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Definition of a core region of RAG-2 that is functional in V(D)J recombination

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ABSTRACT

The products of the RAG-1 and RAG-2 genes cooperate to allow V(D)J recombination in lymphoid and non-lymphoid cells. As one step toward understanding the role of RAG-2, we have constructed mutated RAG-2 genes and examined their ability to support recombination of plasmid substrates in a fibroblast cell line. The mutations define essential and dispensable parts of the RAG-2 gene. Mutations in the N-terminal part eliminate almost all activity. In the central region of the protein, some but not all local alterations still allow recombination. On the other hand, proteins with large deletions from the C-terminal end, including one truncated by 25%, still retain activity, even though this part of the protein is highly conserved between species. Similar results were obtained with substrates that retain either a signal joint or a coding joint, or perform an inversion. Thus all basic features of V(D)J joining are retained in a RAG-2 protein with only the first 75% of the sequence.

INTRODUCTION

V(D)J recombination is the process by which functional immunoglobulin and T cell receptor genes are assembled during the development of the immune system. Extensive studies have identified the sequences needed to target recombination to the correct sites, the structures of the various recombined DNA products, and the presence of broken DNA species that may be intermediates in the reaction (reviewed in 1). However, the factors involved in catalyzing the process have been elusive. A significant advance in this direction was the identification of the RAG-1 and RAG-2 genes (2, 3). Expression of both these genes is found in lymphoid cells at the developmental stage where V(D)J recombination occurs, and their co-expression in non-lymphoid cells such as fibroblasts renders these cells recombinationally active. The products of these genes are thus the only lymphoid-specific factors required for V(D)J recombination (reviewed in 4). The essential role of RAG-1 and RAG-2 in this process is further demonstrated by the properties of mice (or cell lines) in which either of these genes has been disrupted (5, 6). The disruption leads to a total absence of V(D)J recombination, without affecting any other known property.

The detailed functions of the RAG-1 and RAG-2 genes are not yet known. In particular, there has been no compelling evidence to distinguish whether they activate V(D)J recombination indirectly or participate directly in the reaction. One approach to a more detailed understanding of these genes is to test mutated versions for recombinational activity. Recent investigations of a number of RAG-1 mutations (7–9) showed that a large part of the gene, coding for the first 383 amino acid residues, is dispensable for recombination of plasmid substrates. It was also shown that several sequence patterns identified by a homology search—possible motifs for a zinc-finger region, a nuclear localization signal, and a topoisomerase active site near the C-terminal end—are not necessary for activity.

In this work, mutations in RAG-2 are tested similarly for their ability to support recombination of plasmid substrates in fibroblasts. Such substrates have been designed to retain either one of the two novel junctions made by V(D)J recombination, a signal joint or a coding joint. Cells from normal mice make both junctions with similar efficiency, but it is known that under some conditions (for example in cells from mice with the *scid* mutation) there is a strong preference for one junction over the other. We therefore tested the ability of the RAG-2 mutations to form both types of junction.

MATERIALS AND METHODS

Plasmids

The full length RAG-1 and RAG-2 expression plasmids (pJH548 and pJH549 respectively) were described previously (7). Both ends of the open reading frame encoding the RAG-2 protein were modified in plasmid pMS201 by PCR methods. The 5′ end was changed to an NcoI recognition site (CCATGG) which serves as a good eukaryotic consensus translation start signal (10). This modification changed the second amino acid from serine to alanine. The 3′ end was changed to add a series of nine histidines and three copies of an epitope tag immediately after the last encoded amino acid, followed by a stop codon, as used previously for RAG-1 (7). The pCDM8 expression vector (11) was modified to remove the MluI site by fill-in and blunt end ligation.

Subsequent constructs were all derived from pMS201 and retain this C-terminal addition. Plasmids pMS202 through pMS213 were each generated by PCR amplification using pMS201 as

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template and specific synthesized oligonucleotide primers designed to create a deletion of coding region and an insertion of a SalI recognition site. Plasmids pMS214 through pMS218 were prepared by deleting the sequence between pairs of SalI sites generated in the previous constructs.

Extrachromosomal substrate assay

Plasmids were transfected into the fibroblast cell line 3TGR (12) and activity was enhanced by treatment with sodium butyrate as previously described (7). In these experiments each tissue culture dish was pretreated with 2 μg ProNectin F (Stratagene). As described before, plasmid DNA was reisolated 40–48 hours after transfection, digested with the restriction enzyme DpnI, and candidate recombinants were identified by transformation into *E.coli* and selection for resistance to both ampicillin and chloramphenicol.

Hybridization analysis of recombinants

The plasmids in chloramphenicol-resistant colonies were further characterized by colony hybridization with a battery of oligonucleotide probes. The presence of the precise signal joint expected to be found after recombination of pJH200 (and pJH299 inversions) was confirmed by hybridization to an oligonucleotide which spans the new junction. Recombined pJH290 plasmids, which retain a coding joint, were challenged by hybridization to six oligonucleotides, confirming the loss of both signal-coding sequence borders in their original contexts, the loss of the oop transcription terminator, the loss of the spacer sequences within each signal, and the retention of one coding sequence, starting 46 base pairs internal to this coding end. Chloramphenicol resistance confirms retention of the other coding sequence, because the AUG of the chloramphenicol acetyl-transferase gene is 52 base pairs internal to the second coding end. Recombined pJH299 plasmids were also analyzed by hybridization to six oligonucleotides, to confirm the predicted structures of molecules containing an inversion or hybrid joint. The structures of many coding joint, inversion and hybrid joint plasmids were further confirmed by restriction analysis.

Computer analysis

The multiple sequence alignment was assembled using the Pileup program of the GCG sequence analysis software package and subsequently modified manually for display purposes.

RESULTS AND DISCUSSION

Local mutations were made at eleven sites distributed within the RAG-2 gene, and a series of larger deletions in the C-terminal region was also constructed. Figure 1 is a schematic diagram of the mutation sites. The exact alterations, together with the recombination activities of these RAG-2 variants, are presented in Table 1. The mutation sites altered some highly conserved and some less conserved sequences (see discussion below). In the series of small deletions spaced through the coding region, pMS202-210, pMS212, and pMS213, short segments of coding region were replaced by a SalI recognition site (in the frame to encode the amino acids valine and aspartic acid), with the accompanying deletion of several amino acids. These small deletions were distributed through the protein, avoiding cysteines to prevent disruption of disulfide bonds and the possible ensuing long-range effects.

All mutations were tested with pJH200 (13), in which a segment between the signal sequences is deleted upon recombination and a signal joint is retained in the plasmid. Recombination was also measured with pJH290 (14), a similar plasmid in which deletion results in retention of a coding joint. With a few mutations, we also tested recombination of pJH299 (15), in which the DNA between the signal sequences becomes inverted. We describe the results obtained with pJH200 first.

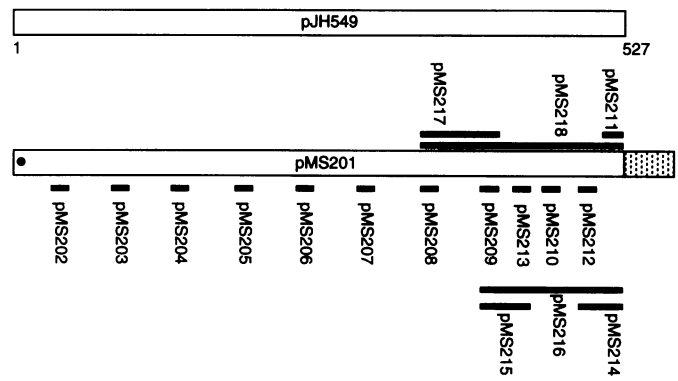

Figure 1. Schematic representation of the RAG-2 coding region with deletion mutations. The top rectangle represents the natural mouse gene indicating amino acids from 1 to 527. The rectangle below represents plasmid pMS201, with one point mutation (dot) and the C-terminal epitope tag (stippled). The solid bars indicate sequences which are deleted from pMS201 in the other plasmids.

Table 1. RAG-2 expression plasmids and recombination activities

Plasmid	RAG-2 sequence	pJH200 plasmids screened	pJH200 recomb. (%)	pJH290 plasmids screened	pJH290 recomb. (%)
pJH549	1-527	390k	1.6	60k	1.1
pMS201	1-527, S2A, epitope tag	120k	4.7	150k	1.7
pMS202	VFFF 28 VDM	930k	0.003	170k	0.004
pMS203	IDSDKH 84 VD	990k	<0.001	200k	0.003
pMS204	IDVVYS 142 VD	590k	0.002	200k	<0.001
pMS205	IDFEFG 185 VD	900k	0.002	125k	<0.001
pMS206	VDLPLG 238 VD	140k	4.6	230k	1.6
pMS207	IEISEM 297 VD	690k	0.001	270k	<0.001
pMS208	SEAFYF 340 VD	780k	0.13	900k	0.05
pMS209	AEANSF 388 VD	110k	3.4	60k	3.7
pMS210	TELNKP 436 VD	630k	0.09	630k	0.04
pMS211	del 518-527 VD	120k	3.8	70k	3.0
pMS212	QTPK 489 VD	55k	1.5	70k	3.1
pMS213	DDESVT 408 VD	62k	0.84	65k	1.9
pMS214	del 489-527 VD	300k	1.6	300k	0.6
pMS215	del 388-413 VD	230k	1.6	180k	1.0
pMS216	del 388-527 VD	240k	0.8	240k	0.4
pMS217	del 340-393 VD	1.1 M	0.12	1.7M	0.05
pMS218	del 340-527 VD	430k	<0.001	350k	<0.001

The plasmids contain the mouse RAG-2 coding region as specified in the second column. Plasmid pJH549 encodes the normal gene. Plasmids pMS202 through pMS218 were all derived from pMS201 and are identical except for the changes noted in one letter code. Specific mutations are listed such that the original sequence, to the left of the number, is replaced by the sequence to the right. For example, in pMS202, the four residues VFFF starting at 28 are replaced by VDM. Extended deletions are indicated by 'del'. Recombinants were selected for chloramphenicol resistance and structures of product plasmids were confirmed by oligonucleotide hybridization (see Methods). All results shown for pJH200, and those for pJH290 assayed with pMS208 and pMS217, are the averages of two or more independent transfections. The other results shown for pJH290 are from single experiments. (k=thousands, M=millions).

The unaltered mouse RAG-2 coding region in pJH549 and the slightly modified version in pMS201 (see Materials and Methods) yielded several percent recombined molecules, with pMS201 giving a somewhat higher level. Plasmid pMS201 was the precursor of all the RAG-2 mutations discussed below.

The short alterations in the amino terminal portion of the protein (pMS202 through pMS205) are incompatible with function; none supported more than 1/1000 of the recombination obtained with the parent plasmid pMS201. Toward the center of the protein sequence, the mutation in pMS206 (residues 238–243) allows normal levels of activity, but pMS207 (residues 297–302) is inactive. The remaining constructs, which introduce small deletions in the C-terminal portion of the protein, retain recombination activity to varying degrees. Some of these latter mutations change identified features in the peptide sequence. The deletions in plasmids pMS209 and pMS213 are located within a strikingly acid-rich sequence, and pMS212 deletes a phosphorylation site proposed to modulate protein stability (16). All three of these plasmids are quite active in recombination, at a level between 20% and 80% of that obtained with the parent plasmid pMS201. On the other hand, the mutation in pMS208 lies within a less well-conserved region, but lowers recombination considerably.

Table 2. Activity of selected RAG-2 mutants with substrate pJH299

RAG-2 mutant	plasmids screened	inversional recombination %
pJH549	1.5M (3)	0.08
pMS208	900k	0.001
pMS216	90k	0.15
pMS217	1.7M (3)	0.002
pMS218	230k	<.001

RAG-2 expression plasmids and notation are described in Table 1. The number of independent transfections when greater than one is noted in parenthesis.

Larger deletions, all within the C-terminal region of RAG-2, were prepared by removing the coding sequence between SalI sites of pairs of the plasmids described above. When these constructs were tested in the assay, high levels of activity, comparable to the parent plasmid, were obtained even after some large segments had been deleted. It is especially striking that pMS216 works so well, despite deletion of 25% of the protein, from residue 388 to the C-terminus. Other deletions within this region also retain activity. Both pMS214 (residues 489–527) and pMS215 (residues 388–413) are quite active. Plasmid pMS211, which deletes a highly conserved basic region of RAG-2 (residues 518–527) also gives a high recombination frequency.

The largest deletion tested, removing residues from 340 to the C-terminus in pMS218, was recombinationally inactive.

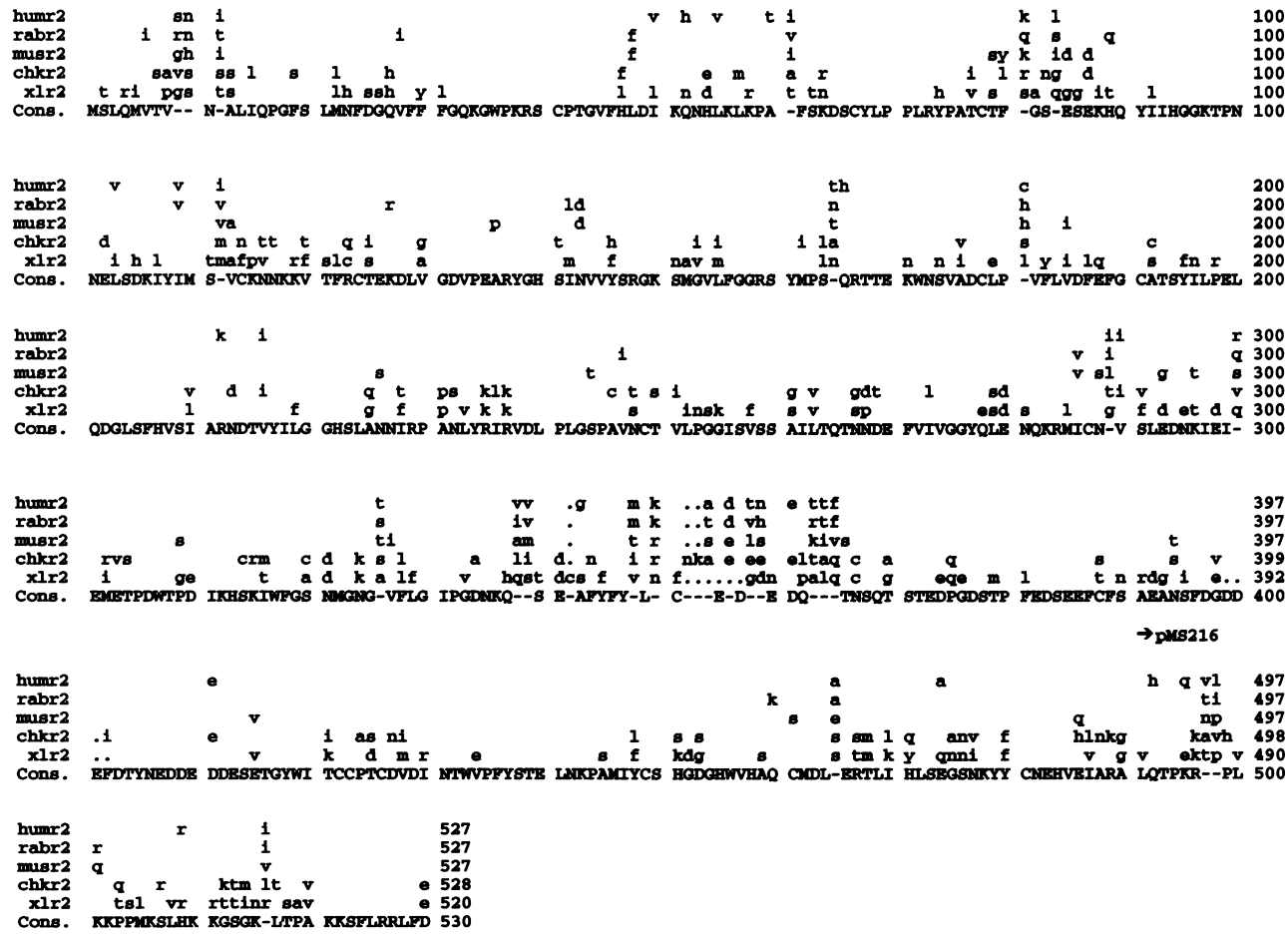

Figure 2. The amino acid sequence of RAG-2 of five species [human (7), mouse (14), rabbit (2), chicken (1), and xenopus (4)] are aligned above that of the consensus. Dashes are at positions where no consensus was obtained. Dots are introduced into the sequences where gaps are necessary to maximize alignment. The beginning of the deletion of plasmid pMS216 is indicated below the consensus.

However, because two smaller deletions that together cover the same region, pMS217 (deleting residues 340–393) and pMS216 (deleting residues 388–527) retain some recombination activity, there does not appear to be any sequence strictly essential for recombination within this whole C-terminal region from residue 340 onward. Instability of the protein product of pMS218 may be one factor contributing to its apparent inactivity. Western blots reveal considerably less RAG-2 protein in cells transfected with pMS218 than with the less extensive C-terminal deletions (data not shown).

The experiments cited above only tested for the formation of a signal joint. We also checked the ability of these mutations to complete coding joints, with the substrate pJH290. As shown in Table 1, the activity of the RAG-2 mutants in forming coding joints was found to parallel their ability to make signal joints.

It seemed possible that inversional recombination might be more demanding of intact RAG-2 function than signal joint or coding joint formation alone. For this reason, four of the C-terminal deletion mutants were also assayed with the plasmid pJH299, in which V(D)J recombination inverts the segment of DNA between the recombination signals. Recombination with this substrate was an order of magnitude lower than with pJH200 or pJH290, but the effects of RAG-2 mutations on inversions (Table 2) were parallel to those found with the deletional substrates. A less frequent event in this plasmid fuses one signal sequence to the coding sequence of the opposite signal, deleting the intervening DNA to yield an alternative recombined junction called a hybrid joint (17). Hybrid joints were also found in each case where recombinants were obtained, but at levels too low to compare meaningfully.

It is helpful to view these results in the context of sequence conservation in the RAG-2 gene. No extensive homology has been found between RAG-2 and any other protein, but the RAG-2 sequence itself is highly conserved across species from Xenopus to human. Figure 2 is a sequence alignment of the RAG-2 proteins predicted from the DNA sequences of five species (3, 18–21). The figure shows a consensus sequence together with the individual differences. The deletion of the C-terminal 25% of RAG-2 removes coding sequence that is strongly conserved among all the five species, but appears not to be required for recombination. This contrasts with the situation in RAG-1, where the most highly conserved region corresponds well with the region necessary for activity. In RAG-1, the nonessential N-terminal region is relatively poorly conserved, while the C-terminal two-thirds of the gene forms a highly conserved, and apparently essential, block. In RAG-2, highly conserved and less well-conserved regions are interspersed throughout the gene, and in particular the extreme C-terminus and the sequence from residue 388 to 450, which are dispensable, are among the best conserved.

The approach we have taken cannot anticipate the consequences of a mutation on protein structure, stability, or functional interactions. From the inactivity of a single RAG-2 variant, one cannot conclude that the altered region is essential for function. For example, pMS210 deletes four residues in the acid-rich sequence near the C-terminus, and reduces the recombination of pJH200 by 50-fold. However, the larger deletion of pMS216 encompasses that of pMS210 and restores substantial activity. Nevertheless, when several mutations in one region all lead to loss of function, it is probable that essential features are being disrupted. This appears to be true of the N-terminal part of RAG-2, where no mutations retain significant activity.

Closely similar results obtained with a parallel collection of RAG-2 mutations have been obtained by Cuomo and Oettinger (accompanying paper). They have similarly shown that a large C-terminal segment of RAG-2 is dispensable for recombination. The results reported here also correlate well with the properties of two mutants studied by Silver *et al.* (8). Their deletion of residues 374 to 414 removes the strikingly acid-rich region, with no apparent effect on recombination of pJH200. The mutation in our pMS215 removes much of the same region, again with survival of recombination (in our case for both signal and coding joint substrates). Also, their replacement mutant DDE 405 NNQ reduced recombination of pJH200 by a factor of four. The nearby mutation in our construct pMS213 (DDESVT 408 VD) creates a short deletion and has a similar effect on activity.

It has been reported recently that the RAG-2 protein undergoes phosphorylation in vivo (16) at two sites, and that phosphorylation at threonine 490 targets the protein for rapid degradation. The deletions in our pMS212, pMS214 and pMS216 all remove this threonine residue. One might anticipate that deletions removing the phosphorylation site would demonstrate increased activity, but this was not observed either in the original publication (16) or in our work.

It is striking that both RAG-1 and RAG-2 can be so extensively deleted, yet remain functional in recombination. Do the deleted regions serve no purpose in the natural setting? This seems unlikely. Rather, the assay used here may not require features of the protein that address other aspects of the rearrangement of antigen receptor genes. Among these could be determinants of interaction with the chromosome and the control of rearrangement of the immunoglobulin and T-cell receptor loci, or the regulation of the recombination activity itself.

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