

RAG-1 interacts with the repeated amino acid motif of the human homologue of the yeast protein SRP1

[V(D)J recombination/nuclear envelope]

PATRICIA CORTES, ZHENG-SHENG YE, AND DAVID BALTIMORE

The Rockefeller University, 1230 York Avenue, New York, NY 10021

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ABSTRACT Genes for immunoglobulins and T-cell receptor are generated by a process known as V(D)J recombination. This process is highly regulated and mediated by the recombination activating proteins RAG-1 and RAG-2. By the use of the two-hybrid protein interaction system, we isolated a human protein that specifically interacts with RAG-1. This protein is the human homologue of the yeast SRP1 (suppressor of a temperature-sensitive RNA polymerase I mutation). The SRP1-1 mutation is an allele-specific dominant suppressor of a temperature-sensitive mutation in the zinc binding domain of the 190-kDa subunit of *Saccharomyces cerevisiae* RNA polymerase I. The human SRP cDNA clone was used to screen a mouse cDNA library. We obtained a 3.9-kbp cDNA clone encoding the mouse SRP1. The open reading frame of this cDNA encodes a 538-amino acid protein with eight degenerate repeats of 40–45 amino acids each. The mouse and human SRP1 are 98% identical, while the mouse and yeast SRP1 have 48% identity. After cotransfection of the genes encoding RAG-1 and human SRP1 into 293T cells, a stable complex was evident. Deletion analysis indicated that the region of the SRP1 protein interacting with RAG-1 involved four repeats. The domain of RAG-1 that associates with SRP1 mapped N-terminal to the zinc finger domain. Because this region of RAG-1 is not required for recombination and SRP1 appears to be bound to the nuclear envelope, we suggest that this interaction helps to localize RAG-1.

Immunoglobulin and T-cell receptor genes are composed of different gene segments, present in multiple copies in germline DNA. During development of B and T lymphocytes, these gene segments are assembled by a mechanism known as V(D)J (variable-diversity-joining region) recombination. This recombination process, which is ordered and highly regulated, leads to the formation of a complete functional gene. The DNA sequence requirements for V(D)J recombination are well-characterized. Gene segments are flanked by highly conserved heptamer and nonamer DNA motifs, separated from each other by a spacer of 12 or 23 bp (1). These signal sequences are necessary and sufficient to direct V(D)J recombination of exogenous substrates (2–4). However, little is known about the proteins involved in V(D)J recombination. Several activities are thought to be components of the recombinase machinery such as DNA binding, endonucleolytic cleavage, and ligase.

By a genetic approach, two genes known as RAG-1 and RAG-2 were identified by their ability to activate V(D)J recombination when cotransfected into fibroblasts that normally are inactive for recombination (5–7). RAG-1 and RAG-2 genes are highly conserved between species and their fundamental role in recombination was demonstrated by the lack of B and T cells in RAG-1 and RAG-2 “knockout” mice

(8, 9). Several additional proteins that recognize the heptamer or nonamer motifs have been identified, but their role in V(D)J recombination has not yet been proven (10). Recently, it has become evident that ubiquitously expressed cellular proteins may be recruited by specific factors to participate in V(D)J recombination. For example, the gene affected by the severe combined immune deficient (scid) mutations (11) and genes involved in double-strand DNA break repair seem to play an important role during V(D)J recombination (12–14).

We have used the yeast two-hybrid system (15) to identify proteins that interact with RAG-1. One of these corresponds to the human homologue of the yeast protein SRP1. This gene was previously cloned from yeast by the ability of a mutant allele to suppress a mutation of RNA polymerase I. Yeast SRP1 contains a central region with eight degenerate 42-amino acid repeats (16). The human homologue has a similar structure, with eight repeats in the central region of the protein. This 42-amino acid motif, known as an arm motif, was first identified in the *Drosophila* segment polarity gene armadillo (17) and recently has been found in several proteins that have diverse cellular functions (18).

Here we describe the human (h) and mouse (m) homologues of SRP1, we show their interaction with RAG-1 in yeast as well as in mammalian cells, and we map the regions responsible for the interaction in both proteins. The possible role of SRP1 in V(D)J recombination is discussed.

MATERIALS AND METHODS

Cells and Antibodies. 293T cells (19) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin, and streptomycin. Anti-influenza hemagglutinin (HA) monoclonal antibody (12CA-5) was from Berkeley antibody company. Anti-glutathione *S*-transferase (GST) antibodies were generated in rabbits and affinity purified. pcDNA1/amp (Invitrogen), p65/GST, and p50-HA expression vectors will be described elsewhere.

DNA Constructs. A pEG202RAG-1 vector encoding full-length RAG-1 was assembled from a *Bam*HI/*Eco*RV PCR product, an *Eco*RV/*Not*I fragment from RAG-1/CDM8, and a *Not*I/*Bam*HI pEG202 vector (15). Ligation of these three fragments generated pEG202/RAG-1. The joining segments were sequenced and the expression of the fusion product RAG-1-LexA was tested by transformation in yeast followed by Western blotting. pEG202/RAG-1 was used to screen a human cDNA library. All the subcloning of RAG-1 and hSRP1 homologue was done by production of in-frame PCR fragments or by directly subcloning from the cDNA with short oligonucleotides to leave the protein in-frame. The junctions of all constructs were sequenced.

For immunoprecipitation experiments, pcDNA1/amp (Invitrogen) was modified by introducing an oligonucleotide containing an initiation codon in-frame with the HA epitope

and a nuclear localization signal (NLS). *EcoRI/Xho I* sites were present downstream of the NLS to allow for in-frame subcloning of the *EcoRI/Xho I* fragments from the yeast library vector pJG4-5. RAG-1-GST fusion protein was produced by subcloning RAG-1 into pEBG1, a modified form of the vector pEF-BOS (20). The vector pEBG1RAG-1 allows expression of GST fusion proteins by in-frame subcloning into *BamHI/Not I* sites. RAG-1 was also assembled in this vector by a three part ligation, an in-frame *BamHI/EcoRV* PCR product, and an *EcoRV/Not I* fragment from the RAG-1/CDM8 vector.

Two-Hybrid Screening. Screening by the two-hybrid system was performed as described (15, 21). Sequencing of the interacting clones was performed with Sequenase according to the manufacturer's directions (United States Biochemical).

Transfections. 293T cells were seeded at a density of 10⁶ cells per 100-mm dish and grown for 1 day before transfection. Cells were transfected with calcium phosphate as described (19). Briefly, each dish was treated with 1 ml of a DNA calcium phosphate precipitate containing 10 μg of each plasmid DNA. After incubation at 37°C for 24 hr, the medium was replaced and cells were harvested 48 hr posttransfection.

Immunoprecipitation. 293T cells were metabolically labeled with [³⁵S]methionine for 4 hr. Cells were harvested from the plate and pelleted at 1000 × g for 7 min. Supernatant was removed and cells were resuspended in 1 ml of RSB (10 mM Tris-HCl, pH 7.4/10 mM NaCl/5 mM MgCl₂/0.5% Nonidet P-40/protease inhibitors). Lysates were incubated on ice and centrifuged at 1000 × g for 10 min. The cytosolic fraction was removed and the pellet of nucleus was resuspended in 1 ml of LSB (20 mM Tris-HCl, pH 7.4/1 M NaCl/0.2 mM MgCl₂/0.1% Nonidet P-40/protease inhibitors), incubated on ice for 20 min, and spun at 90,000 rpm for

10 min in a Beckman Optima TLX ultracentrifuge. The supernatant was used for immunoprecipitation. Preimmune rabbit serum together with 5 μg of RNase and 10 μg of DNase were added to the 1 M extract and incubated for 2 hr. Protein A-agarose (Boehringer) (40 μl) was then added for 1 hr and centrifuged. The supernatant was used for immunoprecipitation. Either 0.5 μg of rabbit polyclonal affinity-purified GST antibodies or 1.0 μg of monoclonal HA antibody was added to the precleared extracts and incubated for 2 hr; protein G-agarose (Boehringer) was then added. Incubation was continued for 1 hr; beads were recovered by low-speed centrifugation and washed six times. Immunoprecipitates were resuspended in SDS loading buffer and samples were run in a 12.5% polyacrylamide gel and visualized by autoradiography.

RESULTS

Isolation of Proteins Interacting with RAG-1. The yeast two-hybrid system as modified by Brent and colleagues (15, 21) was used to identify proteins encoded in a HeLa cell library that interact with RAG-1. Five million transformants were screened and 500 potential positives from galactose/Leu⁻ plates were patched onto galactose 5-bromo-4-chloro-3-indolyl β-D-galactoside plates where colonies producing β-galactosidase turn blue. Eighteen blue colonies were further analyzed. To confirm the interaction between the HeLa cell protein with RAG-1 and not with LexA, the library plasmid was recovered from yeast DNA by transformation of the bacterial strain KC8 (15). DNA prepared from KC8 was used for sequencing and restriction analysis. The library plasmids were reintroduced with a reporter into yeast along with a plasmid expressing either LexA-RAG-1 or just LexA. The 18 positive clones were found to interact with the LexA-RAG-1

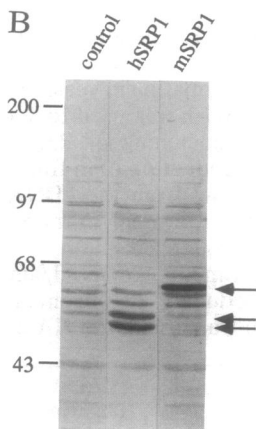
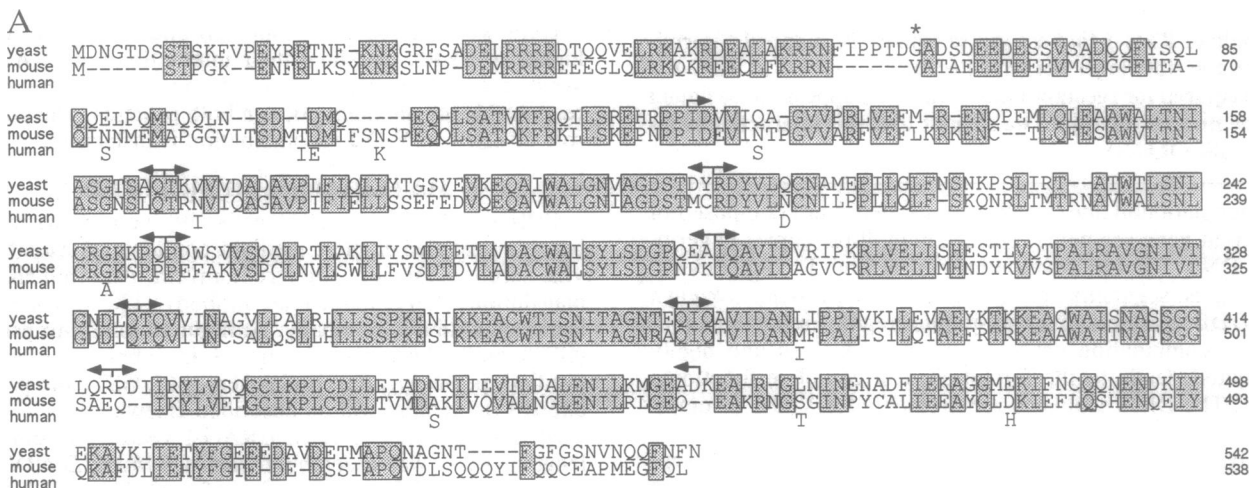


FIG. 1. Sequence and comparison of hSRP1, mSRP1, and yeast SRP1. (A) Alignment of the amino acid sequences. Amino acids conserved in yeast SRP1 and mSRP1 are shown in stippled boxes. Below the mouse sequence is indicated any amino acid in the human sequence that differs from that of the mouse. Asterisk indicates the beginning of the partial hSRP1 clone isolated with the yeast two-hybrid system. Arrows indicate beginning and end of the different repeats of SRP1. (B) Expression of hSRP1 and mSRP1. Nuclear extracts from 293T cells transfected with the indicated cDNAs were separated by electrophoresis through a SDS/12.5% polyacrylamide gel and Western blotted with antibody raised against the partial hSRP1. The hSRP1 cDNA was subcloned into pcDNA1/HAN (hSRP1) and the mSRP1 cDNA into the pcDNA1 (mSRP1). The control extract was transfected with an empty pcDNA1 (control). Arrows indicate proteins immunoreactive with the hSRP1 antibodies. Numbers on left are kDa.

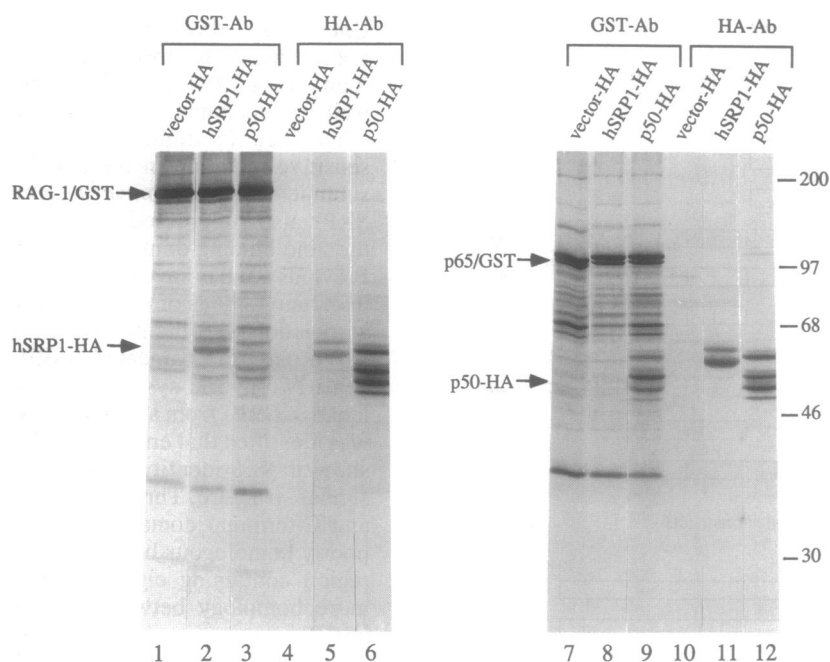


FIG. 2. Interaction of RAG-1 and hSRP1 in mammalian cells. 293T cells transfected with the indicated DNAs were metabolically labeled with [35 S]methionine and nuclear protein extracts were immunoprecipitated with the antibodies indicated at top of the figure, fractionated by SDS/PAGE, and visualized by autoradiography. (Left) Nuclear extracts from cells transfected with RAG-1-GST and either control plasmid vector-HA (lanes 1 and 4), hSRP1-HA (lanes 2 and 5), or p50-HA (lanes 3 and 6). (Right) Nuclear extracts from cells transfected with p65/GST and empty vector-HA (lanes 7 and 10), hSRP1-HA (lanes 8 and 11), or p50-HA (lanes 9 and 12). Arrows identify immunoprecipitated proteins. Migration of molecular mass markers (kDa) is also indicated. GST-Ab, anti-GST antibodies; HA-Ab, anti-HA antibodies.

fusion but not with LexA alone. Dot blot analysis indicated that these 18 clones represented 10 different genes. These genes were subcloned into pcDNA/HAn, a eukaryotic expression vector under the direction of a cytomegalovirus promoter. This vector was modified to express fusion proteins tagged with the influenza virus HA epitope and containing the NLS from simian virus 40 large tumor antigen. The 10 independent cDNAs were cotransfected together with RAG-1-GST into 293T cells, and the interaction of their protein products was analyzed by coimmunoprecipitation with antibodies against the HA epitope. Two of the 10 clones specifically interacted with RAG-1 under the conditions used during the immunoprecipitation (data not shown).

The Human Homologue of Yeast SRP1 Is One of the Proteins That Interact with RAG-1. Sequence analysis of the clones interacting with RAG-1 revealed that one of them corresponded to the human homologue of the yeast nuclear envelope protein SRP1 (16). The hSRP1 cDNA (2.8 kbp) isolated from the HeLa library encoded a 489-amino acid truncated protein (Fig. 1A). This partial human cDNA was used to isolate a 3.9-kbp DNA from a mouse cDNA library (22D6). The sequence analysis of the mouse cDNA showed an open reading frame encoding a protein of 538 amino acids (Fig. 1A), with a predicted molecular mass of 60 kDa. The mouse 3.9-kbp *Not* I fragment was cloned into the *Not* I site of pcDNA I/AMP, a eukaryotic expression vector. Transfection into 293T cells of the vector containing the 3.9-kbp fragment in the correct orientation yielded a protein with an apparent molecular mass of \approx 60 kDa, as determined by Western blotting with rabbit polyclonal antibodies produced against a fusion protein containing the 489 amino acids of the hSRP1 (Fig. 1B).

Transfection of the full-length mSRP1 cDNA had no effect on V(D)J recombination when cotransfected with RAG-1, RAG-2, and pJH288 in 293T, scid, and xrs-6 cells (data not shown). The tissue distribution of mSRP1 transcripts was examined by Northern blot. All mouse tissues analyzed expressed equivalent levels of SRP1 and two mSRP1 mRNA

species of \approx 4.0 and \approx 5.0 kb were consistently observed (data not shown).

RAG-1 Associates with hSRP1 in Mammalian Cells. The above studies showed that RAG-1 interacted with hSRP1 when expressed in yeast. We next investigated whether RAG-1 and hSRP1 could interact in mammalian cells. A DNA construct expressing a RAG-1-GST fusion protein was cotransfected into 293T cells with plasmid DNA encoding the 489-amino acid, truncated hSRP1 tagged with the HA epitope, and a NLS under the direction of a cytomegalovirus promoter. As controls, we used empty vectors or constructs expressing the known interacting proteins NF- κ B p50 and p65 tagged with HA or GST, respectively. We cotransfected a vector encoding RAG-1 or p65 with vectors encoding hSRP1 or p50. Forty-eight hours after transfection, cells were metabolically labeled with [35 S]methionine for 4–5 hr and nuclear extracts were prepared and used in immunoprecipitation reactions (see *Materials and Methods*; Fig. 2). Anti-GST antibody immunoprecipitated hSRP1-HA when this protein was coexpressed with RAG-1-GST (lane 2) but not when coexpressed with p65/GST (lane 8). Conversely, HA antibodies immunoprecipitated RAG-1-GST when cotransfected with hSRP1-HA (lane 5), but not when cotransfected with the unrelated protein p50-HA (lane 6). Under similar conditions, the control p50-HA and p65/GST formed a very stable complex with each other; GST antibody coimmunoprecipitated p65/GST together with p50-HA (lane 9) and HA antibody coimmunoprecipitated p50-HA in a complex with p65/GST (lane 12). These results indicate that RAG-1 and hSRP1 interact specifically in mammalian as well as in yeast cells.

Mapping of SRP1 and RAG-1 Interacting Regions. The regions in hSRP1 and RAG-1 that are involved in SRP1-RAG-1 interaction were mapped by deletion analysis. A detailed deletional analysis of hSRP1 (Fig. 3A) showed that the area of hSRP1 important for its interaction with RAG-1 is located in the region of the protein containing the internal repeats. The data also showed that the N- and C-terminal domains of SRP1 were not essential for its interaction with

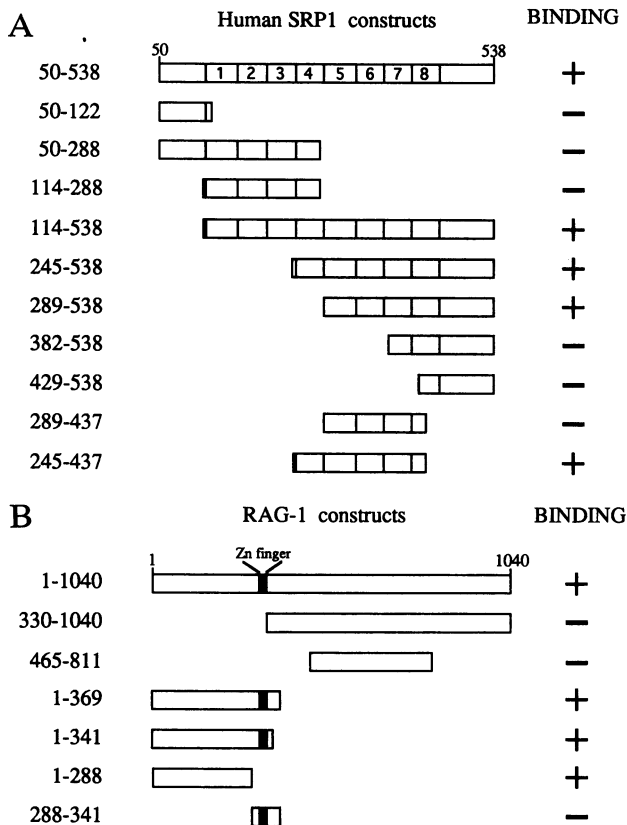


FIG. 3. Mapping the interacting regions between RAG-1 and hSRP1. (A) Mapping the hSRP1 region that interacts with RAG-1. Deletion constructs of hSRP1 were analyzed by their ability to interact with RAG-1 by using the yeast two-hybrid system. Schematic representation of the deletion constructs is shown. SRP1 repeats are indicated (boxes 1–8). Numbers on left indicate amino acid region contained in each construct. Column on right shows the constructs that scored positive (+) or negative (-). (B) Mapping the RAG-1 region that interacts with hSRP1. Deletion constructs of RAG-1 were analyzed by their ability to interact with hSRP1. Schematic representation of the deletion constructs is shown. Zinc finger domain is indicated by a solid bar.

RAG-1. A construct containing only the repeats 4, 5, 6, and 7 (amino acids 245–437) was still able to interact with RAG-1. The additional deletion of the repeat 4 rendered the construct (amino acids 289–437) unable to interact with RAG-1. Interestingly, addition of the C-terminal region gave rise to a construct (amino acids 289–538) with the ability to interact with RAG-1. Constructs containing repeats 7 and 8 and the C-terminal region (amino acids 382–538) or the N-terminal region and repeats 1, 2, 3, and 4 (amino acids 50–288) were unable to interact with RAG-1. These results indicate that a region containing at least four repeats (repeats 4, 5, 6, and 7) is required for the interaction of hSRP1 with RAG-1 and that flanking sequences (the C-terminal region) are capable of influencing this interaction.

A similar analysis of the protein region of RAG-1 involved in the interaction with hSRP1 showed that the N-terminal region of RAG-1 (amino acids 1–288) was sufficient for a stable interaction with hSRP1, while no interaction was observed between RAG-1 constructs containing most of the protein (amino acids 330–1040) but lacking the N-terminal region. Thus, amino acids 1–288, not containing the zinc finger, contain the RAG-1 region responsible for interacting with hSRP1.

DISCUSSION

We used the two-hybrid system modified by Brent and colleagues (15) to identify proteins that interact with RAG-1.

One of the identified genes corresponded to the human homologue of the previously cloned yeast protein SRP1. We found that hSRP1 was able to interact with RAG-1 in yeast as well as in mammalian cells. The yeast SRP1-1 mutation is an allele-specific dominant suppressor of a temperature-sensitive mutation in the zinc binding domain of the A190 subunit of *Saccharomyces cerevisiae* RNA polymerase I. SRP1 also suppressed a temperature-sensitive mutation in the zinc binding domain of the polymerase I A135 subunit. Previous studies have indicated that SRP1 is localized mainly in certain regions of the nuclear periphery, probably in close association with the nuclear envelope (16).

We used the truncated hSRP cDNA obtained from the yeast two-hybrid system to clone a cDNA encoding a full-length mSRP1 from a mouse cDNA library. A 3.9-kbp cDNA was identified that encodes a full-length mSRP1. The mSRP1 showed 48% identity with yeast SRP1 and both shared a similar structure. Three domains could be recognized: the N- and C-terminal domains, which are very hydrophilic ones poorly homologous between yeast and mouse, and a central region containing eight degenerate repeats, which shows more homology between mouse and yeast. Both m- and hSRP1 are very conserved; comparison of both proteins starting from Val-50 in the hSRP1 shows 97% identity. The high conservation between mSRP1, hSRP1, and yeast SRP1 proteins is probably a good indication of the protein's important role in nuclear function. SRP1 was shown to be an essential gene in yeast because disruption of this gene was lethal.

We mapped the regions on hSRP1 and RAG-1 required for their interaction. The minimal interaction region of hSRP1 seems to involve four repeats (repeats 4, 5, 6, and 7 or 5, 6, 7, and 8). This repeat, known as an arm motif and recently found in other proteins, has also been shown to participate in the interaction between the tumor suppressor adenomatous polyposis coli and β -catenin (22, 23). Thus, the suggestion that this repeat is able to mediate specific protein-protein interactions (18) is further supported by our work. The four-repeat region of SRP1 responsible for interacting with RAG-1 is located between amino acids 245 and 437 and is therefore downstream of Pro-219, the amino acid altered in the SRP1-1 suppressor mutant of RNA polymerase I. Genetic evidence suggested that SRP1 and the two subunits A190 and A135 of RNA polymerase I could interact directly through the zinc finger present in both polymerase I subunits, mainly because all the polymerase I temperature-sensitive mutants that were suppressed by SRP1-1 mutant (proline to glutamine at position 219) were located in the zinc finger domain of both polymerase I subunits (16). We thought that perhaps the zinc finger domain of RAG-1 present at the N terminus of RAG-1 protein (between amino acids 290 and 328) might contain the region responsible for interacting with hSRP1. However, deletion analysis of RAG-1 protein showed that the RAG-1 region interacting with hSRP1 is located outside the zinc finger domain, in the N-terminal region of RAG-1 between amino acids 1 and 288. Thus, the interaction between RAG-1 and hSRP1 appears to occur through a different region from that which might have been predicted based on the interaction between polymerase I and SRP1. Since there is no direct evidence for interaction between polymerase I and SRP1, it is possible that they interact through other proteins. Alternatively, the temperature-sensitive mutation in both polymerase I subunits and the SRP1-1 suppressor could alter protein structure and the interaction may occur through different regions of the two proteins (16). The evidence of a link between RAG-1 and hSRP1 and between polymerase I and SRP1 suggests that SRP1 may play an important role in allowing critical nuclear reactions such as recombination and transcription to become localized to the nuclear envelope, a

localization that might bring together all the essential components involved in these complex processes.

The analysis of deletion mutants of RAG-1 clearly showed that RAG-1 interacted with hSRP1 through a region contained between amino acids 1 and 288. However, previous studies indicated that the region of RAG-1 between amino acids 1 and 384 was dispensable for the recombination of exogenous plasmid substrates. Deletion of this complete region decreased by 50% the recombination activity of the protein (24, 25). This suggests that the conditions and requirements for the recombination of endogenous locus versus exogenous substrates may be markedly different.

During recombination of exogenous substrates, the sequences to recombine are present at high concentration and the recombination signal sequences are in very close proximity. The situation is very different during recombination of the endogenous locus, where the recombination signal sequences are kilobases apart from each other. Thus, even when the region of RAG-1 that interacts with hSRP1 appears to have a small effect on the recombination activity of an exogenous substrate, the role of this region in recombination of endogenous genes may be far more relevant and remains to be studied. In the context of endogenous recombination, the interaction of RAG-1 with molecules located in the nuclear envelope could provide the anchoring activity required to localize and perhaps start the formation of a recombination-competent complex.

Important nuclear reactions such as replication and transcription have been suggested and recently shown to occur in specific regions of the nucleus. They seem to localize to the nuclear matrix, forming large complexes known as replication and transcription factories. The factors involved in replication as well as transcription are anchored in a solid matrix and it has been shown that DNA moves through this complex (26, 27). One might envision an analogous situation for V(D)J recombination where recombination of endogenous loci might normally require the association of the recombination machinery to the nuclear envelope, perhaps through interaction of RAG-1 with hSRP1. Localization of both RAG-1 and RAG-2 to the nuclear periphery has been observed in early thymocytes (E. Spanopoulou and D.B., unpublished data).

Note Added in Proof. Since this paper was submitted for publication, an article by Cuomo *et al.* (28) describing a similar observation was published.

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