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rag-1 and rag-2 Are Components of a High-Molecular-Weight Complex, and Association of rag-2 with This Complex Is rag-1 Dependent

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Despite the essential and synergistic functions of the rag-1 and rag-2 proteins in V(D)J recombination and lymphocyte development, little is known about the biochemical properties of the two proteins. We have developed cell lines expressing high levels of the rag proteins and specific, sensitive immunological reagents for their detection, and we have examined the physical properties of the rag proteins in vitro and their subcellular localizations in vivo. rag-1 is tightly associated with nuclear structures, requires a high salt concentration to maintain its solubility, and is a component of large, heterogeneously sized complexes. Furthermore, the presence of rag-1 alters the behavior of rag-2, conferring on it properties similar to those of rag-1 and changing its distribution in the nucleus. We demonstrate that rag-1 and rag-2 are present in the same complex by coimmunoprecipitation, and we provide evidence that these complexes contain more molecules of rag-2 than of rag-1. The demonstration of intracellular complexes containing rag-1 and rag-2 raises the possibility that interaction between these proteins is necessary for their biological function.

The ability of the specific immune system to recognize a vast number of different antigens depends on the generation of a diverse array of immunoglobulin (Ig) and T-cell receptor molecules on B and T cells, respectively. The necessary diversity is created to a large extent by a site-specific recombination reaction known as V(D)J recombination, which assembles the variable regions of Ig and T-cell receptor genes from component variable (V), joining (J), and, in some cases, diversity (D) gene segments. The enzymatic machinery required for V(D)J recombination exists only in developing lymphocytes, and this specificity is due to the lymphocyte-restricted coexpression of two recombination-activating genes, *RAG-1* and *RAG-2*. These genes were isolated by virtue of their ability to confer V(D)J recombination activity on nonlymphoid cells (40, 48, 49). The central role of *RAG-1* and *RAG-2* in lymphoid development was established by gene-targeting experiments which revealed that *RAG-1*- or *RAG-2*-deficient mice are devoid of V(D)J recombination activity, show a complete arrest of B- and T-cell development at the stages corresponding to the earliest V(D)J rearrangements, and hence suffer from severe combined immune deficiency (38, 52).

Studies of V(D)J recombination in cell lines and lymphoid tissues have led to the development of relatively sophisticated models for the reaction (28), but the identities and functions of the components of the enzymatic machinery are only slowly coming to light (2, 4, 26, 55, 56). For example, it remains unknown what factor(s) is responsible for sequence-specific DNA recognition and cleavage of substrate DNA, and it is unclear what roles the rag-1 and rag-2 proteins play in the reaction. Both rag proteins are absolutely required for the earliest detectable step of the reaction (the generation of site-specific DNA breaks [51, 58]), and this evidence and all other evidence (38, 50, 52) are consistent with the idea that the rag proteins function together as the essential lymphocyte-specific components of a V(D)J recombinase enzyme complex. In this regard, it is worth noting that the *RAG-1* and *RAG-2* genes (which lack any sequence similarity to each other) are located within a few kilobases of each other in all species in which they have been characterized (8, 14, 16, 23, 40), with each rag-coding sequence contained in a single exon.

Relatively little has been reported concerning the properties of the rag-1 and rag-2 proteins since the initial description of the genes. Mutagenesis has allowed the definition of minimal regions of the rag proteins required for recombination of exogenous substrates (12, 45, 46, 54), and the yeast two-hybrid system has identified a family of proteins that interact with rag-1 (10, 11). These rag-1-interacting proteins in all likelihood function to transport rag-1 to the nucleus (15). No interaction between rag-1 and rag-2 could be detected with the two-hybrid assay (11), and no proteins that interact with rag-2 have been described. Nothing has yet been reported concerning the biochemical properties of the rag proteins, except for the description of important sites of phosphorylation of rag-2 (32, 33).

We have therefore undertaken a direct physical analysis of the properties of the rag proteins in overexpressing cell lines and in thymus (the richest in vivo source of large numbers of RAG^+ immature lymphocytes). We demonstrate that rag-1 is predominantly found in large, heterogeneously sized complexes, is tightly bound in the nucleus, and requires a high salt concentration to maintain its solubility at physiological pH. Furthermore, the presence of rag-1 substantially affects the behavior of rag-2, conferring on it properties similar to those of rag-1, as well as altering its distribution in the nucleus. These results raise the possibility that the rag proteins are found together in the same complex, and we demonstrate that this is indeed the case by coimmunoprecipitation (co-IP). Additional experiments suggest that the rag-1–rag-2 complex may contain multiple molecules of rag-2 for each molecule of rag-1 and define subpopulations of rag-1 that differ in their affinities for nucleic acids. We hypothesize that complexes containing both

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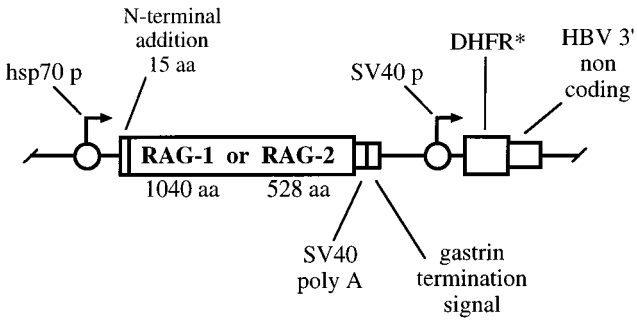

FIG. 1. rag-1 and rag-2 expression constructs used for stable transfection of the B-lymphoma cell line M12. Expression of rag proteins is driven by the *Drosophila* heat shock protein 70 promoter (hsp70 p). The late polyadenylation signal from simian virus 40 (SV40 poly A) and the gastrin termination signal were inserted for transcriptional termination. The murine rag-1 and rag-2 proteins carry an N-terminal addition of 15 aa consisting of a 4-aa epitope tag recognized by the M2 FLAG monoclonal antibody, a 6-aa histidine affinity tag, and a 5-aa enterokinase cleavage site. For selection of integration and subsequent amplification, an expression cassette providing MTX resistance was added. The expression of the mutant DHFR gene (DHFR*) is driven by the SV40 early promoter (SV40 p). The poly(A) signal is provided by the 3′ noncoding region of hepatitis B virus (HBV 3′ noncoding). Shown are pDGS464 (*RAG-1*) and pDGS466 (*RAG-2*) with their *RAG* and DHFR* cassettes in the head-to-tail orientation.

rag proteins are likely to be the active entity in catalyzing V(D)J recombination. If this is the case, then co-IP becomes the first in vitro assay for rag protein function, and our data suggest a method by which this complex might be purified.

MATERIALS AND METHODS

Expression constructs. The coding regions of the murine *RAG-1* and *RAG-2* cDNAs, derived from cDNA clones M2 (region equivalent to nucleotides 95 to 3382 of cDNA clone M6 [49]) and MR2-1 (nucleotides 156 to 1365 [40]), respectively, were modified to contain consensus Kozak translation initiation sequences and inserted into the *Bam*HI-*Sal*I sites of pBB70 (a generous gift from S. Lindquist), downstream of the *Drosophila* heat shock protein 70 promoter, to generate pDGS452 and pDGS453, respectively. Further modifications, starting with pDGS452 and pDGS453, were carried out in parallel, and the names of the corresponding rag-2 plasmids are given below in brackets.

The N-terminal addition to the rag-coding sequences (Fig. 1) was inserted by annealing and ligating the oligonucleotides 5′GATCCACCATGGACTACAAAG ATCATCACCACCATCATCACGATGATGATGACAAG and 5′CTTGTCAT CATCATCGTGATGATGGTGGTGATGATCTTTGTATGTCCATGGTG into the *Nco*I (mung bean nuclease blunted)-*Bam*HI sites of pDGS452 [pDGS453] to yield pDGS455 [pDGS456]. The gastrin termination signal (47) was generated by annealing and ligating the oligonucleotides 5′TCGAGTTAACTTTTTTTTTA ATTTTTATTTTATTTTATTTTTAG and 5′TCGACTAAAAATAAAATA AAATAAAAATTAAAAAAAAAGTTAAC into the *Sal*I site of pDGS455 [pDGS456], such that the inserted sequence was oriented with the *Hpa*I site proximal to the rag-coding sequence, to yield pDGS458 [pDGS459]. A polyadenylation signal was inserted by ligating the 241-bp *Bam*HI-*Bcl*I fragment of simian virus 40 (strain 776; Sigma catalog no. D6643) into the *Hpa*I site of pDGS458 [pDGS459] to yield pDGS461 [pDGS462]. The mutant dihydrofolate reductase (DHFR*) expression cassette from pNUT (a generous gift from R. Palmiter) (5, 42) was inserted as a *Sal*I-*Asp*718 (filled in with Klenow) fragment into the *Sma*I site of pDGS461 [pDGS462] to yield pDGS464 [pDGS466] and pDGS465 [pDGS467]. In pDGS464 [pDGS466] the rag and DHFR* expression cassettes are in the head-to-tail transcriptional orientation (Fig. 1), while in pDGS465 [pDGS467] the cassettes are in the head-to-head orientation. The complete sequences of pBB70 and pDGS452 to pDGS467 are available upon request.

Generation of stably transfected cell lines. Five-microgram portions of *Not*I-linearized expression plasmids pDGS464 to pDGS467, in various combinations, were introduced by electroporation into 5×10^6 M12 cells in 0.4 ml of phosphate-buffered saline (PBS) in 0.4-cm-gap cuvettes (Bio-Rad) by using a Bio-Rad Gene Pulser (200 V, 500 μF; time constant τ, 20 to 25 ms). After 2 days, cells were plated at various densities in 96-well plates in medium containing 100 nM methotrexate (MTX), and single-cell clones were isolated after 3 weeks of selection and expanded. Levels of expression of *RAG-1* and *RAG-2* were determined by Northern (RNA) blotting, and various clones with the highest levels of *RAG* expression were plated in 1 μM MTX in 96-well plates to select for cells with increased levels of DHFR* expression. Single-cell clones surviving the first round of amplification were isolated after 2 weeks and expanded, and *RAG* mRNA levels were again determined by Northern analysis. A second round of amplification, in the presence of 10 μM MTX, was followed by Northern blot and Western blot (immunoblot) analyses to determine the levels of rag-1 and rag-2 expression. A third and last step of amplification was performed with 100 μM MTX. The first round of amplification resulted in a 10- to 100-fold increase in the levels of *RAG-1* and *RAG-2* mRNA expression, and the second round resulted in an additional 10-fold increase. No significant increase in the levels of rag-1 and rag-2 protein expression was observed after the third round of amplification. The clonal cell lines Sr1, Sr2, and Dr3 were derived from the third round of amplification (100 μM MTX) and were thereafter maintained in 10 μM MTX. The cell lines were derived from cells transfected with the following constructs: Sr1 with pDGS464, Sr2 with pDGS466, and Dr3 with both pDGS465 and pDGS467. No systematic difference in the levels of rag-1 and rag-2 expression was observed between plasmids with head-to-tail (pDGS464 or pDGS466) and those with head-to-head (pDGS465 or pDGS467) arrangements of the expression cassettes.

αrag-1 and αrag-2 antibodies. Amino acids (aa) 56 to 123 of murine rag-1 or aa 70 to 516 of murine rag-2 were expressed in bacteria as fusion proteins with a mutant *Pseudomonas* exotoxin (6), and the fusion proteins were used to immunize rabbits. The anti-rag-1 (αrag-1) and αrag-2 antibodies were affinity purified by using bacterially expressed rag polypeptides, expressed as fusion proteins with maltose-binding protein (pMAL; New England Biolabs). The concentration of the affinity-purified antibodies was determined by measuring the optical density at 280 nm, using a conversion factor of an optical density at 280 nm of 1.40 equals 1 mg/ml. The purity of the purified immunoglobulin was confirmed by sodium dodecyl sulfate-polyacrylamide gel electropheresis (SDS-PAGE) followed by silver staining. The generation and affinity purification of the polyclonal αrag-1 and αrag-2 antibodies will be described in detail elsewhere (27).

Cell culture and heat shock induction. Wild-type M12 cells were grown at 37°C in 5% CO_2 in RPMI 1640 (Gibco-BRL)–8% heat inactivated newborn calf serum–50 μM β-mercaptoethanol (β-ME)–100 U of penicillin per ml–100 μg of streptomycin sulfate per ml. MTX-resistant cell lines were grown in a similar medium except that the serum was dialyzed and MTX (Calbiochem) was added as indicated.

For induction of the rag-1 or rag-2 protein, cells from cultures with a density of 0.5×10^6 to 1.0×10^6 cells per ml were centrifuged (the supernatant was saved for the recovery period [see below]), and the cell pellet was resuspended in medium to a final volume of 0.5 ml, transferred to a 1.5-ml Eppendorf tube, and heat shocked either at 43°C for 30 min or at 45°C for 6 min in a water bath. The cells were then diluted back into the original medium and allowed to recover for 5 to 6 h or as indicated.

In vivo V(D)J recombination assay. Cells were transiently transfected either by a standard DEAE-dextran transfection method (cell lines Mx1A and Mx1B) (22, 31) or by electroporation (cell line Dr3) as follows. Cells (0.5×10^6) were washed once in 0.2 ml of HBS^- (30 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.5], 140 mM NaCl, 5 mM KCl), resuspended in 20 μl of HBS^- containing 1 μg of p12x23 (29), transferred to a 0.1-cm-gap cuvette (Bio-Rad), and electroporated with a Bio-Rad Gene Pulser (1,000 Ω 250 μF, and 100 V; time constant τ, ca. 17 ms). After a 10-min incubation, the cells were transferred into tissue culture medium.

The V(D)J recombination assay was performed as described earlier (29, 40). Briefly, cells were transiently transfected with either an inversional (p12x23) or a deletional (31) recombination substrate. The next day, if applicable, the cells were heat shock induced, and 1 day later the recombination substrates were harvested by rapid alkaline lysis and transformed into bacteria, which were then selected for either Amp^r or Amp^r Cam^r. The percent recombination was calculated as 100 times the number of Amp^r Cam^r colonies divided by the number of Amp^r colonies. The harvested recombination substrates were not *Dpn*I digested (a procedure typically used to destroy those plasmid molecules that have not replicated in the mammalian cell and therefore were not available for recombination [22]) prior to transformation into bacteria, and thus the actual percent recombination may be higher than that reported in Table 1.

Immunocytochemistry. To improve cell adherence to the glass slides, the slides were cleaned for 5 min in 3.7% HCl, rinsed with deionized water and then with 95% ethanol, allowed to air dry, dipped for 2 min in a freshly prepared solution of 2% 3-aminopropyltriethoxysilane (Sigma) in acetone, allowed to air dry, rinsed in deionized water followed by 95% ethanol, and allowed to air dry.

When indicated (see Results), cultured cells were heat shock induced (45°C, 6 min) and allowed to recover for 5 h. The cells were washed in ice-cold PBS and adjusted to 0.25×10^6 cells per ml. Thymocyte suspensions from 3-week-old C57BL/6 mice were washed in ice-cold PBS and adjusted to 10^6 cells per ml. Cell suspensions (0.2 ml) were cytospun (Shandon; Cytospin 2) onto glass slides. Samples were fixed in 3.7% formaldehyde in PBS for 20 min, rinsed in PBS, blocked in $NPBS^+$ (PBS containing 0.1% Nonidet P-40, 2% nonfat dry milk, 5% bovine serum, and 0.02% sodium azide) for 30 min, incubated with the αrag antibody in $NPBS^+$ at 2 μg/ml overnight, washed with NPBS (PBS containing 0.1% Nonidet P-40), incubated with a fluorescein isothiocyanate-labeled goat anti-rabbit antibody in $NPBS^+$ at 7.5 μg/ml for 2 h, washed with NPBS, counterstained with propidium iodide (PI) in PBS at 0.8 μg/ml for 5 min, rinsed with PBS, and mounted. Photographs were taken under either 496-nm (for fluores-

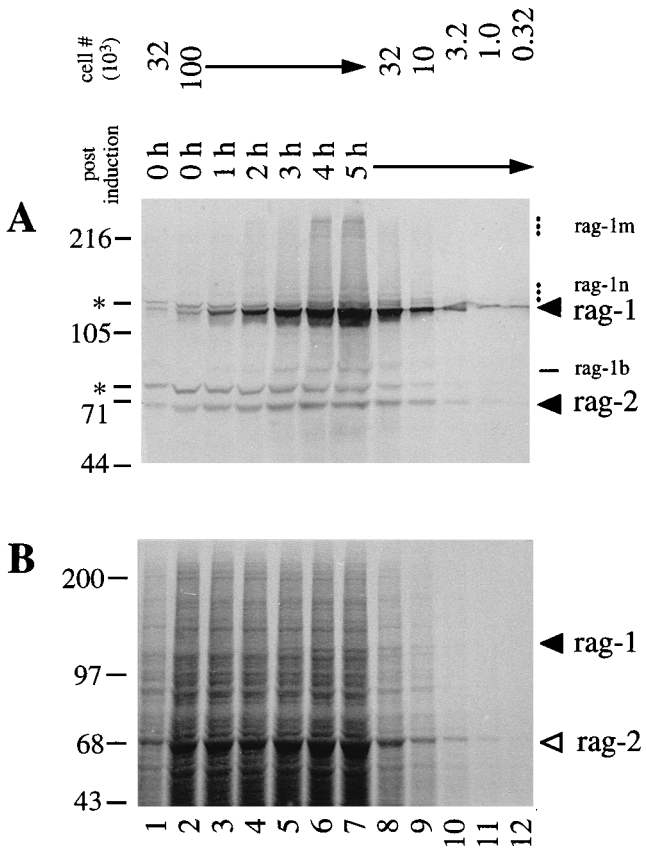

FIG. 2. Time course of induction of rag-1 and rag-2 in the double-expresser cell line Dr3. Cells were harvested at different time points after heat shock induction as indicated. Total-cell lysates were separated on an SDS–6% polyacrylamide gel and analyzed by Western blot analysis (A) and Coomassie blue staining (B). Lanes 8 to 12 represent successive threefold dilutions of lane 7, while lane 1 is a threefold dilution of lane 2. The molecular masses of marker proteins run in the same gels are indicated at the left (in kilodaltons). rag-1 was found to have an apparent molecular mass of approximately 125 kDa (calculated, 120 kDa), and rag-2 was found to have an apparent molecular mass of approximately 65 kDa (calculated, 60 kDa). rag-1b (approximately 85 kDa) is a predominant rag-1 breakdown product. rag-1m (several bands above 200 kDa) and rag-1n (a ladder of three to five bands between approximately 125 and 160 kDa) are higher-molecular-mass forms of rag-1. The two bands marked with asterisks are due to reactivity of cellular proteins with the biotin-avidin complex used as a third layer in the Western blot analysis (data not shown). Induced rag-1 is visible by Coomassie blue staining in lanes 5 to 8, whereas the rag-2 signal (open triangle) is masked by comigrating proteins.

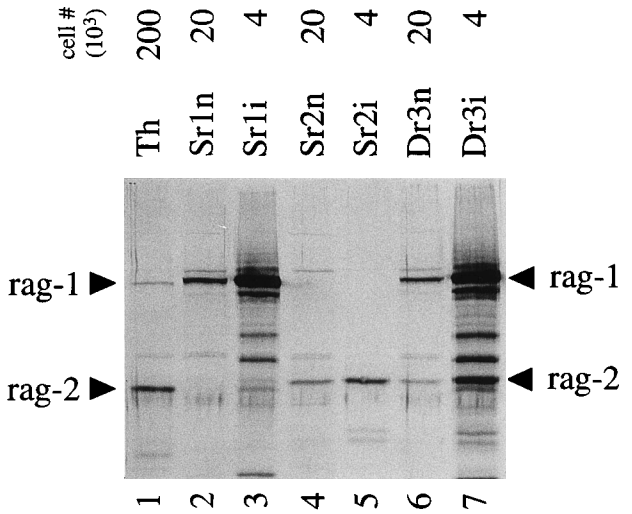

FIG. 3. Levels of rag protein expression in thymus and rag-expressing cell lines. Total-cell lysates were separated on an SDS–6.5% polyacrylamide gel and subjected to Western blot analysis. The following samples were compared: thymocytes from 21-day-old C57BL/6 mice (Th), rag-1-single-expresser cell line Sr1 without (Sr1n) and with (Sr1i) induction, rag-2-single-expresser Sr2 without (Sr2n) and with (Sr2i) induction, and double-expresser Dr3 without (Dr3n) and with (Dr3i) induction. All induced cells were allowed to recover for 5 h after the induction. The numbers of cell equivalents loaded in each lane are indicated in thousands. The rag proteins from the transfected cell lines are slightly larger than those from thymus because of the 15-aa N-terminal addition described in the legend to Fig. 1. Populations of cells were obtained and split in two portions, one of which was used in this experiment and the other of which was used for the experiment shown in Fig. 4.

cein isothiocyanate) or 554-nm (for PI) light, using Kodak TMY 135 black-and-white print film.

Western blot analysis. SDS-PAGE was performed with the Bio-Rad Mini-Protean II system. Gels were stained with either Coomassie blue or Silver Stain Plus (Bio-Rad). Proteins were transferred by electroblotting (Bio-Rad Mini Trans-Blot) from gels to polyvinylidene difluoride membranes according to the manufacturer's instructions with the following modifications to the transfer for improved efficiency of transfer of rag-1: the transfer was set up without equilibrating the gels, and electrophoresis buffer without SDS was used as the transfer buffer (25 mM Tris base plus 192 mM glycine, without pH adjustment). The membranes were blocked in $TTBS^+$ (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20, 3% nonfat dry milk) for 30 min, incubated with αrag-1 and αrag-2 antibodies in $TTBS^+$ at 2 μg/ml overnight, and developed by using a Vectastain ABC-AP system (Vector), with signals visualized with the phosphatase substrate 5-bromo-4-chloro-3 indolylphosphate/nitroblue tetrazolium (BCIP/NBT).

Preparation of cell extracts. Tissue culture cells were heat shock induced, allowed to recover for 6 h, swollen in hypotonic buffer (10 mM HEPES [pH 8.0], 2 mM β-ME), and frozen in liquid nitrogen as pellets. Extracts were maintained throughout either on ice or at 4°C. Buffer A (20 mM HEPES [pH 8.0], 150 mM NaCl, 1 μM leupeptin, 1 μM pepstatin, 1 μM bestatin) was added to 150×10^6 cells and a total volume of 1.5 ml. Cells were Dounce homogenized with 15 strokes with a tight-fitting pestle, lysates were centrifuged at $166{,}000 \times g$ for 10 min, the supernatant was saved, and the pellet was resuspended in buffer A to a total volume of 1.5 ml with Dounce homogenization. The salt concentration was brought to 2 M by adding 1 ml of 5 M NaCl, and the viscous samples were incubated for 25 min on ice and further homogenized with 10 strokes in the Dounce homogenizer. The high-salt lysates were centrifuged at $166{,}000 \times g$ for 10 minutes, and the supernatant was saved; to the pellet, buffer A and 5 M NaCl were added to a total volume of 1.5 ml and a final salt concentration of 2 M NaCl. Ultrasonication with a microtip (Branson; Sonifier 250) was used to resuspend the pellet until the sample viscosity was low. Insoluble matter was pelleted at $166{,}000 \times g$ for 10 min, the supernatant was saved, and the pellet was resuspended for analysis.

IP. Antibodies used in IP experiments were coupled to CNBr-activated Bio-Gel A-15m (Bio-Rad) agarose beads (20) to the following amounts of antibody per ml of resin: 1.6 mg of nonspecific rabbit IgG, 1.2 mg of αrag-1, and 0.8 mg of αrag-2. The temperature of the samples throughout the IP was kept below 4°C, since in reactions performed at room temperature the efficiency of co-IP was significantly reduced (data not shown). High-salt extracts were prepared at concentrations of 100 mg of tissue or 50×10^6 cells per ml in buffer A1 (50 mM Tris-HCl [pH 7.2], 1% Nonidet P-40, 0.5% deoxycholic acid, 0.01% SDS, 1 M

TABLE 1. Levels of V(D)J recombination in rag-1–rag-2-double-expresser cell lines

Cell line[a]	Induction[b]	Substrate[c]	% Recombination[d]
Mx1A	n	pJH200	13.5, 14.1
	i	pJH200	12.5, 21.2
Mx1B	n	pJH200	10.2, 10.8
	i	pJH200	15.6, 18.7
Dr3	n	p12 × 23	11.8

[a] Dr3 (10 μM MTX) was derived from Mx1A (1 μM MTX). Mx1A and Mx1B (1 μM MTX) were derived from the same parental cell line (100 nM MTX).
[b] Cells were processed without (n) or with (i) heat shock induction.
[c] p12 × 23 is an inversion substrate, and pJH200 is a deletion substrate.
[d] Calculated as described in Materials and Methods. In all cases a minimum of 800 Amp^r Cam^r colonies was obtained. Wild-type M12 cells, not expressing rag-1 and rag-2, had a recombination frequency of less than 0.06%.

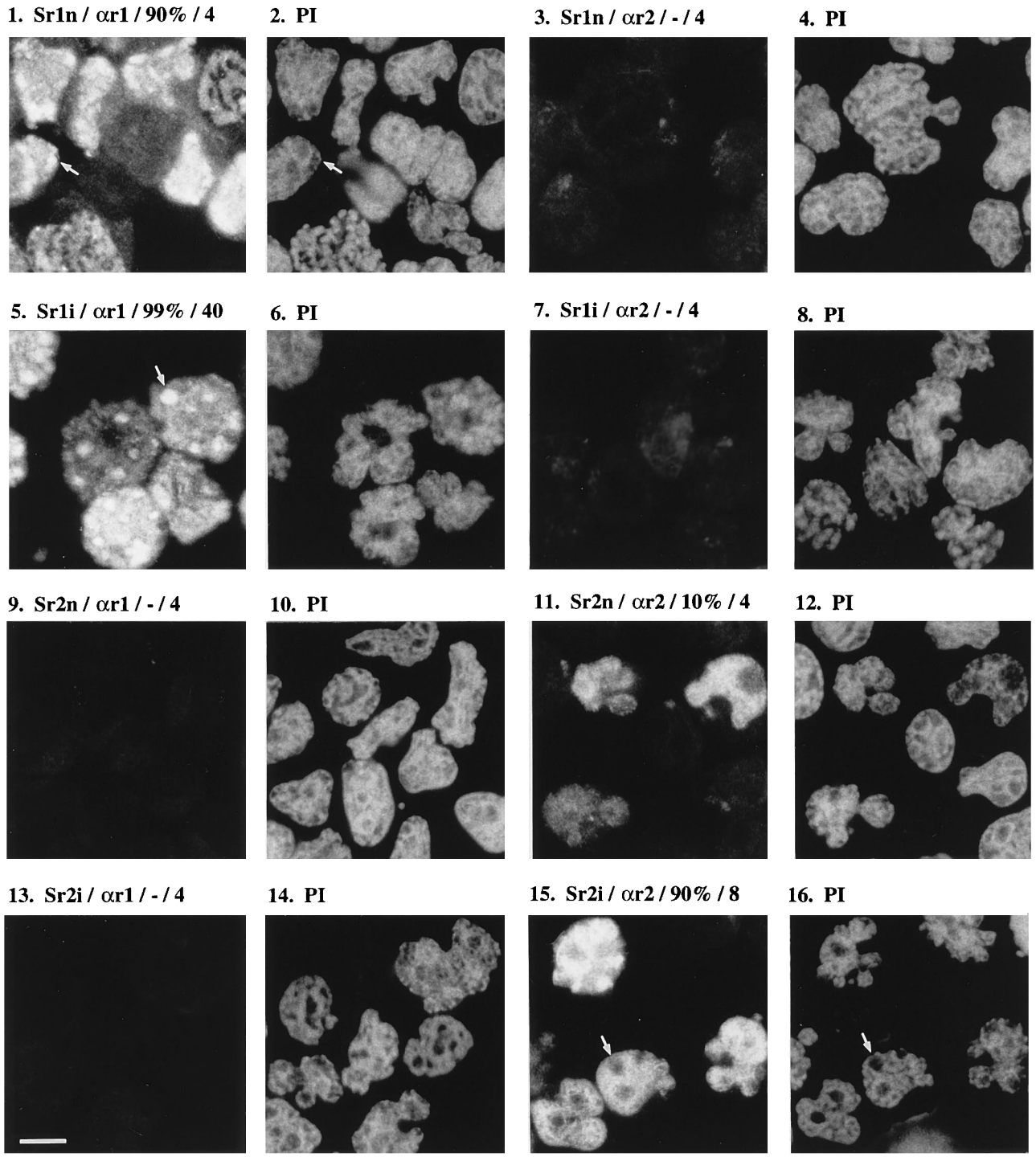

FIG. 4. Immunofluorescence labeling of rag-1 and rag-2. The cells analyzed were derived from identical samples as described for Fig. 3. Cells were stained with either αrag-1 antibodies (αr1) (panels 1, 5, 9, 13, 17, 21, and 29), αrag-2 antibodies (αr2) (panels 3, 7, 11, 15, 19, 23, and 31), or rabbit IgG (panels 25 and 27). The approximate percentage of positively staining cells is indicated above each panel. Because of significant differences in the intensities of the fluorescent signals (most pronounced in rag-1-expressing cells without [panels 1 and 17] and with [panels 5 and 21] induction), the photographs for some panels were intentionally underexposed, with the factor of underexposure indicated as the last number above the panels. PI staining of the matching areas is shown to the right of each fluorescence photograph (even numbers, panels 2 to 32). Bar in panel 13, 10 μm. Abbreviations for cell lines are as described for Fig. 3.

NaCl) by ultrasonication until samples were of low viscosity, and the extracts were then centrifuged at 38,000 × *g* for 15 min. The supernatants, prior to their use for IP, were either precleared with 30 μl of rabbit IgG-beads per ml for 1 h or adjusted to 1.2 M NaCl and 0.5% polyethylenimine (PEI) to precipitate nucleic acids and then incubated for 5 min and centrifugated at 14,000 × *g* for 5 min. The precleared or PEI-treated extracts were split into samples equivalent to 30 mg of tissue, 15×10^6 cells (Sr1n, Sr2n, and Dr3n cells), or 1.5×10^6 cells (Dr3i cells), the volume was adjusted to 350 μl with buffer A1, and 10 μl of the

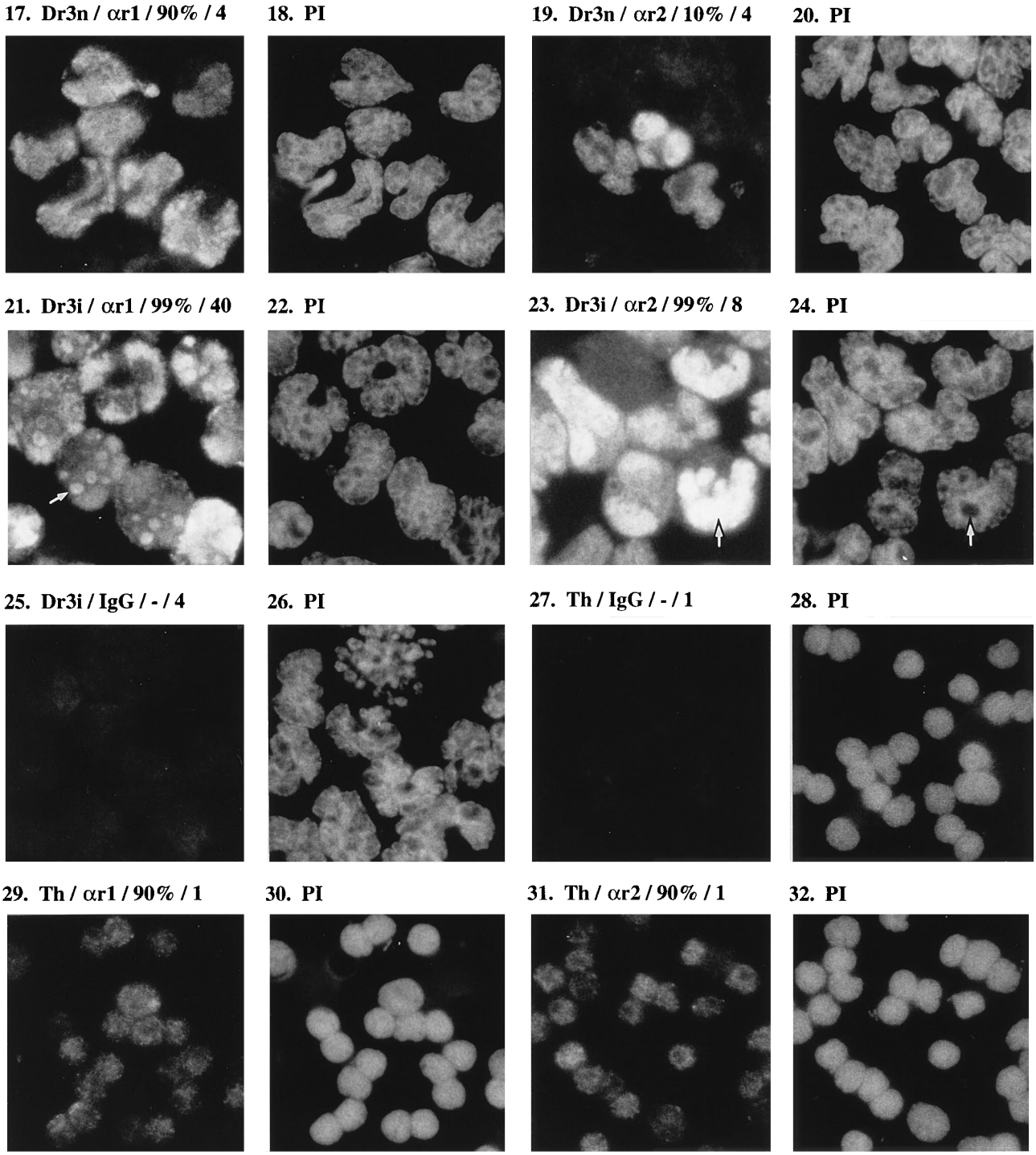

FIG. 4—*Continued*.

corresponding antibody-agarose beads was added. After a 3-h incubation with end-over-end mixing, the beads were washed alternately with buffers A1 and A0 (same as A1 but without NaCl) for a total of six washes; this was followed by elution with sample buffer.

RESULTS

Cell lines stably expressing high levels of biologically active rag-1 and rag-2. To study the murine rag-1 and rag-2 proteins in a context similar to their normal physiological environment, expression vectors (Fig. 1) containing the *RAG* cDNAs were stably transfected into the murine B-lymphoma cell line M12 (25). The vectors provide for inducible rag expression from the *Drosophila* heat shock protein 70 promoter and contain a mutant DHFR (DHFR*) expression cassette for selection of transfected cells in MTX. Heat shock-inducible expression of the rag proteins in M12 cells has been reported previously (41).

M12 cells stably transfected with *RAG-1*, *RAG-2*, or both expression vectors were selected initially in 100 nM MTX, and selection for clones with increased levels of DHFR* expression (and hence, presumably, amplification of the expression vectors) was achieved with successive rounds of selection in increasing concentrations of MTX (see Materials and Methods).

The double-expresser cell line Dr3 showed substantial levels of rag-1 and rag-2 expression prior to induction (Fig. 2A, lane 2 [and compare with thymus in Fig. 3, lanes 1 and 6]). After heat shock induction and recovery at 37°C, rag protein levels increased steadily for 5 h (Fig. 2A, lanes 2 to 7) and appeared to level off between 5 and 6 h postinduction (data not shown). Levels of inducibility, judged by serial dilution of the 5-h-postinduction extract (Fig. 2A, lanes 7 to 12), were estimated to be 50- to 100-fold for rag-1 and 5- to 10-fold for rag-2. On the basis of the signal intensity of rag-1 obtained by Coomassie blue staining (Fig. 2B, lane 7) in comparison with molecular weight standards (not shown), the rag-1 protein was estimated to be expressed at levels of ca. 5 mg per 10^9 induced cells, or, considering a molecular mass of 120 kDa for rag-1, induced cells were estimated to contain an average of 2.5×10^7 rag-1 molecules (corresponding to 2.5×10^5 to 5.0×10^5 molecules per uninduced cell). The levels of expression for rag-2 could not be estimated because rag-2 could not be identified by Coomassie blue staining.

In the single-rag-expressing cell lines Sr1 and Sr2, the levels of expression and fold inducibility were similar to those found in Dr3 (Fig. 3). Uninduced Dr3 cells were estimated to contain 30- to 50-fold more rag-1 and 3- to 5-fold more rag-2 than thymocytes on a per-cell basis (note, however, that a Dr3 cell is roughly 5- to 10-fold larger in volume than a thymocyte [see Fig. 4]). This suggests that the rag-1/rag-2 ratio is 10-fold higher in uninduced Dr3 cells than in thymocytes and (depending on the experiment) becomes 20- to 100-fold higher in induced DR3 cells than in thymocytes.

To confirm that the expressed rag proteins were biologically active, V(D)J recombination substrates were transiently transfected into rag-1–rag-2-double-expresser cell lines that subsequently either were or were not heat shock induced. The lines assayed included two clones isolated after selection in 1 μM MTX (Mx1A and Mx1B) and Dr3 (which was derived from Mx1A). Recombination frequencies were measured by isolating substrate molecules 48 h after transfection and introducing them into bacteria, where properly rearranged molecules confer resistance to chloramphenicol (see Materials and Methods). All lines yielded very high levels of V(D)J recombination, with recombination frequencies consistently above 10% (Table 1). These values were more than 100-fold above the background level observed with the M12 parental cell line (recombination frequency, <0.06%). They are also substantially higher than the levels observed in similar assays of a wide variety of cell lines expressing endogenous rag-1 and rag-2 (9, 31) and are higher than the levels obtained with other rag expression systems (12, 31, 41, 46, 54). Heat shock induction 24 h after transfection resulted in only a variable and small (less than 2-fold) increase in recombination, substantially lower than the 5- to 100-fold increase seen for rag protein expression (Fig. 2A). This might be explained by one or more of the following factors: the relative timing of substrate transfection and heat shock induction may not be optimal, the rag proteins expressed after heat shock induction may have a lower specific activity than the constitutively expressed proteins, and with high levels of the rag proteins, the rate of recombination may become limited by the availability of some other essential factor. The results make it clear, however, that the rag proteins expressed from these vectors are active in performing V(D)J recombination.

Localization of the rag-1 and rag-2 proteins. To determine the subcellular localization of the rag proteins, cytospun cells were stained for rag-1 or rag-2 by immunofluorescence labeling with affinity-purified polyclonal antisera raised to portions of the rag proteins (Fig. 4). PI was used as a nuclear counterstain (primarily detecting DNA) in all samples (Fig. 4, even-numbered panels). Both rag-1 and rag-2 were found to localize predominantly to the nucleus in uninduced transfected cells and in thymocytes, consistent with previously reported results (13, 54). Heat shock induction resulted in an increase in staining intensity, as expected (note that the photographs of induced cells have been deliberately underexposed compared with those of uninduced cells as indicated above each panel in Fig. 4), and in an increase in the amount of rag protein detected in the cytoplasm (compare the immunofluorescence and PI staining profiles in Fig. 4, panels 1, 2, 5, and 6, panels 11, 12, 15, and 16, and panels 17 to 24).

The staining profiles for rag-1 and rag-2 were dissimilar in at least one regard. In both Sr1 and Dr3 cells, rag-1 partially colocalized with regions of the nucleus that stained poorly with PI (arrows in Fig. 4, panels 1 and 2). This was most obvious after heat shock induction, with these cells exhibiting circularly shaped regions that stain intensely with the αrag-1 antibody but poorly with PI ("PI holes"; arrows in panels 5 and 21). In contrast, in both the uninduced and induced rag-2-single-expresser Sr2, PI holes corresponded to areas poorly stained with the αrag-2 antibody (arrows in panels 15 and 16). Interestingly, when coexpressed with rag-1 in Dr3 cells, rag-2 was found at substantial levels in areas corresponding to the PI holes, resulting in a pattern of more evenly stained nuclei (most clearly seen after heat shock induction [arrows in panels 23 and 24]). Thus, rag-2 appears to be relocalized in the presence of rag-1. While this might be an artifact of the cell lines in combination with the effects of heat shock treatment, data presented below support the alternative explanation that the abundant rag-1 protein in these areas serves to relocalize a portion of the abundant rag-2 protein by virtue of direct or indirect interactions between the two rag proteins.

The specificities of the αrag-1 and αrag-2 antibodies were confirmed by the very faint signals obtained when rag-1-single-expresser Sr1 cells were stained with αrag-2 antibodies (Fig. 4, panels 3 and 7) and when the rag-2-single-expresser Sr2 cells were stained with αrag-1 antibodies (panels 9 and 13). The use of purified rabbit IgG as a first antibody gave similarly low signals, both in double-expresser Dr3 cells and with thymocytes (panels 25 and 27).

The proportion of cells expressing rag-1 was estimated to be 90% for both Sr1 and Dr3, and upon induction it increased to approximately 99% in both cases. rag-2 was found to be expressed at high levels in only approximately 10% of the cells for both Sr2 and Dr3. Upon induction, the proportion of rag-2-expressing cells increased to an estimated 90% of Sr2 cells and 99% of Dr3 cells. In thymus cells, signals for both rag-1 and rag-2 were found in an estimated 90% of the cells, consistent with the large fraction of thymocytes that are actively carrying out V(D)J recombination (3, 43).

rag-2 is extracted more readily from cells in the absence of rag-1. Both rag proteins were difficult to extract efficiently from induced double-expresser Dr3 cells, with only a small fraction of the proteins present in a 150 mM NaCl cytoplasmic extract, as expected, and less than half of the protein extracted by subsequent Dounce homogenization in the presence of 2 M NaCl (Fig. 5A, lanes 2 and 3). Additional extraction with 2 M NaCl with extensive ultrasonication released more rag-1 and

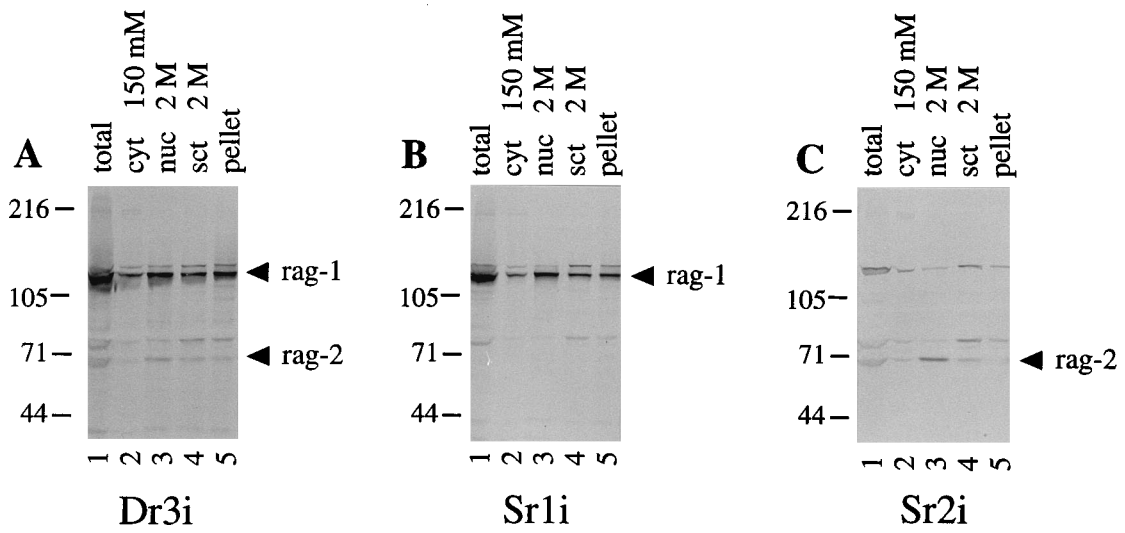

FIG. 5. Extraction of rag proteins from induced expresser cell lines, monitored by Western blot analysis. Double-expresser Dr3 cells (A), rag-1-single-expresser Sr1 cells (B), and rag-2-single-expresser Sr2 cells (C) were induced and allowed to recover for 6 h. The following cellular fractions, obtained by consecutive extraction, were separated on an SDS–6% polyacrylamide gel: total-cell lysates (total), 150 mM NaCl cytoplasmic extract (cyt 150 mM), 2 M NaCl nuclear extract (nuc 2 M), reextraction of pelleted nuclear material in the presence of 2 M NaCl combined with ultrasonic treatment (sct 2 M), and the insoluble debris (pellet). Each lane contains the equivalent of 10^5 cells. Extraction of noninduced cells gave a very similar distribution of the rag proteins in the various fractions (data not shown). Numbers on the left of each panel are molecular masses in kilodaltons.

rag-2 proteins, but significant portions of both proteins remained in the insoluble pellet (Fig. 5A, lanes 4 and 5). Resistance to high-salt extraction is a feature characteristic of nuclear matrix proteins such as lamin or topoisomerase II (24, 34), and the results suggest that at least a portion of the rag proteins in these cells is tightly bound to nuclear structures.

rag-1 from single-expresser Sr1 cells showed a distribution throughout the fractions that was almost identical to that seen with Dr3 cells (compare Fig. 5A and B). In contrast, the bulk of rag-2 from single-expresser Sr2 cells was released from the nuclei in the first high-salt fraction, and almost no rag-2 was retained in the insoluble nuclear debris (Fig. 5C, lanes 3 and 5). The presence of rag-1 therefore appears to alter the extraction properties of rag-2, and again a plausible explanation for this observation is a direct or indirect interaction of the rag-1 and rag-2 proteins, in which the tighter retention of rag-1 in the nucleus is imposed upon rag-2.

These results raised the possibility that rag-1 might be poorly soluble at low salt concentrations in crude extracts, and this was confirmed in experiments that tested the solubility of the rag-1 protein at 100 and 600 mM NaCl as a function of pH (Fig. 6). When high-salt-extracted rag-1 (Fig. 6, lane 1) was diluted to 100 mM NaCl at low or physiological pH, rag-1 was efficiently pelleted by centrifugation at 14,000 $\times$ *g* (material remaining in the supernatant is shown in lanes 9 to 12). An increase of either the pH to 11 or the salt concentration to 600 mM significantly improved the solubility of rag-1. The solubility of rag-2 from Sr2 cells has not been examined in this manner, but 600 mM NaCl is sufficient to maintain its solubility (Fig. 5C, lane 3). Because of these observations, subsequent experiments with the rag proteins were performed in the presence of at least 600 mM NaCl or strong ionic detergents.

In other experiments, we have found that the extraction and solubility properties of the rag proteins are similar in thymus and uninduced cells (data not shown). This suggests that the results shown in Fig. 5 and 6 are not artifacts of the overexpression system used.

Glycerol gradient fractionation experiments support the idea of an interaction between rag-1 and rag-2. Ultracentrifugation of high-salt nuclear extracts pelleted significant quantities of rag-1 protein, and size exclusion chromatography of extracts resulted in severe quantitative losses of both rag proteins and suggested that the proteins were in aggregates or complexes with significant size heterogeneity (data not shown). To examine this further, nuclear or total-cell extracts containing the rag proteins were analyzed on high-salt glycerol density gradients. On high-salt gradients containing 2% SDS and 1.5% β-ME, rag-1 (120 kDa) cofractionated primarily with the 120- and 135-kDa subunits (apparent molecular mass) of the marker protein thyroglobulin (Fig. 7A, lane 4). rag-2 (60 kDa) was found in fractions containing the Ig γ heavy chain (ca. 55 kDa) or the thyroglobulin subunits (Fig. 7A, lanes 3 and 4). Thus, under strong denaturing and reducing conditions, the rag proteins behaved predominantly as monomers of the predicted molecular weights.

In the absence of SDS (and regardless of the presence or absence of β-ME), however, this was not the case (Fig. 7B to F). rag-1, from both the induced double-expresser Dr3 cells (Fig. 7B) and rag-1-single-expresser Sr1 cells (Fig. 7C), spread down the gradient into later fractions, with significant amounts colocalized with nonreduced thyroglobulin (670 kDa) and some found in the pellet at the bottom of the gradient. Thus, even in the presence of a very high salt concentration, rag-1 was found to a significant extent in high-molecular-weight complexes. rag-2 behaved similarly when coexpressed with rag-1, with rag-2 first appearing in the fraction with the marker protein ovalbumin (44 kDa) but also appearing in all later fractions (Fig. 7B). In contrast the majority of rag-2 from the induced single-expresser Sr2 cells was found in the fraction with ovalbumin. The difference in the behavior of rag-2 in the presence and in the absence of rag-1 further supports the idea of direct or indirect interactions between the two rag proteins, with rag-1 imposing its tendency to form complexes upon rag-2.

To determine if the rag proteins behaved similarly when isolated from cells not subjected to heat shock treatment or from a physiological source, high-salt cellular extracts from either uninduced Dr3 cells (Fig. 7E) or murine thymus (Fig.

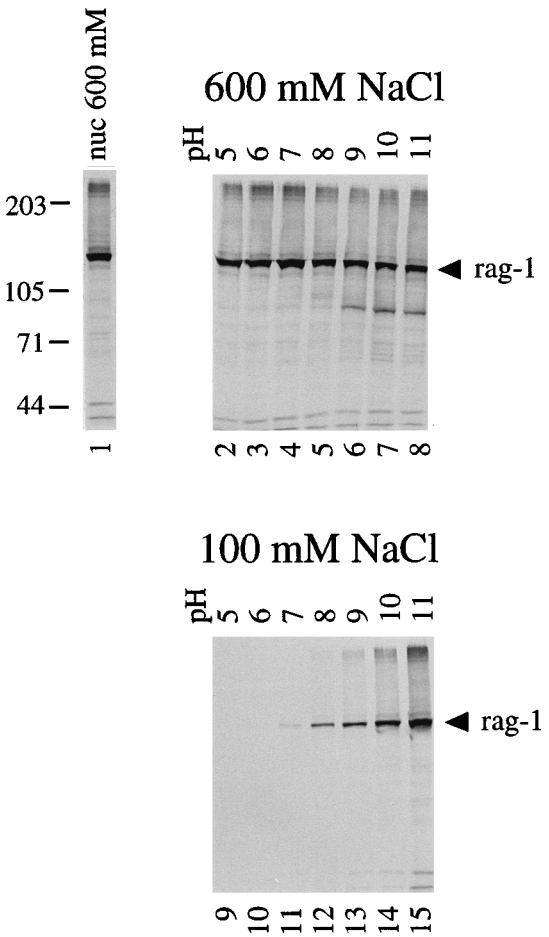

FIG. 6. Solubility of rag-1 in the presence of 100 or 600 mM NaCl as a function of pH. A Western blot analysis is shown. A nuclear extract (nuc 600 mM) from induced (6-h recovery) rag-1-single-expresser cells (Srli) was prepared by ultrasonication in the presence of 600 mM NaCl, pH 6.8. The nuclear extract was diluted into various buffers to obtain the indicated salt (600 or 100 mM NaCl) and pH (from 5 to 11) conditions. The samples were spun (15 min at 14,000 × *g*), and the supernatants were precipitated with trichloroacetic acid and separated on an SDS–6.5% polyacrylamide gel. Each lane contains the equivalent of 10^5 cells. Numbers on the left are molecular masses in kilodaltons.

7F) were fractionated on high-salt glycerol density gradients. In both cases, the results were similar to those obtained with induced Dr3 cells, with rag-1 and rag-2 present in high-molecular-weight complexes. These results indicate that the observed properties of the rag proteins are not a consequence of heat shock induction or the N-terminal addition introduced by the expression construct, and they suggest that the observed tendency to form complexes represents an inherent property of the rag proteins.

Interaction of the rag-1 and rag-2 proteins confirmed by co-IP. The affinity-purified αrag antibodies were capable of immunoprecipitating the cognate rag proteins from high-salt detergent extracts from thymus or induced or uninduced expresser cell lines (Fig. 8A). In addition, the αrag-1 antiserum clearly coimmunoprecipitated rag-2 from all extracts containing both proteins (Fig. 8A, lanes 2, 11, and 14). Co-IP of rag-1 with αrag-2 antiserum was also detected in extracts from uninduced (Dr3n) and induced (Dr3i) double-expresser cells but much more faintly (lanes 12 and 15). The specificities of the IP and co-IP signals were confirmed by the absence of any detectable direct IP or co-IP signal for (i) rag-1 with the rag-2-single-expresser cell line Sr2 (Fig. 8A, lanes 8 and 9), (ii) rag-2 with the rag-1-single-expresser cell line Sr1 (lanes 5 and 6), and (iii) either rag protein from any source with nonspecific rabbit IgG (lanes 1, 4, 7, 10, and 13).

By comparing the rag IP signal intensities (Fig. 8A) with the signals representing the total quantity of rag proteins prior to IP (Fig. 8B), the following approximate efficiencies of IP were estimated: 5% for rag-1 and rag-2 from thymus, 15% for rag-1 and 5% for rag-2 from Dr3n cells, and 30% for both rag-1 and rag-2 from Dr3i cells. By making a similar comparison, the efficiency of rag-2 co-IP with rag-1 (relative to the total amount of rag-2) could be estimated: 1% from thymus, 5% from Dr3n cells, and 30% from Dr3i cells.

Although silver staining does not stain all proteins with equal efficiency, we used the intensities of silver-stained rag-1 and rag-2 proteins obtained by IP (Fig 8C, lanes 2 and 3) to make a crude estimate of the number of rag-1 and rag-2 molecules present on average in each uninduced double-expresser Dr3 cell. By comparison with the signal intensities of 100 ng of the marker proteins phosphorylase B and myosin heavy chain (not shown), IP of rag-1 from 5×10^6 Dr3n cells was estimated to yield approximately 75 ng of protein. Because the IP efficiency is roughly 15%, 10^6 Dr3n cells can be calculated to contain approximately 100 ng of rag-1 or approximately 5×10^5 rag-1 molecules per cell. This number is almost identical to the earlier estimate based on Coomassie blue signals of rag-1 (Fig. 2). For rag-2, we estimate that IP yielded 25 ng of protein from 5×10^6 Dr3n cells, which, considering the IP efficiency of about 5%, corresponds to approximately 100 ng per 10^6 cells or approximately 10^6 rag-2 molecules per Dr3 cell. These crude estimates suggest that the observed signal intensities in the Western blot analysis shown in Fig. 8 (e.g., Fig. 8B, lane 4) provide a reasonable quantitative representation of the ratio of rag-1 to rag-2 molecules (perhaps overestimating rag-2 by as much as twofold), and they allow us to estimate that thymocytes contain on average 10^4 to 10^5 rag-1 and rag-2 molecules per cell, with rag-2 clearly in excess (Fig. 8B, lane 1).

Because of the high salt concentration and sonication used to prepare our nuclear extracts, they contain large amounts of DNA and RNA. We were interested in determining how this affected the IP and co-IP behaviors of the rag proteins, and therefore we used PEI to precipitate nucleic acids from high-salt cell lysates prior to IP. The PEI-induced precipitation was performed in the presence of 1.2 M NaCl, a salt concentration that allows ionic interaction between PEI and nucleic acids but should prevent interaction of virtually any protein with nucleic acids or with PEI (7). Precipitation of both RNA and DNA under these conditions was confirmed by ethidium bromide staining of agarose gels containing PEI precipitates (data not shown). rag-2 was not significantly affected by the PEI treatment, with regard to either the amount in the extract (Fig. 8B, lanes 1, 4, and 5, versus Fig. 8D, lanes 1, 4, and 7) or the amount detected by IP or co-IP (Fig. 8A, lanes 2, 3, 11, 12, 14, and 15, versus Fig. 8D, lanes 2, 3, 5, 6, 8, and 9). In contrast, PEI precipitation of nucleic acids depleted rag-1 from extracts to either a small extent (approximately 10%) from thymus (Fig. 8B, lane 1, versus Fig. 8D, lane 1) or a considerable extent (50 to 70%) from Dr3n and Dr3i cells (Fig. 8B, lanes 4 and 5, versus Fig. 8D, lanes 4 and 7). Even more substantial reductions in the rag-1 signal intensities were observed when PEI-treated lysates were used for IP with the αrag-1 antibodies: rag-1 IP signals were below the level of detection with PEI-treated extracts from thymus and Dr3n cells (Fig. 8D, lanes 2 and 5), while they were reduced at least fivefold with a PEI-treated extract from Dr3i cells (Fig. 8A, lane 14, versus Fig.

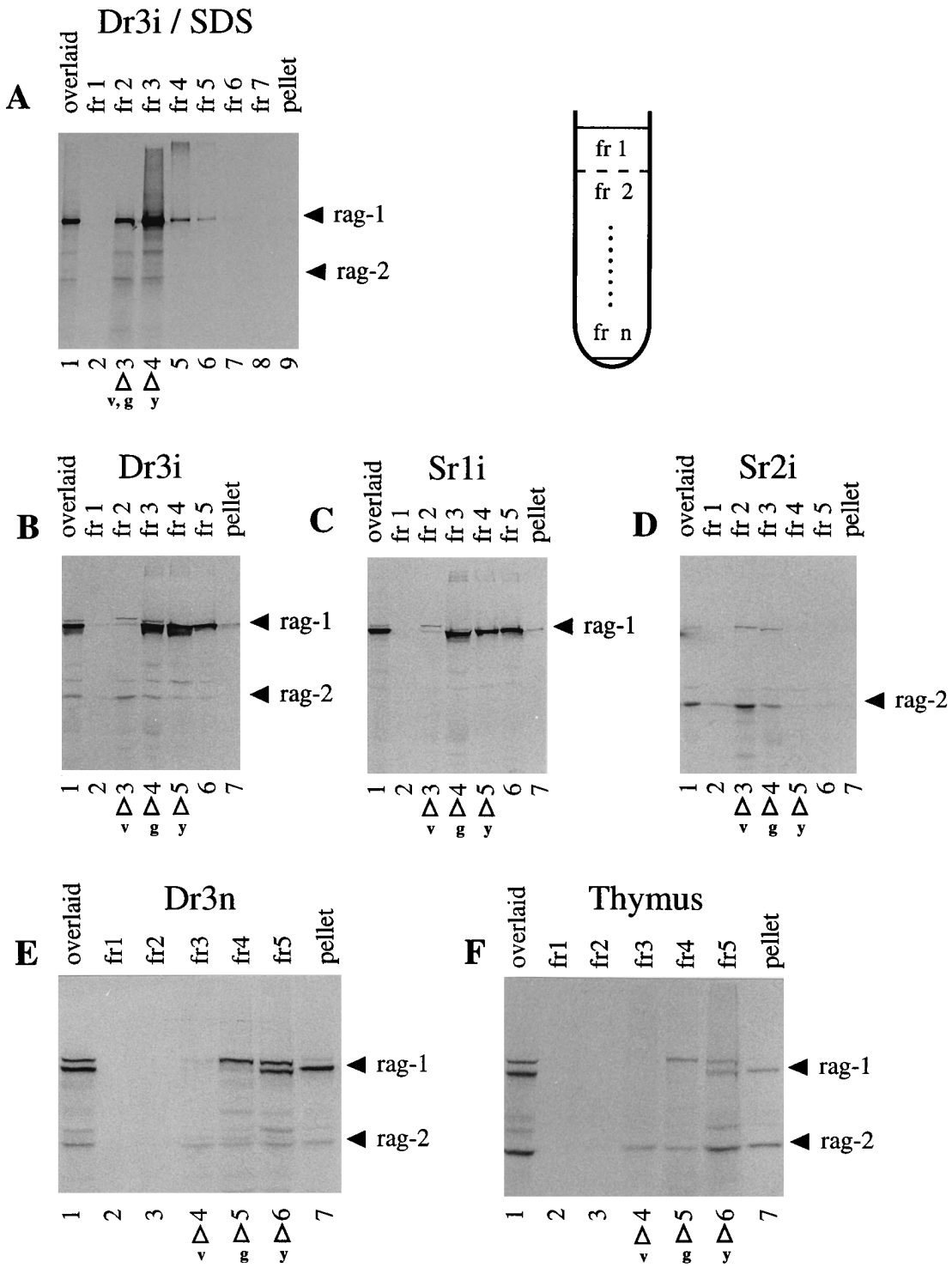

FIG. 7. Fractionation of the rag proteins on high-salt glycerol density gradients. A Western blot analysis is shown. (A) A nuclear extract from induced double-expresser cells (Dr3i) was prepared in the presence of 600 mM NaCl and with ultrasonication. The extract was adjusted to 2% SDS, 1.5% β-ME, and a volume of approximately 0.4 ml, boiled for 5 min, and overlaid onto a 3.6-ml, 15 to 45% linear glycerol gradient (600 mM NaCl, 2% SDS, 1.5% β-ME). After an 18-h centrifugation at 20°C and 200,000 × *g*, the gradient was separated into fractions (fr 1 to fr 7 and pellet). The equivalent of 10^5 cells was analyzed in each lane of an SDS–7% polyacrylamide gel. (B to D) Nuclear extracts from induced double-expresser cells (Dr3i) (B), induced rag-1-single-expresser cells (Srli) (C), or induced rag-2-single-expresser cells (Sr2i) (D) were prepared in the presence of 2 M NaCl without ultrasonication. The extracts were adjusted to approximately 0.25 ml and overlaid onto 3.6-ml, 15 to 50% linear glycerol gradients (2 M NaCl). After a 14-h centrifugation at 4°C and 166,000 × *g*, the gradients were separated into fractions (fr 1 to fr 5 and pellet). The equivalent of 0.5×10^5 cells was analyzed in each lane of an SDS–6% polyacrylamide gel. (E and F) Total cellular extracts from double-expresser cells (Dr3n) (E) and mouse thymus (C57BL/6, 20 days postpartum) (F) were prepared in the presence of 600 mM NaCl with ultrasonication. The extracts were adjusted to approximately 0.25 ml and overlaid onto 2.8-ml, 15 to 50% linear glycerol gradients (600 mM NaCl). After a 16-h centrifugation at 4°C and 166,000 × *g*, the gradients were separated into fractions (fr 1 to fr 5 and pellet). An equivalent of 0.25 mg of cells (Dr3n) (by weight of wet cellular pellet) or thymic tissue was analyzed in each lane of an SDS–6% polyacrylamide gel. Ovalbumin (v) (44 kDa), gamma globulin (g) (158 kDa), and thyroglobulin (y) (670 kDa) were mixed into all the extracts prior to loading on the glycerol gradients. Duplicate SDS-polyacrylamide gels were stained with Coomassie blue (data not shown), and fractions with the highest intensities of the marker signals are indicated with open triangles. Typically a smaller quantity of the markers trailed into the next lower fraction (fr n + 1). Note that the rag proteins sediment slightly lower in the gradients relative to the marker proteins in panels E and F than in panels B, C, and D. Additional glycerol gradient data (not shown) strongly suggest that this is due to the lower salt concentration in the gradients in panels E and F and not to a difference in the properties of the rag proteins.

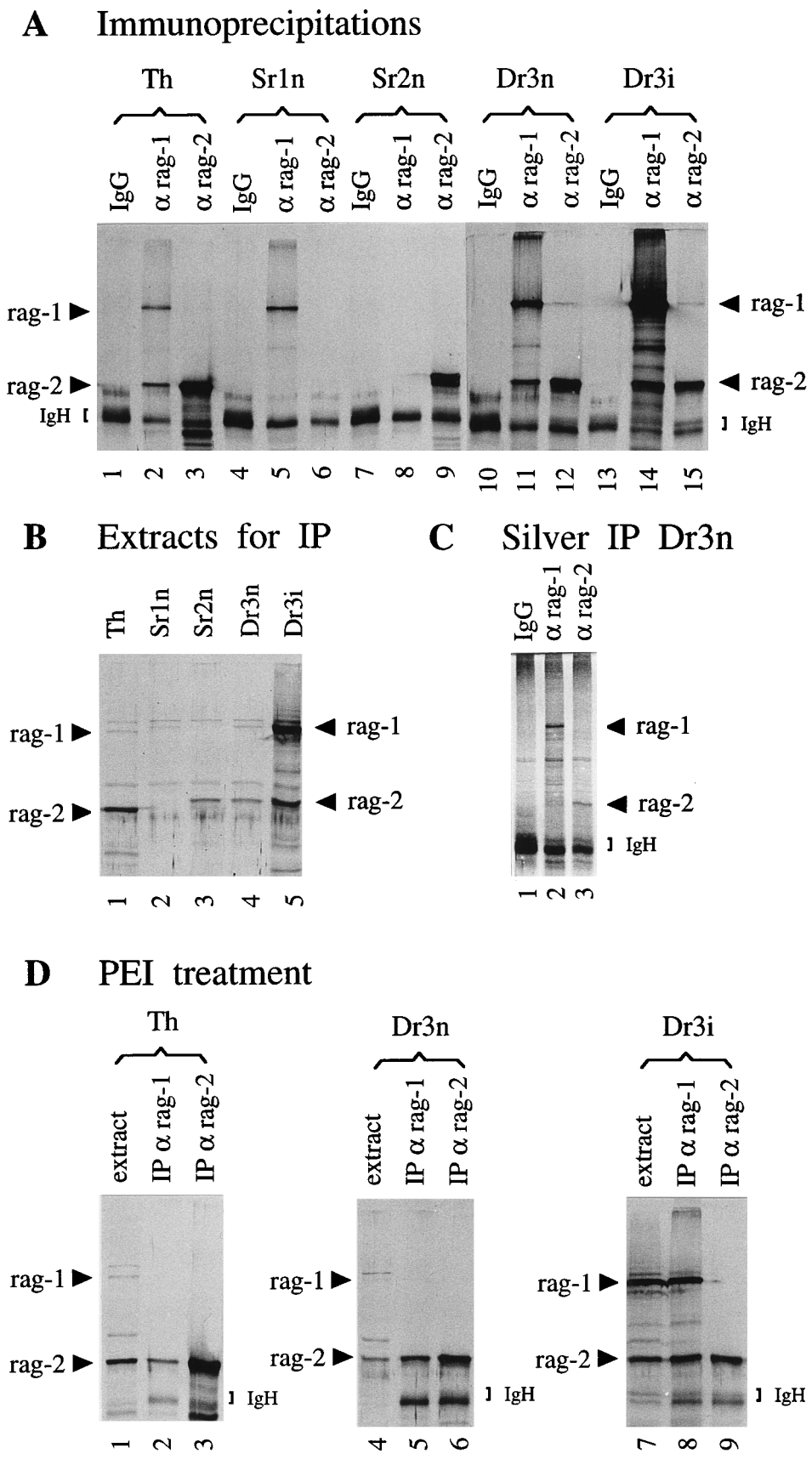

FIG. 8. IP of rag-1 and rag-2 from thymus and rag-expressing cell lines. (A, B, and D) Western blots; (C) silver-stained SDS-polyacrylamide gel. (A) Total extracts of thymic tissue (Th) and cells (noninduced rag-1-single-expresser cells [Sr1n], noninduced rag-2-single-expresser cells [Sr2n], noninduced double-expresser cells [Dr3n],

8D, lane 8). This is particularly striking in light of the fact that the amount of rag-2 that could be coimmunoprecipitated with rag-1 was not reduced in these same samples. It thus appears that precipitation of nucleic acids with PEI removes a portion of rag-1 from the extract and renders some of the remaining rag-1 refractory to IP but does not affect the population of rag-1 molecules that participate in complexes with rag-2. We interpret these results as suggesting that our extracts contain several distinct populations of rag-1 which differ in their affinities for nucleic acids and their participation in complexes containing rag-2.

DISCUSSION

The intimate connection between V(D)J recombination and the rag proteins strongly suggests that an understanding of the biochemical and functional properties of these proteins will be vital for detailed studies of the reaction mechanism. We have begun that process here by determining the extraction, solubility, density gradient fractionation, and cellular localization properties of the rag proteins. Furthermore, we have demonstrated that the rag proteins associate in a stable complex, and we have begun to characterize the properties of that complex. These results have important implications for the design of schemes to purify the rag proteins and of in vitro experiments involving them, and they provide an initial insight into the possible nature of the enzymatic complex that catalyzes V(D)J recombination.

As a first step in these studies, we derived cell lines that express high levels of the biologically active rag proteins and have demonstrated that expression levels were stable in the rag-double-expresser cell line Dr3 over a period of more than 12 months of continuous culture (data not shown). The large number of rag-1 and rag-2 molecules in these cells (10^5 to 10^6 per cell prior to heat shock induction) is paralleled by high levels of recombination activity, making this cell line an appealing alternative to Abelson murine leukemia virus-transformed pre-B-cell lines for the study of the rag proteins. An increase in expression of the rag proteins by a factor of 10 to 100 upon heat shock induction makes Dr3 cells, as well as the single-rag-1 (Sr1) and single-rag-2 (Sr2) cell lines, rich sources of murine rag proteins for further in vivo and in vitro biochemical characterization.

Basic physical parameters of the rag proteins. rag-1 is difficult to extract efficiently from nuclei (Fig. 5), is insoluble at moderate or low salt concentrations at physiological pH (Fig. 6), and is found predominantly in large complexes in crude extracts even in the presence of high salt concentrations (Fig. 7). rag-2, which can be extracted readily on its own under physiological conditions, behaves similarly only in the presence of rag-1. These results suggest that rag-1 is capable of strong interactions with other nuclear components in a rag-2-independent fashion, while strong interactions involving rag-2 are largely rag-1 dependent. This conclusion is further strengthened by the differences seen in the immunofluorescence localization of rag-2 in the absence or presence of rag-1 (Fig. 4).

We propose that rag-1 interacts with a variety of nuclear components and serves as a central organizing molecule for the formation of an active V(D)J recombination enzyme complex. Small portions of rag-1, when expressed in and purified from bacteria, bind nonspecifically to DNA (44), as does full-length rag-1 protein immobilized on a specific antibody column (data not shown). In addition, use of the yeast two-hybrid system has revealed that rag-1 interacts with two related nuclear membrane proteins, Rch-1 (11) and hSRP-1 (10). The region of rag-1 that participates in these interactions has been mapped, in one case, to a portion that lies outside the minimal essential region for enzymatic activity, prompting the authors to suggest that the interaction plays a role in localizing rag-1 (10). The significance of these interactions with regard to V(D)J recombination is unclear, however, since Rch-1 and hSRP-1 have recently been shown to be closely related to a *Xenopus* protein, named importin, that is essential for transport to the nucleus. It is interesting that these reports (10, 11) describe the existence of novel (but as yet uncharacterized) cDNA clones that encode proteins that interact with rag-1. In contrast, no proteins that interact with rag-2 in this assay have been reported.

Numerous in vitro studies have been performed to identify proteins that interact with the *cis*-acting DNA sequence required for V(D)J recombination. This sequence, termed the recombination signal sequence (RSS), consists of highly conserved heptamer and somewhat less well conserved nonamer elements separated by a spacer of either 12 ± 1 or 23 ± 1 bp. A variety of heptamer (1, 19, 35, 36, 53)-, nonamer (18, 30)-, or RSS (17, 37, 39)-binding factors have been reported, but the relevance of any of these factors to V(D)J recombination remains unclear, and in one case, it has been demonstrated that a factor initially identified as a heptamer-binding protein does not have the appropriate sequence specificity (21, 57). Neither rag-1 nor rag-2 has ever been identified in such RSS-binding experiments. If specific recognition of the RSS is rag-1 and/or rag-2 dependent (perhaps mediated directly by one or both of the rag proteins), our data provide an explanation for the failure thus far to identify RSS-binding factors with a clear physiological relevance to V(D)J recombination: the procedures used in those studies would almost certainly have left most of the rag proteins in the "insoluble" nuclear fraction or rendered the rag proteins insoluble. If RSS binding is dependent on a complex containing both rag-1 and rag-2, efforts to identify RSS-binding factors by Southwestern (DNA-protein) screens of expression libraries would also fail to identify relevant clones (17, 35, 53). For similar reasons, our data provide some guidelines for attempts to establish an in vitro V(D)J recombination system. It is worth noting, however, that we have not extensively investigated the ability of nonionic detergents to alter the properties of the rag proteins.

and induced double-expresser cells [Dr3i]) were prepared in the presence of 1 M NaCl and with ultrasonication. The extracts were precleared with rabbit IgG (coupled to agarose beads). For the IP, antibodies were used as indicated (rabbit IgG, αrag-1, or αrag-2, all coupled to agarose beads). The antibody beads were washed, and bound material was eluted with sample buffer (also eluting off some of the coupled antibodies, visible on the Western blots in form of heavy chain [IgH]). Eluates equivalent to either 4 mg of tissue (Th), 2×10^6 cells (Sr1n, Sr2n, and Dr3n), or 2×10^5 cells (Dr3i) were separated in each lane of an SDS–6.5% polyacrylamide gel. (B) Total extracts (after the IgG preclearing [same as used for IP]) equivalent to either 0.04 mg of tissue (Th) or 2×10^4 cells (Sr1n, Sr2n, Dr3n, and Dr3i) were separated in each lane of an SDS–6.5% polyacrylamide gel. (C) The equivalent of 5×10^6 cells from the same samples as in panel A, lanes 10, 11, and 12, were analyzed on an SDS–6.5% polyacrylamide gel and subjected to silver staining. (D) Total extracts (without IgG preclearing) were supplemented to 1.2 M NaCl and 0.5% PEI. The PEI-treated supernatants (extract) were used for IP, which was performed as described for panel A. The equivalent of either 0.04 mg of tissue (Th extract), 4 mg of tissue (Th IP αrag-1 and Th IP αrag-2), 2×10^4 cells (Dr3n extract and Dr3i extract), 2×10^6 cells (Dr3n IP αrag-1 and Dr3n IP αrag-2), or 2×10^5 cells (Dr3i IP αrag-1 and Dr3i IP αrag-2) was analyzed on an SDS–6.5% polyacrylamide gel.

The interaction between rag-1 and rag-2. rag-1 substantially altered the behavior of rag-2, making it less readily extracted from nuclei and more prone to participate in large complexes as well as causing it to partially relocalize into nuclear structures that contain large amounts of the rag-1 protein. These observations are consistent with the possibility that protein complexes containing both rag-1 and rag-2 exist in vitro and in vivo. We were able to directly demonstrate the existence of such complexes in vitro by co-IP of the rag proteins (Fig. 8). The rag proteins may interact directly with one another or by virtue of interactions with one or more other factors. It is interesting that the yeast two-hybrid assay failed to detect a direct rag-1–rag-2 interaction, prompting the speculation that yeast cells may lack another factor(s) important for the interaction (11). The complexes containing rag-1 and rag-2 are stable in detergent extracts containing 1 M NaCl but are relatively temperature labile since co-IP (but not direct IP) of the rag proteins was substantially reduced if IP was performed at room temperature instead of at 4°C (data not shown). The temperature-sensitive nature of the complex suggests that the observed interaction is not an in vitro artifact due to irreversible aggregation of the rag proteins. Co-IP of the rag proteins is the first direct in vitro assay for a rag protein function and will be an essential assay in monitoring the purification of rag protein complexes.

A pronounced asymmetry in the yields of co-IP was observed; i.e., rag-2 was coimmunoprecipitated much more efficiently with rag-1 than rag-1 was with rag-2. There are at least two, not mutually exclusive, explanations for this: (i) the ratio of rag-1 to rag-2 molecules in the complexes is less than 1, and (ii) the polyclonal αrag-2 antibodies bind to one or more regions of rag-2 that are important for its participation in the complex. In the latter case, inefficient co-IP of rag-1 with αrag-2 antibodies would be the result of inefficient recognition of complexed versus free rag-2 molecules and possibly would also be due to disruption of the complexes by the αrag-2 antibodies. When an extract of induced double-expresser Dr3 cells was subjected to five consecutive IPs with αrag-2 antibodies (efficiently depleting the rag-2 that could be immunoprecipitated with αrag-2 antibodies), subsequent co-IP of rag-2 with αrag-1 antibodies resulted in a yield about 50% of that obtained with a nondepleted control extract (data not shown). This suggests that indeed the αrag-2 antibodies preferentially precipitate rag-2 protein that is not engaged in rag-1–rag-2 complexes. It also suggests that the αrag-2 antibodies do not efficiently disrupt the complexes. It is worth noting that the αrag-1 antibodies were raised to a small, nonessential N-terminal region of rag-1, while the αrag-2 antibodies were raised to a much larger portion of rag-2 (see Materials and Methods), including much of the region shown to be essential for activity (12, 45).

Precipitation of nucleic acids with PEI resulted in a substantial depletion of rag-1 (50 to 70%) from high-salt cellular extracts from the uninduced or induced rag-double-expresser Dr3 cells and in a small (10%) depletion from thymic extracts. In contrast, rag-2 was not depleted from the extracts by PEI treatment, and remarkably, the amount of rag-2 that could be coimmunoprecipitated with αrag-1 antibodies was also not diminished (Fig. 8). These results suggest that a subpopulation of rag-1 molecules associates very tightly (stable at 1.2 M NaCl) with nucleic acids and that a quite distinct subpopulation is present in complexes with rag-2. In addition, the results (particularly lanes 2 and 5 of Fig. 8D) provide additional support for the hypothesis of an asymmetric stoichiometry of rag-1 and rag-2 in the complexes, with the ratio of rag-1 to rag-2 being smaller than 1. In this regard, it is interesting that the ratio of rag-1 to rag-2 in the thymus is also less than 1 (Fig. 3, lane 1; Fig. 8B, lane 1).

The percentage of the total rag-2 in cellular extracts engaged in complexes with rag-1 is likely to be substantially higher than our estimated efficiencies of co-IP (which range from 1% in the thymus to 30% in the induced double-expresser cell line). At least two factors make it likely that these numbers are underestimates: (i) the efficiencies of rag-1 IP were well below 100% (ranging from 5% in the thymus to 30% in the induced double expresser), and (ii) some portion of the complexes may have dissociated in the high-salt detergent buffer used in the IP experiments. Given the substantial effect of rag-1 on the fractionation properties of rag-2 in the glycerol gradient analyses (Fig. 7), a significant fraction of the total rag-2 may be engaged in complexes with rag-1.

We estimate the average number of rag-1 and rag-2 protein molecules in thymocytes to be in the range of 10^4 to 10^5 molecules per cell. If the rag proteins are part of the V(D)J recombination enzyme complex, this indicates a surprising discrepancy between the large number of potentially active rag molecules (and hence enzymatic complexes) and the presumably small number of recombination events that occur in a developing thymocyte. The explanation for this discrepancy will likely be found at various regulatory levels, such as rag protein modification, efficiency of assembly of active recombination enzyme complexes, and substrate accessibility.

A picture of a close partnership between rag-1 and rag-2 has therefore emerged, with the *RAG-1* and *RAG-2* coding sequences each contained in one exon, positioned in very close proximity to each other in the genome (40), and with both essential for successful V(D)J recombination. Our data provide further support for this concept by demonstrating a direct or indirect interaction of the rag-1 and rag-2 proteins in cell extracts and a strong influence of rag-1 on the behavior of rag-2 both in vitro and in vivo. It is tempting to speculate that it is the rag-1 and rag-2 present jointly in complexes, rather than noninteracting rag proteins, that are actually biologically active, likely as the lymphocyte-specific components of the V(D)J recombination enzyme complex. The purification of rag-1–rag-2 complexes will lay the groundwork for additional insight into the biochemical functions of the rag-1 and rag-2 proteins, allow determination of whether the interaction between the rag proteins is direct or indirect, and potentially reveal other proteins involved in high-molecular-weight rag protein complexes.

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