Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6100–6104, July 1993
Immunology

Dispensable sequence motifs in the *RAG-1* and *RAG-2* genes for plasmid V(D)J recombination

(recombination activating genes/cysteine-rich motifs/topoisomerase homology)

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Contributed by David Baltimore, February 22, 1993

ABSTRACT **As a probe of whether *RAG-1* and *RAG-2* gene products activate other genes or form part of the recombinase itself, certain mutants of the *RAG* genes were assayed for their ability to activate variable–diversity–joining region [V(D)J] recombination in a plasmid substrate in fibroblasts. The results indicate that the N-terminal one-third of *RAG-1*, including a zinc-finger-like domain, and an acidic domain of *RAG-2* are dispensable for activating V(D)J recombination in a fibroblast, although they contribute quantitatively. In contrast, deletion of the C-terminal segment of *RAG-1*, which has homology to a topoisomerase-like protein from yeast, abolished recombination activation. These results do not support the hypothesis that the *RAG* gene products are transcription factors and suggest the possibility that they are parts of the recombination machinery.**

The *RAG-1* and *RAG-2* genes were identified and cloned by virtue of their ability to induce 3T3 fibroblasts to conduct the normally lymphocyte-specific process of variable–diversity–joining region [V(D)J] recombination, the proper joining of antibody gene segments by DNA recombination (1–3). Their indispensable role in V(D)J recombination was verified by the lack of such recombination in either B or T cells after ablative mutation of either the *RAG-1* or *RAG-2* genes (4, 5). The biochemical processes carried out by the two proteins, however, remain to be elucidated. They could be developmental switch genes for transcriptionally activating V(D)J recombination, analogous to *MyoD* and other myogenic genes that activate events of muscle development (6). More likely, the RAG proteins themselves could be part of the recombination machinery. By this latter interpretation, the *RAG-1* and *RAG-2* gene products would be the only components of the V(D)J recombinase machinery lacking in 3T3 fibroblasts, and supplying them by transfection would activate recombination directly. Models that fall between these two extremes are also possible; for example, it is possible that one of the *RAG* genes is an activator of the expression of other genes and the other *RAG* gene functions as part of the recombinase machinery directly.

Although the protein sequences translated from the DNA of the two genes do not give any direct indication of their function, there are recognizable motifs that could imply functional abilities. The RAG-1 protein sequence contains a Cys-His motif shared by at least 20 other proteins (7, 8). This motif (aa 290–328 of RAG-1) may have two zinc fingers separated by one residue, but the first potential finger differs, to our knowledge, from all others previously described (9–11). Some proteins with the motif, including VZ61, IE110, CG30, and RPT-1, are putative transcription factors (12–15), although none at this point has been shown unequivocally to be a DNA-binding transcriptional activator or repressor and no function for this motif has been determined.

RAG-2 contains a very acidic stretch of sequence (aa 270–410) (3). This stretch is reminiscent of the acidic activating domain of some transcription factors (16). The zinc-finger-like domain of RAG-1 and the acidic stretch of RAG-2 suggest that the proteins could function as transcription factors, either separately or as a complex.

There is also evidence from the protein sequence suggesting that RAG-1 is a component of the recombinase machinery. The C-terminal half of RAG-1 has sequence similarity to the yeast protein HPRI (17), which has sequence similarity to yeast topoisomerase I (18). Topoisomerases catalyze transesterification reactions and are, therefore, similar to recombinases.

To establish which structural features of the RAG-1 and RAG-2 proteins are important for V(D)J recombination, mutants were constructed and assayed for their ability to promote the recombination of an extrachromosomal test substrate in fibroblasts. We find the features of RAG-1 and RAG-2 that suggest that they may be transcription factors are ones that are not necessary for V(D)J recombination activity, although the region of RAG-1 that is similar in sequence to HPR1 is necessary for function. These data suggest that the *RAG* genes are not likely to be transcription factors but, rather, might be components of the V(D)J recombination machinery.

MATERIALS AND METHODS

Plasmids. The cDNA expression vector for the wild-type RAG-1 cDNA and for all the RAG-1 mutants is identical to the M2 cDNA expression vector based on CDM8 as described (2) except that the 3′ untranslated sequences of the RAG-1 cDNA were removed starting from the *Xcm* I site 10 bp downstream of the stop codon. The expression vector for the wild-type RAG-2 cDNA, in which all mutations were introduced, is identical to the RAG-2 cDNA CDM8 vector as described (3). To construct the *RAG-1* gene-containing retrovirus, the RAG-1 cDNA was inserted into the recombinant retroviral vector pMFG (P. Robbins, L. Spain, and R.C.M., unpublished data) such that the AUG of *RAG-1* was converted into an *Nco* I site. In addition the *Xcm* I site just downstream of the *RAG-1* stop codon was converted into a *Bam*HI site for cloning into pMFG. The retrovirus containing the *RAG-2* gene was also constructed using the retroviral vector pMFG (see above). In this case, the AUG of *RAG-2* was reconstructed to allow its cloning into an *Nco* I site, and the *Ase* I site at the stop codon was adapted to provide a full stop codon and a *Bam*HI site for cloning into pMFG. (Complete structures are available from the authors on request.)

Mutagenesis. All site-directed mutagenesis employed the Bio-Rad Muta-Gene kit according to the manufacturer's

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Abbreviation: V(D)J, variable–diversity–joining region.

instructions with the following changes. The wild-type RAG-1 or RAG-2 expression vectors in CDM8 (described above) were introduced into the *dut ung Escherichia coli* BW 3I3/P3 (a gift of B. Seed, Harvard University School of Medicine). After recovery of the uracil-substituted single-stranded template and polymerization of a second strand from a primer containing a mutation, the DNA was transformed into *E. coli* XS127 (*res*$^-$ *mod*$^+$). To create deletions of the N terminus of the RAG-1 protein, the silent mutations T39G and A42G were introduced to create unique *Nar* I and *Kas* I restriction sites, which were used as starting points for all deletions extending downstream. To construct the RAG-1 protein internal deletion of aa 299–330, the silent mutation T894G creating a *Bam*HI site was introduced. To construct the RAG-2 protein internal deletion of aa 374–414, the silent mutation T1116C creating an *Xma* I site was introduced. The structure of the mutants was confirmed by restriction mapping, where possible, and sequencing. Two isolates of each mutation were obtained to reduce the possibility of unintentional second-site mutations having a significant effect.

Transient V(D)J Recombination Assays. 3T3 cells infected with the *RAG-1*- or *RAG-2*-containing MFG retroviruses were prepared by first cotransfecting the ecotropic retroviral packaging cell line ψcre (19) with the appropriate retroviral vector and pSV2Neo. Supernatants from the highest-titer neomycin-selected ψcre producer colonies were used to infect 3T3 cells. A 3T3 cell population infected with the *RAG-2* gene-containing retrovirus was transfected with 6 μg of the wild-type or mutant RAG-1 cDNA expression vector and 10 μg of the V(D)J recombination reporter construct pJH200 (20). For *RAG-2* mutants, a 3T3 cell population infected with the *RAG-1*-containing retrovirus was transfected with 8 μg of the mutant cDNA expression vector and 10 μg of pJH200. Subsequent steps were as described (3), except that the *Dpn* I-digested DNA was transformed into *E. coli* MC 1061 by a calcium chloride procedure (21). The colony filter hybridization with signal joint oligonucleotide probe was as in ref. 3, except that the filter membranes used were Hybond-N (Amersham).

Transfection of 293T Cells. 293T cells were transfected by a modified calcium phosphate procedure (W. Pear and D.B., unpublished data).

Western Blot Analysis. Extracts were prepared from transfected 293T cells 48 h after DNA was added and fractionated by electrophoresis through a SDS/12.5% polyacrylamide gel. The gel contents were transferred to nitrocellulose, probed with anti-RAG-1 monoclonal antibody (E.S., unpublished data) diluted 1:200 with 3% (wt/vol) nonfat dry milk, and developed with horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody (Amersham) diluted 1:1000. The membrane was visualized with the ECL chemiluminescence kit (Amersham) according to the manufacturer's instructions.

Indirect Immunofluorescence. The indirect immunofluorescence was a variation of that described in ref. 22 and will be described elsewhere (E.S., unpublished data).

RESULTS

Passage and Expression of *RAG-1* and *RAG-2* in Retroviral Vectors. To create cell populations that stably express either RAG-1 or RAG-2, the RAG cDNAs were cloned into a retroviral vector, MFG (P. Robbins, L. Spain, and R.C.M., unpublished results). The resulting plasmids, MFGR1 (the *RAG-1* retrovirus) and MFGR2 (the *RAG-2* retrovirus), were separately transfected into the retroviral packaging line ψcre (19) with pSV2neo as a selectable marker and clones that produced the highest levels of virus were selected (data not shown).

Populations of 3T3 cells infected with supernatants derived from the producer lines of MFGR1, MFGR2, or both MFGR1 and MFGR2 showed greater than single copy integration by Southern blot (Fig. 1). To examine whether the cells infected with the viruses were able to make functional *RAG-1* and *RAG-2* gene products, a transient recombination assay was performed in which the plasmid pJH200 (20)—which confers chloramphenicol resistance to *E. coli* only after the recombination signal sequences have been joined in a V(D)J recombination event—was transfected into the virally infected 3T3 cell populations. Forty-eight hours later, episomal DNA was prepared from the transfected cells and transformed into bacteria to score for recombinants. Only bacterial colonies that were doubly resistant to ampicillin and chloramphenicol and hybridized to an oligonucleotide probe containing a signal joint were counted. Transient recombination assays on 3T3 populations infected with both MFGR1 and MFGR2 gave a high frequency of recombination, whereas transient assays in populations singly infected with either MFGR1 or MFGR2 gave no recombination (Table 1). From this result it is evident that the retroviral vectors provide sufficient levels of expression of both *RAG* genes to promote V(D)J recombination within the test substrate.

Recombination Activity of Mutants. To assess the recombination activity of *RAG-1* mutants, a population of 3T3 cells previously infected with MFGR2 was cotransfected with a plasmid expression vector encoding the mutant and the recombination test substrate pJH200. The MFGR2-infected 3T3 population gave an overall recombination frequency of 0.9% when cotransfected with the expression plasmid encoding wild-type *RAG-1*, CDM8R1, and pJH200. In similar experiments, MFGR1-infected 3T3 cells gave an overall recombination frequency of 0.21% when cotransfected with the wild-type *RAG-2* expression plasmid CDM8R2 and pJH200. By comparison, 3T3 cells infected with both MFGR1 and MFGR2 gave a recombination frequency of 6.09% when transfected with pJH200. The lower recombination frequencies observed when transfecting one of the *RAG* genes may reflect lower expression of a transfected gene than a resident

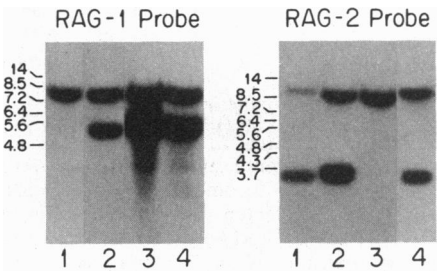

FIG. 1. Gel transfer hybridization showing passage and titer of *RAG-1*- and *RAG-2*-containing retroviral vectors. Genomic DNA from 3T3 cells infected with supernatant containing the *RAG-1*- or *RAG-2*-encoding retroviruses MFGR1 or MFGR2 was digested with the restriction enzyme *Nhe* I and separated by electrophoresis through an agarose gel. The gel contents were transferred to a nylon membrane and hybridized with either a *RAG-1* or *RAG-2* coding sequence probe. The integrated proviruses are expected to be cut twice with *Nhe* I, once in each long terminal repeat. (*Left*) The *RAG-1* probed blot. Lanes: 1, uninfected 3T3 DNA; 2, uninfected 3T3 DNA with MFGR1 plasmid DNA added to a level of one copy per genome; 3, unselected 3T3 population infected with the MFGR1 virus; 4, unselected population of 3T3 cells infected with both the MFGR1 and MFGR2 viruses. (*Right*) The *RAG-2* probed blot. Lanes: 1, unselected population of 3T3 cells infected with the MFGR2 virus (underloaded relative to the other lanes judging from the 8.7-kb endogenous RAG-2 band); 2, DNA from the same unselected population of 3T3 cells infected with both MFGR1 and MFGR2 that was shown in *Left*, lane 4; 3, DNA from uninfected 3T3 cells; 4, DNA from uninfected 3T3 cells with plasmid of the MFGR2 virus added to the level of one copy per 3T3 genome.

Table 1. V(D)J recombination activity of wild-type and mutant *RAG-1* and *RAG-2* genes in 3T3 cells

Protein	No. of colonies Amp$^+$/Cam$^+$	Oligo$^+$	Amp$^+$	% rec	% total rec
		RAG-1 mutants			
RAG-1	215, 89	214, 88	855, 493	1.00, 0.71	0.90
No RAG-1	0, 5	0, 0	617, 598	0, 0	0.00
Δ15–79	35, 26	35, 26	262, 261	0.53, 0.41	0.47
Δ15–135	46, 51	45, 50	528, 502	0.34, 0.40	0.37
Δ15–172	101, 167	99, 166	678, 887	0.58, 0.75	0.68
Δ15–273	78, 45	77, 45	565, 245	0.55, 0.73	0.60
Δ15–330	61, 27	61, 27	486, 380	0.50, 0.28	0.41
Δ15–469	1, 0	0, 0	419, 374	0, 0	0.00
Δ15–502	3, 0	0, 0	637, 514	0, 0	0.00
Δ299–330	35, 17	34, 15	924, 442	0.15, 0.14	0.14
Δ699–end	3, 3	0, 0	369, 449	0, 0	0.00
Δ994–end	1, 1	0, 0	501, 508	0, 0	0.00
Δ1009–end	167, 282	165, 281	505, 391	1.31, 2.87	1.99
Δ1023–end	352, 362	349, 358	604, 640	2.31, 2.24	2.27
KK142,143IQ	92, 154	91, 152	413, 698	0.88, 0.87	0.87
C290S	7, 3	6, 3	331, 347	0.07, 0.03	0.05
H295D	30, 84	30, 82	423, 943	0.28, 0.35	0.33
D299E	110, 119	107, 117	467, 551	0.92, 0.85	0.88
H307N	24, 19	24, 17	682, 600	0.14, 0.11	0.13
H307N,C310S	11, 8	10, 7	457, 322	0.09, 0.09	0.09
R311S	63, 36	62, 36	561, 408	0.44, 0.35	0.40
P326G	3, 15	3, 15	236, 584	0.05, 0.10	0.09
C328S	8, 1	8, 1	475, 133	0.07, 0.03	0.06
Y330F	49, 43	49, 43	342, 343	0.57, 0.50	0.54
T335V	42, 64	42, 64	277, 392	0.61, 0.65	0.63
D336N	98, 64	98, 63	629, 312	0.62, 0.81	0.68
Y998F	39, 23	37, 23	205, 247	0.72, 0.37	0.53
		RAG-2 mutants			
RAG-2	16, 23	14, 23	231, 466	0.24, 0.20	0.21
No RAG-2	0, 5	0, 0	266, 537	0, 0	0.00
Δ374–414	30, 20	28, 19	589, 407	0.19, 0.19	0.19
S40A	33, 6	32, 6	653, 218	0.20, 0.11	0.17
T121A	9, 8	8, 8	288, 178	0.11, 0.18	0.14
K312Q	7, 5	6, 4	154, 192	0.16, 0.08	0.12
DDE405NNQ	4, 4	4, 4	271, 348	0.06, 0.05	0.05
		Double infection			
MFGR1+2	144	143	94	6.09	

RAG-1 wild-type and mutant expression plasmids were transiently transfected with the V(D)J recombination reporter pJH200 into a population of 3T3 cells infected with the retrovirus MFGR2 that expresses RAG-2 activity. Forty-eight hours later, episomal DNA was prepared and transformed into bacteria, and the bacteria were plated on ampicillin (Amp) or ampicillin plus chloramphenicol (Amp/Cam) plates. All mutants were isolated independently in duplicate, and separate plasmid preparations were assayed in this manner. The protein column shows the structure of the protein assayed. The Amp$^+$/Cam$^+$ and Amp$^+$ columns show the number of bacterial colonies growing on plates with those drugs. The Oligo$^+$ column shows the number of colonies on the Amp/Cam plates that hybridized with the signal joint oligonucleotide probe. The percent recombination (% rec) column shows the percent of signal joint hybridizing colonies to Amp$^+$ colonies, taking into account that 25-fold more bacteria were plated on the Amp/Cam plates than on the Amp plates. In the Amp$^+$/Cam$^+$, Oligo$^+$, Amp$^+$, and % rec columns, each of the two numbers shown refers to an independently picked mutant that was assayed; the first number always refers to the first mutant. The % total rec column was calculated by pooling the Oligo$^+$ colonies and Amp$^+$ colonies to calculate a final percent recombination. *RAG-2* mutants were treated identically, except they were transiently co-transfected into a population of 3T3 cells infected with the *RAG-1*-expressing retrovirus MFGR1. For the double infection, a 3T3 cell population infected with both the MFGR1 and MFGR2 retroviruses was transfected with pJH200. The V(D)J recombination frequency was calculated as above. Δ, deletion.

one, some miscoordination of the expression of the various elements, or inefficient double transfection into the virally infected cells. The low value, however, was well above the background—which was <0.01%—and allowed the effects of mutations to be measured.

A series of deletion mutants of the RAG-1 N terminus from aa 15 toward the C terminus of the protein were assayed by cotransfection of pJH200 and the expression plasmids encoding the mutants into 3T3 cells infected with MFGR2 (Table 1). Deletions of aa 15–79, 15–135, 15–172, 15–273, and 15–330 resulted in recombination frequencies ≈50% of the wild-type levels. Deletion of aa 15–330 completely encompasses the zinc-finger-like region, implying its dispensability for recombination within the pJH200 plasmid. Deletion of aa 299–330, which spanned all but the first two cysteine residues of the zinc-finger-like region, showed ≈15% of the activity of wild-type RAG-1. Deletions of aa 15–469, 15–502, 15–581, and 15–757 were all completely nonfunctional in the recombination assay.

Deletions from the C terminus of the 1041-aa RAG-1 protein presented a different picture. Deletion of aa 1023–end (aa 1041) and aa 1009–end gave approximately double the wild-type rate of recombination, whereas deletions of aa 994–end and aa 699–end were both completely nonfunctional in the transient recombination assay. Thus there is critical sequence near the C terminus of RAG-1, the region related to topoisomerase.

In addition, the results of point mutants targeted to various structural features of RAG-1 were examined in the transient recombination assay (Table 1). KK142,143IQ, a double mutant in the putative nuclear localization signal (2), showed a recombination frequency indistinguishable from wild type. The set of point mutations in the Cys-His motif common to RAG-1 and at least 20 other proteins all had similar effects: C290S, H307N, H307N,C310S, P326G, and C328S all showed levels of recombination 10- to 20-fold below wild-type levels, but nevertheless all did produce V(D)J recombination products that hybridized to a signal joint oligonucleotide probe. Point mutations H295D, D299E, R311S, Y330F, T335V, and D336N—in residues that are mostly conserved among human, murine, chicken, and *Xenopus* RAG-1 in the region of the zinc-finger-like motif—resulted in recombination frequencies ranging from ≈33% to equal to wild-type levels. A point mutant, Y998F, in a tyrosine residue suggested to be homologous to the tyrosine in the active site of type I and type II topoisomerases (17), gave ≈50% of the level of recombination of the wild-type protein.

The recombination activity of several mutants of RAG-2 was also determined by transient assay (Table 1). A deletion mutant of RAG-2, aa 374–414, which encompassed virtually all of the acidic stretch of RAG-2, gave a recombination level indistinguishable from that of wild-type RAG-2 when co-transfected with pJH200 into a population of 3T3 cells infected with MFGR1. As another test of the importance of the acidic stretch of RAG-2, the triple point mutant made by substituting NNQ for the wild-type sequences DDE (aa 405–407) in the acidic stretch was constructed. This mutant showed a 4-fold reduction in recombination frequency compared to wild-type RAG-2 but still was able to activate recombination.

Various other point mutants of RAG-2 were constructed. Mutant T121A removed a potential cAMP-dependent protein kinase target site, S40A changed a region of very weak sequence similarity to *E. coli* isoleucine tRNA ligase, and K312Q altered a sequence of weak similarity to the *E. coli* *Eco*RI methylase. None of these point mutants had dramatic effects on recombination levels.

The various mutants were also examined for recombination levels in the highly transfectable cell line 293T both as a way of confirming the results in 3T3 cells and because 293T

cells were used to assay the stability of the mutant proteins (see below). The recombination frequencies of the mutants of RAG-1 and RAG-2 in 293T cells were in good agreement with the data obtained in 3T3 cells (data not shown).

Stability and Expression of Mutants. To evaluate the stability of the mutant proteins, plasmids encoding wild-type *RAG-1* and the *RAG-1* mutants were transfected into 293T cells, and protein extracts were made 48 h after transfection for Western blot analysis. Probing the filters with a monoclonal antibody raised against the C-terminal half of RAG-1 (E.S., unpublished data) revealed that the wild-type protein, point mutants, and N-terminal deletion mutants were expressed and all migrated at the expected molecular masses (Fig. 2). Furthermore, all of the N-terminal deletion mutants with the exception of the aa 15–757 deletion were expressed at higher levels than wild type, suggesting that a sequence in the RNA or protein between aa 15 and aa 79 reduces steady-state protein levels. None of the C-terminal mutants was detected with the monoclonal antibody, suggesting that its epitope is between aa 1024 and the C terminus of the protein (aa 1041).

Subcellular Localization of Mutants. The subcellular localization of a subset of the *RAG-1* mutants was determined by indirect immunofluorescence. To achieve high enough expression to allow detection, these mutants were subcloned into the MFG retrovirus and cotransfected with the ecotropic replication-competent retrovirus ZAP into 293T cells. Viral supernatant was collected 2 days later and used to infect 3T3 cells. These cells were passaged once and then permeabilized and stained with anti-RAG-1 monoclonal antibody followed by rhodamine-conjugated anti-mouse immunoglobulin. Cells infected with virus carrying the wild-type *RAG-1* gene displayed a punctate nuclear staining (Fig. 3). The deletion mutant of RAG-1 aa 299–330 and the RAG-1 mutant in the putative nuclear localization site KK142,143IQ showed the same distribution as the wild-type protein. The *RAG-1* point mutants C290S and H307N in the zinc-finger-like region also showed the wild-type pattern (data not shown). Other mutations in the RAG-1 zinc-finger-like region displayed different patterns of immunofluorescence. The majority of cells infected with a virus carrying the double point mutant H307N,C310S had a diffuse nuclear pattern of staining, although some cells showed the same punctate nuclear staining as cells expressing the wild-type protein. The cells expressing mutant P326G showed uniform nuclear staining

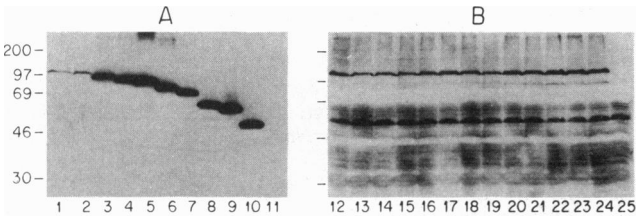

FIG. 2. Expression of the *RAG-1* deletion and point mutants. The insoluble pellets from extracts resulting from transient transfection of 293T cells with expression plasmids for various *RAG-1* mutants were fractionated by electrophoresis through SDS/PAGE gels and transferred to nitrocellulose membranes. The membranes were probed with a monoclonal antibody (E.S., unpublished data) and visualized with a secondary horseradish peroxidase-conjugated antibody and the chemilluminescent ECL system (Amersham). (*A*) Deletion mutants. Lanes: 1, wild-type RAG-1; 2, deletion of aa 298–330; 3, deletion of aa 15–79; 4, deletion of aa 15–135; 5, deletion of aa 15–172; 6, deletion of aa 15–273; 7, deletion of aa 15–330; 8, deletion of aa 15–469; 9, deletion of aa 15–502; 10, deletion of aa 15–581; 11, deletion of aa 15–757. (*B*) Point mutants (a longer exposure than in *A*). Lanes: 12, KK142,143IQ; 13, C290S; 14, H295D; 15, D299E; 16, H307N; 17, H307N,C310S; 18, R311S; 19, P326G; 20, C328S; 21, Y330F; 22, T335V; 23, D336N; 24, Y998F; 25, no *RAG-1*.

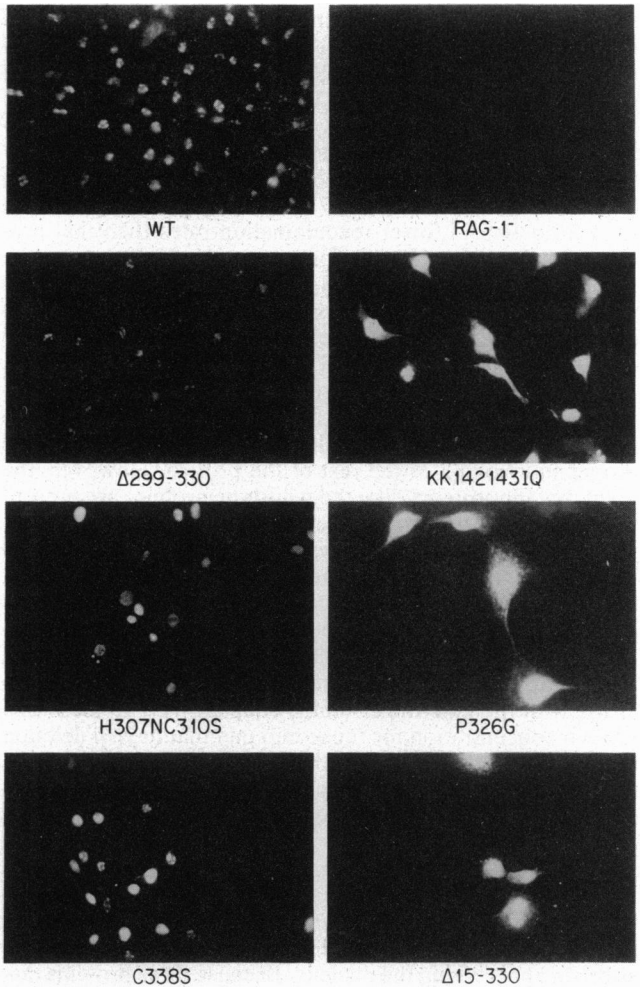

FIG. 3. Indirect immunofluorescence of wild-type and mutant RAG-1 proteins. Wild-type *RAG-1* and various mutants were cloned into the retrovirus vector MFG. These plasmids were cotransfected into 293T cells with a plasmid containing the ecotropic helper virus ZAP. Forty-eight hours later, the supernatant from these cells was used to infect 3T3 cells. Indirect immunofluorescence of the infected 3T3 cells was performed using RAG-1 monoclonal antibody (E.S., unpublished data) and a rhodamine-conjugated secondary antibody. Wild-type RAG-1-infected cells and cells infected with the helper virus alone (Rag-1$^-$) are shown in the top panels. The other mutants are as marked.

superimposed on a nuclear punctate pattern with perhaps some cytoplasmic staining; some of the mutant C328S-expressing cells showed a diffuse nuclear staining pattern, whereas other cells showed the wild-type pattern. Cells expressing the *RAG-1* mutant with the large N-terminal (aa 15–330) deletion showed staining throughout the nucleus and cytoplasm, and some cells had in addition bright nuclear spots.

DISCUSSION

Several lines of experimental evidence presented here suggest that RAG-1 and RAG-2 are likely to be components of the V(D)J recombination machinery, rather than transcription factors that activate a set of genes whose products form the V(D)J recombinase. (*i*) The region of *RAG-1* with sequence similarity to the yeast topoisomerase-like gene *HPR1* is required for recombination. HPR1 suppresses intrachromosomal recombination in yeast; perhaps RAG-1 directs the same recombination machinery toward the substrates for V(D)J recombination. (*ii*) The structural feature of RAG-1

resembling a DNA-binding motif of transcription factors, the zinc-finger-like region, is not necessary to activate V(D)J recombination in fibroblasts. Furthermore, the acidic stretch of RAG-2, which resembles the acidic activation motif of some transcription factors, is also not necessary for recombination activity in fibroblasts.

The point mutants in the motif resembling a zinc finger in RAG-1 (C290S, H307N, H307N,C310S, P326G, and C328S) gave 5- to 10-fold lower recombination rates than that produced by the aa 15–330 deletion mutant that lacked the entire motif. The mutants produced accurately recombined products with correct signal joints and, therefore, displayed no defect other than a low rate, a result that would not be expected if the "zinc finger" were involved as the DNA recognition domain of a transcription factor. The point mutations in the Cys-His motif might cause alterations in the folding of that region that could interfere physically with the proper functioning of the rest of the protein. However, the point mutant proteins, like the wild-type protein, are present in cells at lower levels than the aa 15–330 deletion mutant, as judged by Western blot analysis (Fig. 2). Also, aa 299–330 deletion mutant has a recombination rate only slightly higher than the point mutants, although this deletion removes most of the zinc-finger-like motif but is expressed at levels comparable to the wild-type RAG-1 protein (Fig. 2, lane 2). Thus, a reduced efficiency of recombination by all mutants interfering with the zinc-finger motif, coupled with an increased protein concentration for those mutants that have a deletion spanning at least aa 15–79, is the probable explanation of the recombinant percentages produced by these mutants. It appears that this motif may help RAG-1 protein to function, but it is clearly not absolutely essential, suggesting that it is not directly involved in recognition or catalysis.

The subcellular localization of overexpressed RAG-1 protein and of several of the mutants was determined by indirect immunofluorescence. The wild-type RAG-1 protein localized as bright spots within the nucleus. Because three proteins that contain the zinc-finger-like motif, IE110, Ro/SSA, and PML, have been reported to localize in speckles in the nucleus (23–25), the subcellular localization of several mutants in the zinc-finger-like region has been determined. Some mutations in the zinc-finger-like region did change the pattern of nuclear localization. However, the observation that the aa 299–330 deletion mutant, which lacks all but the first two cysteines of the motif, still displayed the wild-type pattern eliminates the possibility that the motif is responsible for the speckled nuclear distribution of the protein. The mutants in the zinc-finger region that change the spatial pattern of expression in the nucleus, H307N,C310S, P326G, and C328S, probably do so because the folding of the N terminus of the protein is perturbed. The mutations in the putative nuclear localization sequence, KK142,143IQ, do not affect the subcellular localization of the protein, indicating that other domains contribute to the nuclear localization of the RAG-1 protein. The large N-terminal deletion (aa 15–330), which is functional in a recombination assay, does cause a substantial relocation of much of the protein to the cytoplasm, so sequences specifying subcellular localization are likely to be found in that region. We found no consistent relationship between subcellular localization and recombinational activity.

It should be noted that the recombination assay used in experiments presented here utilizes pJH200, an extrachromosomal substrate that forms a signal joint (a heptamer–heptamer fusion) after recombination. It is conceivable that the zinc-finger-like motif of RAG-1 or the acidic region of RAG-2 is required either for recombination in its proper chromosomal context or to form coding joints.

Mutations at the C-terminal end of RAG-1 are informative. Deletion of the last 18 aa or 32 aa results in increased recombination activity. Because our antibody reagents cannot detect RAG-1 mutants missing the C terminus, the mechanism by which activity is increased is not clear and could be either enhanced expression or more efficient intrinsic activity in promoting recombination. Deletion of more sequence from the C terminus of the protein results in no recombination activity at all, suggesting that the C terminus is required for RAG-1 activity. Because this is the region of the protein with sequence similarity to the yeast topoisomerase-like protein HPR1, these results support the speculation that RAG-1 may have a mechanism of action similar to that of HPR1. The suggestion has been made based on structural similarities between HPR1 and various topoisomerases that HPR1 may in fact be a topoisomerase (17, 18). This analysis suggested that Tyr-532 of HPR1 would be at the active site and be the amino acid that covalently bonds to the phosphodiester backbone of DNA. However, a point mutant at the tyrosine residue of RAG-1 that corresponds to Tyr-532 of HPR1 does not abolish V(D)J recombination (RAG-1 Y998F, see Table 1). Clearly, if there are functional analogies between HPR1 and RAG-1, these remain to be elucidated.

We thank Dr. Chris Roman for valuable comments on the manuscript. E.S. was supported by a long-term European Molecular Biology Organization fellowship. This work was supported by National Institutes of Health Grants CA-51462 to D.B. and HL-37569 to R.C.M.

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