If R1T, like R1c, is more soluble than full-length RAG1, it should be a useful reagent for in vitro studies. We have also demonstrated that the 22-amino-acid region from positions 216 to 237 is a critical portion of the N terminus for enhancement of recombination activity and that an intact BIIa motif is essential for this effect. It is significant that the R1A and R1T proteins yield approximately equal recombination efficiencies despite the fact that R1T is expressed at higher levels (Fig. 3B and Table 2). This finding suggests that the first 215 amino acids of RAG1 may make an additional contribution to its ability to perform recombination. It is clear, however, that R1T is significantly more active than R1c and that this cannot be explained by levels of protein expression.

Sadofsky et al. originally reported that the RAG1 core (amino acids 384 to 1008) construct used in these experiments performed recombination as efficiently as full-length RAG1 (25). Others, however, have reported that various N-terminal truncation mutants show reduced activity relative to full-length RAG1: (i) a 2-fold decrease with a protein lacking amino acids 15 to 330 (29), (ii) a 3- to 5-fold decrease with RAG1 proteins that start at amino acid 231 or 243 (12), (iii) an 8-fold reduction with a RAG1 protein containing amino acids 332 to 1008 (25), and (iv) a 4- to 20-fold reduction with a RAG1 protein containing amino acids 331 to 1040 (12). Therefore, our results are consistent with a number of previous studies. We speculate that the discrepancy with the results of Sadofsky et al. (25) may in some way relate to their use of sodium butyrate to boost RAG expression levels and thereby increase recombination efficiency.

In the first set of experiments described here, we tested the activity of full-length and core RAG1 in different cell lines. These experiments reveal a difference in recombination activity, regardless of the cell line used. They also show similar results for two different methods of transient transfection (calcium phosphate and DEAE-dextran), suggesting that our results are not a consequence of the use of a particular method of transfection.

The N-terminal truncation mutants studied here are more highly expressed than R1A (Fig. 3B), suggesting that there may be motifs present in the N-terminal portion of RAG1 that regulate protein stability. A previous study reported a substantial increase in protein levels when amino acids 15 to 79 were deleted (29). This finding is consistent with the increase in protein levels that we observe with the truncation expressed by pCJM118 (amino acids 134 to 1008). It is known that RAG1, like RAG2, is a phosphoprotein (30), and it is possible that like RAG2 levels (17), RAG1 levels are regulated posttranslationally. The N-terminal amino acids of RAG1 between positions

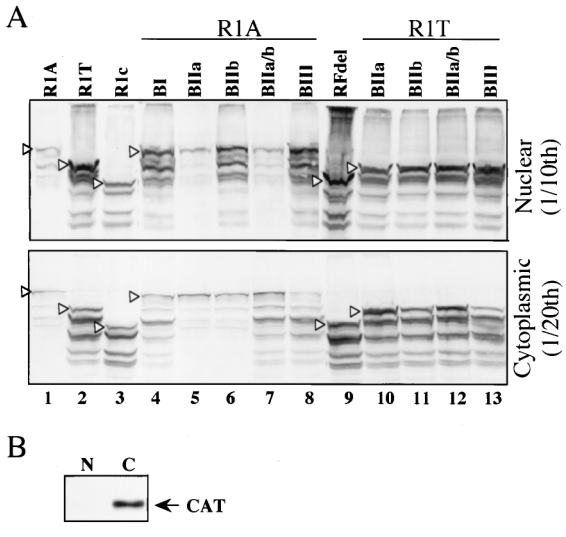

FIG. 6. Subcellular fractionation of RAG1 proteins. (A) COS cells were cotransfected with pR2A, pSJ, and a plasmid expressing the indicated form of RAG1. Duplicate transfections were pooled, and the cellular protein was separated into cytoplasmic and nuclear fractions as described in Materials and Methods. Duplicate samples were electrophoresed on SDS-polyacrylamide gels. One was Western blotted and detected with the α-Myc antibody and alkaline phosphatase; the second was stained for total protein with Coomassie blue to ensure equivalent loading between lanes (not shown). The upper panel contains 1/10 of the nuclear fraction per lane, while the lower panel contains 1/20 of cytoplasmic fraction per lane. Arrowheads mark the expected positions of the RAG1 proteins. (B) COS cells were transfected with pCAT/E3 (25), which expresses a Myc epitope-tagged CAT protein. Cells were then fractionated as described above. The first lane contains 1/100 of the nuclear fraction (N), and the second lane contains 1/200 of the cytoplasmic fraction (C). As expected, this protein is found predominantly in the cytoplasmic fraction.

15 and 79 would be a likely place to look for targets of such regulation.

N-terminal basic motifs and enhanced RAG1 activity. Mutation of the BIIb or BIII motif had no significant effect on the recombination activity of RAG1, and having these regions intact could not compensate for mutation of BIIa. It therefore seems unlikely that BIIb or BIII makes a significant contribution to the effect observed here. Mutation of BIIa, however, significantly and reproducibly reduced the recombination activity of RAG1. This observation conflicts with a previous study that found that mutation of BIIa had no discernible effect on the recombination activity of RAG1 (30). The reasons for this discrepancy are unclear but could relate to the use of a different mutation of the BIIa region (KRKR to IVKL [positions 221 to 224] instead of to IVSR as used here), the use of full-length RAG1 instead of R1A, or the use of a different assay (PCR based) for V(D)J recombination.

There are at least two different types of models for the mechanism of action of the 22-amino-acid region containing BIIa. First, while this region does not appear to act by enhancing nuclear localization (our data and reference 29), it might help target RAG1 to a compartment of the nucleus where recombination occurs more efficiently. For example, it has been suggested that V(D)J recombination might occur at the periphery of the nucleus, perhaps in association with the nuclear envelope or matrix (30). Since nuclear localization factors analogous to Srp-1 are found in both the nucleus and the cytoplasm (reviewed in reference 21), it is possible that such a factor binds to BIIa and facilitates transport of RAG1 to a particular nuclear compartment. However, because BI, BIIa, and BIII contribute equally to RAG1's ability to bind Srp-1 (30), enhanced recombination is unlikely to be explained merely on the basis of affinity for Srp-1.

The second type of model suggests that the BIIa region enhances catalysis of the recombination reaction, for example, by contributing to more efficient higher-order complex formation or breakdown. It is possible that the BIIa region contributes to the disassembly or remodeling of early forms of the recombination complex to allow the formation of joints. It has been shown that signal ends accumulate in cell lines and primary lymphoid cells that have persistent high levels of full-length RAG1 expression. When RAG protein expression decreases, the signal ends disappear, and there is a subsequent accumulation of signal joints (18, 22). This finding has led to the speculation that a complex containing RAG1 and RAG2 remains bound to the signal ends after cleavage and must be released from the ends to allow signal joint formation (18, 22). In support of this view, under appropriate circumstances, RAG1 and RAG2 core proteins remain tightly bound to signal ends after cleavage in vitro (1). Therefore, it is conceivable that R1c is bound even more tightly to signal ends than full-length RAG1 and that the BIIa region is responsible for this difference. However, because the BIIa region enhances signal joint and coding joint formation equally (data not shown), the effect of this region is unlikely to be confined to the resolution of signal ends. Another possibility (30), raised by the observations that full-length RAG1 has affinity for RNA (30) and that nuclear localization signals, such as BIIa, can mediate RNA binding (for a review, see reference 13), is that the BIIa region assists in recognition of regions of single-stranded DNA that are thought to arise during the process of DNA cleavage to yield hairpin-terminated coding ends (3, 23).

Comparison of the N-terminal regions of RAG1 from different species shows that BI, BIIa, and BIIb, but not BIII, have been relatively well conserved through evolution (Fig. 1B). In BIIa, all species contain at least three contiguous basic residues in the region corresponding to amino acids 220 to 223 of the mouse RAG1 protein, and it is this feature that is altered by the BIIa mutation examined here. Further mutagenesis will be required to determine the exact features of the BIIa region that are important for the effects described here.

Our results indicate that there is more to RAG1's ability to perform the V(D)J recombination reaction than the amino acids found in the C-terminal two-thirds of the protein. The data demonstrate that a region outside the RAG1 core has a significant effect on the activity of the protein in extrachromosomal assays. In vitro experiments are under way to determine if this region contributes primarily to events leading up to cleavage or to postcleavage steps of the reaction.

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