[32] RAG and HMGB1 Proteins: Purification and Biochemical Analysis of Recombination Signal Complexes

By SERGE BERGERON, DIRK K. ANDERSON, and PATRICK C. SWANSON

Abstract

Two lymphoid cell-specific proteins, called RAG-1 and RAG-2, initiate the process of antigen receptor gene rearrangement, termed V(D)J recombination, by assembling a protein–DNA complex with two recombination signal sequences (RSSs), each of which adjoins a different receptor gene segment, and then introducing a DNA double strand break at the end of each RSS. The study of RAG–RSS complex assembly and activity has been facilitated by the development of methods to purify the RAG proteins and members of the HMG-box family of high mobility group proteins such as HMGB1 that promote RAG binding and cleavage activity *in vitro*. This chapter describes the purification of recombinant truncated and full-length RAG-1 and RAG-2 expressed transiently in mammalian cells, as well as the purification of bacterially expressed full-length HMGB1. In addition, it details several experimental procedures used in our laboratory to study RAG–RSS complex formation and function *in vitro*.

Introduction

The antigen-binding variable domains present in immunoglobulins and T-cell receptors owe much of their structural diversity to a process known as V(D)J recombination, which is responsible for assembling the exons encoding these domains from component variable (V), diversity (D), and joining (J) gene segments (for review, see Bassing *et al.*, 2002). V(D)J recombination is initiated when a multiprotein complex minimally containing two lymphoid cell-specific proteins called RAG-1 and RAG-2 (the ''RAG complex'') binds a pair of recombination signal sequences (RSSs), each of which adjoins a different gene segment, and then catalyzes a DNA double strand break at each RSS (for reviews, see Fugmann *et al.*, 2000; Gellert, 2002). Each RSS contains a conserved heptamer and nonamer sequence, separated by either 12 or 23 bp of intervening DNA whose composition is more varied (12-RSS and 23-RSS, respectively). Rearrangement preferentially occurs between two RSSs whose spacer length differs (the ''12/23 rule''), a restriction that promotes productive exon assembly.

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Four DNA ends are generated by RAG-mediated cleavage: two blunt, 5′-phosphorylated signal ends and two coding ends terminating in DNA hairpin structures. These recombination intermediates arise through sequential strand cleavage and strand transfer steps in which the RAG complex first nicks the RSS at the heptamer and then joins the resulting 3′-OH to the opposing DNA strand via direct transesterification. After DNA cleavage, signal ends are typically ligated heptamer to heptamer to yield a precise signal joint. In contrast, the joining of coding ends is usually imprecise, as hairpin opening, which is most likely catalyzed by a complex containing Artemis and the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) (Ma *et al*., 2002), and subsequent end processing by enzymes that remove nucleotides or add them [terminal deoxynucleotidyltransferase, TdT (Komori *et al*., 1993)] often lead to gain or loss of nucleotides at the junction. Signal and coding joint formation is mediated by ubiquitously expressed proteins that comprise the nonhomologous end-joining (NHEJ) pathway of DNA double strand break repair, including Ku70, Ku80, XRCC4, and DNA ligase IV (Bassing *et al*., 2002).

Once RAG-1 and RAG-2 were identified as essential components of the V(D)J recombination apparatus (Oettinger *et al*., 1990; Schatz *et al*., 1989), efforts focused on purifying the RAG proteins for biochemical analysis. However, these early efforts were hampered by poor solubility of the full-length RAG proteins. Subsequent studies delineated portions of RAG-1 and RAG-2 essential for supporting V(D)J recombination in cell culture-based assays (Cuomo and Oettinger, 1994; Kirch *et al*., 1996; Sadofsky *et al*., 1993, 1994; Silver *et al*., 1993). These catalytically active ''core'' forms of RAG-1 and RAG-2 proteins were found to be more soluble than their full-length counterparts, a feature that facilitated their eventual purification. With these proteins in hand, the Gellert laboratory completed a series of elegant biochemical experiments to elucidate the mechanism of RAG-mediated RSS cleavage (McBlane *et al*., 1995; van Gent *et al*., 1996). Later studies revealed that binding of the RAG complex to isolated recombination signals (especially a 23-RSS) and RAG-mediated synapsis and cleavage of RSS pairs according to the 12/23 rule is promoted by ''architectural'' DNA binding proteins of the HMG-box family of high mobility group proteins (Hiom and Gellert, 1998; Sawchuk *et al*., 1997; van Gent *et al*., 1997). In this respect, the RAG complex exhibits similarities to several recombinases and transcription factors that interact with, and have their activity stimulated by, HMG-box proteins (Thomas and Travers, 2001). Whether HMG-box proteins facilitate V(D)J recombination *in vivo* remains unclear, as mice deficient in HMGB1 exhibit no apparent defects in lymphocyte development, although its absence causes lethal hypoglycemia within 24 h of birth (Calogero *et al*., 1999).

However, it must be noted that *in vitro*, RAG activity is comparably stimulated by either HMGB1 or the related HMGB2 protein (Swanson, 2002a), raising the possibility that the two proteins may play redundant roles in V(D)J recombination *in vivo*, an issue that has not yet been formally addressed. Nevertheless, HMG-box proteins are often included in biochemical assays of the RAG complex to exploit the benefit of enhanced RAG activity imparted by its association with these proteins. Although most biochemical assays have been performed with ''core'' RAG proteins, the growing recognition that ''noncore'' portions of RAG-1 and RAG-2 help regulate the activity of the RAG complex *in vivo* Akamatsu *et al*., 2003; Dudley *et al*., 2003; Liang *et al*., 2002; Talukder *et al*., 2004) naturally necessitated identifying methods to purify the full-length proteins for use in biochemical assays. We and others reported the successful purification of full-length forms of RAG-1 and RAG-2 and their preliminary biochemical characterization (Elkin *et al*., 2003; Jiang *et al*., 2004; Swanson *et al*., 2004; Tsai and Schatz, 2003). This chapter describes methods to purify core and full-length RAG-1 and RAG-2 from transiently transfected 293 cells, as well as bacterially expressed full-length HMGB1, and details several procedures used to characterize the binding and cleavage activity of the RAG complex *in vitro*.

Expression Vectors

Baculovirus expression vectors containing murine core RAG-1 (residues 384–1008) and RAG-2 (residues 1–387), fused at the amino terminus to a maltose-binding protein (MBP) domain and at the carboxy terminus to a polyhistidine sequence and three copies of the myc epitope tag, were obtained from the laboratory of M. Gellert and have been described previously (McBlane *et al*., 1995). DNA fragments encoding these fusion proteins were subcloned into the mammalian expression vector pcDNA1 (Invitrogen, Carlsbad, CA) using *Bam*HI and *Not*I. Plasmid constructs containing full-length murine RAG-1 or RAG-2 cDNAs were prepared in the laboratory of S. Desiderio and were used for generating DNA fragments encoding noncore portions of RAG-1 and RAG-2. Using this source material for subcloning or as templates for PCR, pcDNA1 expression constructs encoding various forms of wild-type and mutant core and full-length RAG-1 and RAG-2 fusion proteins have been generated (see Table I). The pcDNA1 plasmids are propagated in *Escherichia coli* strain MC1061/P3 (Invitrogen) and are typically purified from overnight 500-ml bacterial cultures (Luria broth supplemented with 30 μg/ml carbenicillin and 7.5 μg/ml tetracyclin) using a Qiagen plasmid maxi kit according to the manufacturer's instructions (Qiagen, Hercules, CA).

TABLE I
FORMS OF RAG-1 AND RAG-2 PURIFIED FROM 293 CELLS AFTER TRANSIENT TRANSFECTION WITH PCDNA1 RAG EXPRESSION PLASMIDS

Designation	Residues[a]	Tags[b]	Mutation(s)	Reference
cMR1hm	384–1008(R1)	MBP, His_9, $(c\text{-}Myc)_3$	None	Swanson and Desiderio (1998)
cMR1hm384/393	384–1008(R1)	MBP, His_9, $(c\text{-}Myc)_3$	Ala(384–393)	Swanson and Desiderio (1998)
cMR1hm394/403	384–1008(R1)	MBP, His_9, $(c\text{-}Myc)_3$	Ala(394–403)	Swanson and Desiderio (1998)
cMR1hm404/413	384–1008(R1)	MBP, His_9, $(c\text{-}Myc)_3$	Ala(404–413)	Swanson and Desiderio (1998)
cMR1hm414/423	384–1008(R1)	MBP, His_9, $(c\text{-}Myc)_3$	Ala(414–423)	Swanson and Desiderio (1998)
cMR1hm424/433	384–1008(R1)	MBP, His_9, $(c\text{-}Myc)_3$	Ala(424–433)	Swanson and Desiderio (1998)
cMR1hm434/443	384–1008(R1)	MBP, His_9, $(c\text{-}Myc)_3$	Ala(434–443)	Swanson and Desiderio (1998)
cMR1hm594/596	384–1008(R1)	MBP, His_9, $(c\text{-}Myc)_3$	Ala(594–596)	Swanson and Desiderio (1998)
cMR1hm607/611	384–1008(R1)	MBP, His_9, $(c\text{-}Myc)_3$	Ala(607–611)	Swanson and Desiderio (1998)
cMR1hm615/618	384–1008(R1)	MBP, His_9, $(c\text{-}Myc)_3$	Ala(615–618)	Swanson and Desiderio (1998)
cMR1hm995/998	384–1008(R1)	MBP, His_9, $(c\text{-}Myc)_3$	Ala(995–998)	Swanson and Desiderio (1998)
cMR1hm999/1003	384–1008(R1)	MBP, His_9, $(c\text{-}Myc)_3$	Ala(999–1003)	Swanson and Desiderio (1998)
cMR1hm1004/1008	384–1008(R1)	MBP, His_9, $(c\text{-}Myc)_3$	Ala(1004–1008)	Swanson and Desiderio (1998)
cMR1	384–1040(R1)	MBP	None	Swanson and Desiderio (1999)
cM2R1hm	384–1008(R1)	MBP_2, His_9, $(c\text{-}Myc)_3$	None	Swanson and Desiderio (1999)
cM2R1	384–1040(R1)	MBP_2	None	Swanson and Desiderio (1999)
cM2R1m	384–1008(R1)	MBP_2, $(c\text{-}Myc)_3$	None	Swanson (2001)
cMR1hm(wtNBD, mtAS)	384–1008(R1)	MBP, His_9, $(c\text{-}Myc)_3$	D600A, D708A or E962A	Swanson (2001)
cMR1hm(mtNBD, mtAS)	384–1008(R1)	MBP, His_9, $(c\text{-}Myc)_3$	Ala(384–393) and D600A, D708A, or E962A	Swanson (2001)
cM2R1m(wtNBD, mtAS)	384–1008(R1)	MBP_2, $(c\text{-}Myc)_3$	D600A, D708A or E962A	Swanson (2001)
cMR1m	384–1008(R1)	MBP, $(c\text{-}Myc)_3$	None	Swanson (2002a)
FLMR1	1–1040(R1)	MBP	None	Swanson *et al.* (2004)
cMR2hm	1–387(R2)	MBP, His_9, $(c\text{-}Myc)_3$	None	Swanson and Desiderio (1998)
cMR2h	1–418(R2)	MBP, His_9	None	Swanson and Desiderio (1998)
cM2R2hm	1–387(R2)	MBP_2, His_9, $(c\text{-}Myc)_3$	None	Swanson and Desiderio (1999)
cMR2	1–387(R2)	MBP	None	Swanson (2002a)
FLMR2	1–527(R2)	MBP	None	Swanson *et al.* (2004)

[a] Murine sequence of RAG-1 (R1) or RAG-2 (R2).
[b] MBP, maltose-binding protein; MBP_2, tandem MBP domains; c-Myc, SEQKLISEEDLRA epitope tag.

The prokaryotic expression construct pET11d-hHMGB1, encoding full-length human HMGB1 tagged at the amino terminus with polyhistidine, was generated in the laboratory of R. Roeder (Ge and Roeder, 1994). To gain insight into how HMGB1 promotes RAG-mediated RSS synapsis and cleavage *in vitro*, we have prepared and analyzed a large panel of truncated and mutant forms of hHMGB1 in RAG-binding and cleavage assays (Bergeron *et al.*, 2005). Generation of the various HMGB1 expression constructs is detailed extensively in that study and is not described further here.

Protein Expression and Purification

RAG Proteins

The human embryonic kidney fibroblast cell line 293 is used to transiently express the RAG proteins for purification. The cells are maintained under standard humidified conditions (37° and 5% CO_2) in 1× Dulbecco's modified Eagle medium (containing high glucose, L-glutamine, and pyridoxine hydrochloride, but not sodium pyruvate; Invitrogen Life Technologies) supplemented with 50 units/ml penicillin G and 50 μg/ml streptomycin (BioWhittaker, Walkersville, MD) and 10% fetal bovine serum (FBS; v/v). We have used FBS from HyClone (Logan, UT), Invitrogen, and BioMeda (Foster City, CA) with comparable results. The final medium is sterile filtered through a 0.45-μm cellulose acetate membrane before use (Corning 500-ml filter system). Cells are grown to confluence in 10-cm culture dishes, resuspended in the same medium, and seeded into fresh medium (1:5 dilution) the day before transfection. The next morning, the medium is aspirated off and replaced with 10 ml fresh medium and returned to the incubator for 3 h. For transient transfection, instead of using the calcium phosphate precipitation method employed previously (Swanson, 2002b), we now use a polyethylenimine (PEI) transfection procedure adapted from one described by Durocher *et al.* (2002) to reduce media consumption, as a glycerol shock step with a medium change is not necessary with this procedure. An aqueous stock solution of PEI (Polysciences Inc., Warrington PA) is prepared (1 μg/μl, brought to pH 7.0 by the addition of HCl) and stored in aliquots at −80°. The stock solution is prepared immediately after opening a new bottle of PEI, as the transfection efficiency declines considerably if solutions are prepared from powdered PEI opened and stored at ambient temperature and atmosphere for longer than 4 weeks (unpublished observations). Plasmid DNA is added to 0.9% NaCl (w/v; sterile filtered) at a final concentration of 10 μg/ml. For coexpression of RAG-1 and RAG-2, 5 μg of each

RAG-expression vector is added; otherwise 10 μg of an individual plasmid is added. To this mixture, PEI is added at a final concentration of 30 μg/ml. After vortexing briefly, samples are incubated at 25° for 10 min, and then 1 ml of the DNA–PEI mixture is added to each dish of 293 cells. Typically, for each RAG protein preparation, two 7-ml solutions of DNA–PEI are assembled for transfection of 14 dishes of 293 cells. After incubation for 48 h, the medium is aspirated, and seven dishes are harvested in 5 ml of sterile phosphate-buffered saline–EDTA (137 m*M* NaCl, 27 m*M* KCl, 43 m*M* KH_2PO_4, 14 m*M* Na_2HPO_4, 2 m*M* EDTA) per plate and collected in a 50-ml conical tube on ice. The tubes are centrifuged at 274*g* for 10 min at 4° (Beckman GH3.8 rotor, 1300 rpm) and the supernatant is aspirated. Cell pellets are frozen in a dry ice/ethanol bath and stored at −80° until use.

For protein purification, all steps of the following protocols are performed at 4°. Individually expressed core MBP-RAG-1 and coexpressed MBP-RAG-1 and MBP-RAG-2 are purified using the same procedure. Briefly, each cell pellet is thawed on ice and resuspended in 3.75 ml buffer A [10 m*M* sodium phosphate (pH 7.4), 0.5 *M* NaCl, 1 m*M* dithiothreitol (DTT), 0.25% Tween 20 (v/v)], loaded into a Dounce tissue grinder (Wheaton Science Products, Millville, NJ), and subjected to 20 strokes of a type A pestle. The lysate is clarified by centrifugation at 85,000*g* (Beckman SW55Ti rotor, 30,000 rpm) for 40 min at 4° and the supernatants collected from two pellets are passed over 1 ml of amylose resin (New England Biolabs, Ipswich, MA) packed in a Poly-Prep chromatography column (Bio-Rad, Hercules, CA) equilibrated in buffer A by gravity flow. The column is washed with 10 ml buffer A (the final five lacking Tween 20), and the MBP-RAG protein(s) is eluted with buffer A containing 10 m*M* maltose (also lacking Tween 20). Protein-containing samples are dialyzed (Spectra/Por 25,000 MWCO, Spectrum Laboratories Inc., Rancho Dominguez, CA) against buffer R [25 m*M* Tris–HCl (pH 8.0), 150 m*M* KCl, 2 m*M* DTT, and 10% glycerol (v/v)]) for 3 h. Aliquots are snap frozen in liquid nitrogen and stored at −80° until use. For individually expressed MBP-RAG-2, we find that an alternative purification protocol, adapted from Spanopoulou and colleagues (1996), yields protein that is more active in cleavage assays than the method described earlier. Briefly, each thawed cell pellet (two total) is resuspended in 1.5 ml cold RSB buffer [10 m*M* Tris–HCl (pH 7.4), 10 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.5% IGEPAL CA-630 (NP-40; v/v)], and allowed to swell for 5 min. Next, 2.25 ml cold buffer LSB [20 m*M* Tris–HCl (pH 7.4), 1 *M* NaCl, 0.2 m*M* $MgCl_2$, 1 m*M* PMSF, 0.2% IGEPAL CA-630 (NP-40; v/v)] is added and the sample is rocked at 4° for 1 h. The lysate is clarified by centrifugation as described earlier, and the pooled supernatants are applied to a column packed with amylose resin equilibrated in a 1:1.5 ratio of

RSB:LSB (RSB/LSB buffer). The column is washed with 5 volumes of RSB/LSB buffer and then with 4 volumes of buffer WB [20 m*M* Tris–HCl (pH 7.4), 0.5 *M* NaCl, 5 m*M* $MgCl_2$]. The MBP-RAG-2 fusion protein is eluted in buffer WB containing 10 m*M* maltose and dialyzed as described earlier.

HMGB1

The *E. coli* strain BL21(DE3)pLysS is transformed with a modified version of the pET11d-hHMGB1 expression construct. Bacteria are grown at 37° to an OD_{600} of 0.5 in Luria broth supplemented with 50 μg/ml carbenicillin and 20 μg/ml chloramphenicol, and protein expression is induced by adding isopropyl-ß-D-thiogalactopyranoside to a final concentration of 1 m*M*. The culture is then incubated at 30° for 4 h, after which the cells are collected by centrifugation and the cell pellet is frozen at −80°. The conditions for the induction phase are derived empirically, as we observe that incubation at 30° yields considerably more protein than at 25 or 37°, and that limiting incubation to 4 h versus overnight also benefits overall expression.

A pellet from a 250-ml culture is resuspended with 7 ml of binding buffer [40 m*M* Tris–HCl (pH 8.0), 0.5 *M* KCl, 0.25% Tween 20 (v/v), 50 m*M* imidazole, 1 m*M* PMSF, 10 μM leupeptin, and 1 μM pepstatin A] and sonicated on ice using a Fisher Model 500 sonic dismembrator (10 cycles of 10-s pulse, 10-s pause, at 20% power; Fisher Scientific, Pittsburg, PA). The lysates are centrifuged at 46,000*g* (Beckman SW55Ti rotor, 22,000 rpm) for 40 min at 4°. Supernatants are recovered, passed through 0.45-μm PVDF syringe filters (Millex HV, Millipore, Billerica, MA), and purified by immobilized metal affinity chromatography (IMAC). Yield and purity were compared between the ProBond nickel-chelating resin (Invitrogen) and the chelating Sepharose fast flow (FF) resin (Amersham BioSciences-GE Healthcare, Piscataway, NJ), both of which utilize the iminodiacetic acid-chelating group. In our hands, FF reproducibly yields as much as fivefold more HMGB1 protein than the ProBond resin (with similar purity) and is therefore utilized in the purification protocol described later.

Briefly, FF slurry is loaded into an empty Poly-Prep chromatography column (1 ml bed volume), charged with 5 ml of nickel solution (100 m*M* $NiSO_4$, 40 m*M* Tris–HCl, pH 8.0), and washed with 10 ml of binding buffer. After applying the supernatant, the column is washed with 15 ml of binding buffer. To eliminate the void volume, 800 μl of elution buffer [40 m*M* Tris–HCl (pH 8.0), 0.5 *M* KCl, 500 m*M* imidazole, 1 m*M* PMSF, 10 μM leupeptin, 1 μM pepstatin A] is applied to the column and the flow through is discarded. The proteins are then eluted with 1.2 ml of elution buffer.

Analysis of HMGB1 purified by IMAC reveals the presence of several proteins. The major species are similarly detected by immunoblotting using a rabbit polyclonal anti-HMGB1 antibody raised to residues 166–181 (Pharmingen, San Diego, CA), strongly suggesting that all are derived from HMGB1. Furthermore, the same immunoblotting pattern is observed with unpurified whole bacterial lysates, even if protease inhibitors are added to the purification buffers, indicating that these products are present in bacteria prior to purification. Based on an earlier report suggesting that full-length HMGB1 is difficult to fully denature (Stros, 1998), we initially thought the products represented partially denatured proteins, but then decided to explore further purification using ion-exchange chromatography (IEC). Given the highly acidic nature of the C-terminal tail of HMGB1, we speculated that if it is shortened or eliminated by degradation, full-length HMBG1 could be resolved from degradation products by IEC on a positively charged matrix, as the full-length protein would be expected to bind most tightly to it. This turns out to be true, but two minor high molecular contaminants (~80 kDa) present after IMAC are not removed by this procedure, necessitating incorporation of an additional purification step using a negatively charged matrix. All purification steps involving IEC are performed using a BioLogic LP chromatography system (Bio-Rad). All washes and gradients are prepared using buffer A [40 m*M* Tris–HCl (pH 8.0), 1 m*M* DTT] and buffer B [40 m*M* Tris–HCl (pH 8.0), 1 m*M* DTT, 1 *M* KCl]. To purify full-length HMGB1 from bacterial contaminants, the eluate obtained from the Ni^{2+}-FF chromatography step is diluted to 65 m*M* KCl using buffer A and loaded onto the negatively charged Econo-Pac High S cartridge (Bio-Rad; 1 ml bed volume). Bound proteins are washed with 9 ml of 80 m*M* KCl and eluted using a KCl gradient of 80 m*M* to 0.65 *M* developed with buffer B (25 ml at 1.0 ml/min). Eluted protein fractions centered around 300 m*M* KCl are pooled, diluted twofold with buffer A, and loaded onto a positively charged Econo-Pac High Q cartridge (Bio-Rad; 1 ml bed volume). Bound proteins are washed with 10.5 ml of 0.22 *M* KCl (prepared by mixing buffers A and B) and eluted using a KCl gradient of 0.22 to 0.7 *M* developed with buffer B (40 ml at 1.0 ml/min). Protein fractions containing full-length HMGB1 eluting around 360 m*M* KCl are pooled, dialyzed overnight at 4° against 1 liter of dialysis buffer [25 m*M* Tris–HCl (pH 8.0), 150 m*M* KCl, 2 m*M* DTT, 10% glycerol], snap frozen in liquid nitrogen, and stored at −80° until use. The purity of the isolated proteins after each step of this purification procedure is shown in Fig. 1. HMGB1 purified using this protocol has the expected molecular weight as determined by mass spectroscopy. The purification of other truncated and mutant forms of HMGB1 is described elsewhere (Bergeron *et al.*, 2005).

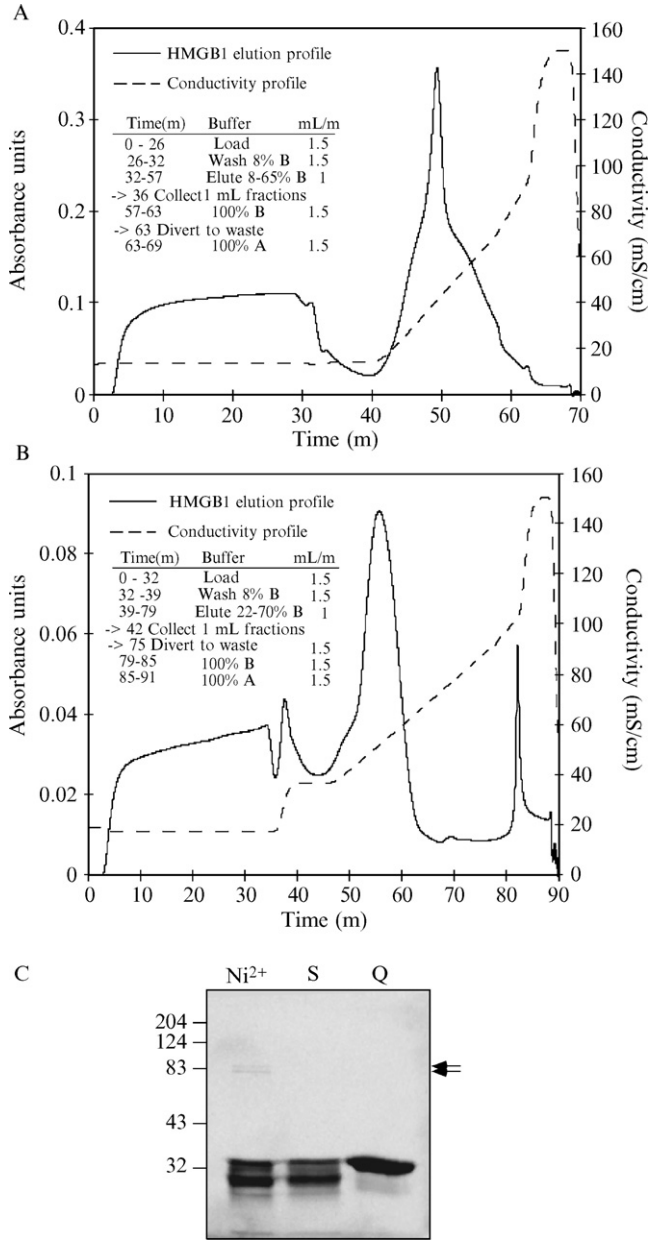


FIG. 1. Purification of bacterially expressed full-length, polyhistidine-tagged human HMGB1. (A) Eluate collected after IMAC was subjected to IEC using an Econo-Pac High S cartridge according to the conditions listed (inset). Conductivity and HMGB1 elution

Oligonucleotide Probe Preparation

Oligonucleotides are synthesized commercially (IDT Inc., Coralville, IA) and those over 35 bases in length are purified further by polyacrylamide gel electrophoresis by the vendor. Standard intact 12-RSS and 23-RSS substrates are assembled from two 50-mer (DAR39 and DAR40; 12-RSS) or two 62-mer (DG61 and DG62; 23-RSS) oligonucleotides (McBlane *et al*., 1995). The ''top'' strand (in this case, DAR39 [5′-GATC TGGCCTGTCTTACACAGTGCTACAGACTGGAACAAAAACCCT GCAG-3′] or DAR61 [5′-GATCTGGCCTGTCTTACACAGTGGTAG TACTCCACTGTCTGGCTGTACAAAAACCCTGCAG-3′]) contains a 16 nucleotide coding sequence followed by consensus heptamer and nonamer sequences (underlined, separated by either 12 or 23 nucleotides), and 6 nucleotides 3′ of the nonamer. To prepare the substrate, top strand DNA (3.33 pmol) is 5′ end labeled in a reaction (20 μl final volume) containing [γ-^{32}P]ATP (Perkin Elmer Life Sciences, Boston, MA; 6000 Ci/mmol, 6.66 pmol) and T4 polynucleotide kinase (10 units; Invitrogen). After incubation at 37° for 40 min, the kinase is heat inactivated by incubation at 65° for 20 min. A small portion of the labeled strand is removed and the remaining DNA is annealed to a fivefold excess of its unlabeled complement. The same procedure can be used to assemble RSS substrates containing a preformed nick introduced at the 5′ end of the heptamer from three oligonucleotides (McBlane *et al*., 1995). To evaluate the completeness of annealing and to remove excess unannealed DNA, the unannealed and annealed samples are loaded onto a 10% nondenaturing polyacrylamide gel [19:1 acrylamide:methylene(bis)acrylamide, prepared in 1× TBE using a SE400 gel apparatus] (Hoefer Inc., San Francisco, CA; 0.75-mm spacers and a 15-well comb) and fractionated at 250 V for 1.5–2 h at 25°. The gel is wrapped in Saran wrap and exposed briefly to film (BioMAX MR, Kodak).

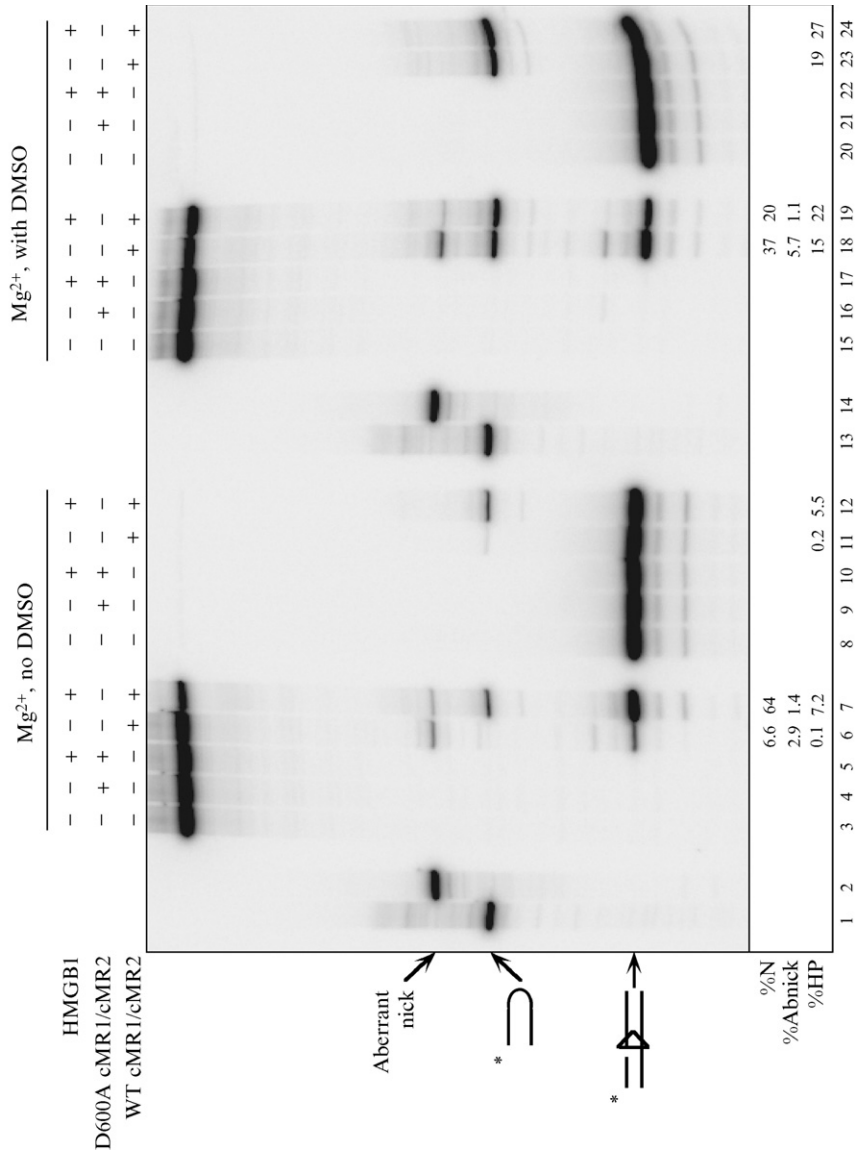
To elute the DNA from the gel, an electroelution apparatus is used according to the manufacturer's instructions (Hoefer GE200 SixPac Gel Eluter). Using the developed film as a template, the annealed DNA is excised from the gel and placed into an elution tube containing 1× TAE (40 m*M* Tris-acetate, 1 m*M* EDTA; 300 μl]. Typically the gel slice is about

profiles are shown as dashed and solid lines, respectively. Fractions 11–24 were collected. (B) Fractions pooled from A were subjected to IEC using an Econo-Pac High Q cartridge according to the conditions listed (inset). Fractions 14–23 were pooled and dialyzed as described in the text. (C) Protein samples collected after IMAC (Ni^{2+}) and after subsequent S and Q IEC purification steps were fractionated by SDS–PAGE and stained with SYPRO Orange. High molecular weight contaminating proteins removed by IEC using the S cartridge are indicated by arrows.

7 mm long and 5 mm wide, which is then split into two pieces lengthwise. It is difficult to load more than this gel volume into a collection tube and still obtain efficient electroelution. The tube is capped with a blotting paper disk and a porous polyethylene plug, and then the bottom nub is cut off. The tube is placed into a 1.5-ml microcentrifuge tube containing 4× TAE (150 μl). The sample is subjected to electrophoresis at 60 V for 40 min with a 5-s reversed field pulse at the end of the run. The solution remaining around the gel slice is removed carefully with a micropipette and added to the solution in the collection tube. The pooled sample is loaded onto a G25 Sephadex desalting column (NAP-5, Amersham BioSciences) equilibrated in 10 m*M* Tris–HCl (pH 8.0). Fractions are collected (two or three drops each) and checked for radioactivity using a hand-held survey meter, and the activity of desired fractions is measured using an LSC6500 liquid scintillation counter (Beckman Coulter, Fullerton, CA). Using fresh [γ-^{32}P] ATP, the most radioactive fractions typically contain 50,000–100,000 cpm/μl ($\sim 1 \times 10^7$ cpm/pmol substrate).

In Vitro Cleavage Assays

The cleavage activity of RAG proteins is evaluated using an *in vitro* assay developed in the Gellert laboratory (McBlane *et al.*, 1995). In our laboratory, cleavage reactions are typically assembled by mixing ~50 ng of each individually expressed RAG protein or ~100 ng of coexpressed RAG protein (in a total of 4 μl dialysis buffer [20 m*M* Tris–HCl (pH 8.0), 150 m*M* KCl, 10% glycerol, 2 m*M* DTT]) and RSS substrate (~0.02 pmol) in a 10-μl reaction containing sample buffer [25 m*M* morpholinepropanesulfonic acid (MOPS)-KOH (pH 7.0), 60 m*M* potassium glutamate, 100 μg/ml bovine serum albumin, and 1 m*M* $MgCl_2$ or $MnCl_2$). In some reactions shown in Fig. 2, full-length HMGB1 (300 ng) and/or dimethyl sulfoxide (DMSO; 20%, v/v) has been included. Reactions are incubated for 1 h at 37°, quenched by adding 2 volumes of sample loading solution (95% formamide, 10 m*M* EDTA), and heated to 95° for 2 min. An aliquot of the sample (5 μl) is fractionated on a 15% polyacrylamide [19:1 acrylamide:methylene(bis)acrylamide] sequencing gel containing 7 *M* urea, and the cleavage products are visualized using a phosphorimager (Storm 860; Molecular Dynamics-GE Healthcare, Piscataway, NJ). Reaction products obtained after incubating an intact or nicked 23-RSS substrate with a wild-type or catalytically inactive (D600A) RAG complex in the presence of Mg^{2+} under various conditions are shown in Fig. 2. Two major products are observed after cleavage of an intact 23-RSS with a wild-type RAG complex in the absence of HMGB1: a small product that comigrates with the 16-mer nicked substrate and a product that comigrates with the sequence predicted


Mg2+, no DMSO
Mg2+, with DMSO
HMGB1
D600A cMR1/cMR2
WT cMR1/cMR2
Aberrant nick
%N
%Abnick
%HP
6.6 64
2.9 1.4
0.1 7.2
0.2 5.5
37 20
5.7 1.1
15 22
19 27
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

from aberrant nicking at position 27 (equivalent to nicking the substrate as a 12-RSS). Aberrant nicking of a 12-RSS substrate is not usually seen at high levels (Swanson, 2002a). In the presence of HMGB1, the amount of the nicked product is increased, but the aberrantly nicked product is less abundant, and a third product is observed that comigrates with the predicted 32 nucleotide hairpin sequence (which is not denatured on this gel). Interestingly, in cleavage reactions containing Mg^{2+}, RAG-mediated hairpin formation is increased in the presence of DMSO. Under these conditions, the addition of HMGB1 primarily promotes the correct placement of nicks, but does not enhance hairpin formation dramatically. By substituting $MnCl_2$ for $MgCl_2$ in the cleavage reaction, RAG-mediated hairpin formation can be readily visualized in the absence of HMGB1 or DMSO (McBlane *et al*., 1995).

Electrophoretic Mobility Shift Assays (EMSA)

Binding of the RAG complex to RSS substrates is evaluated by an electrophoretic mobility shift assay using a Hoefer SE400 apparatus. Before starting the experiment, a 4% polyacrylamide gel [29.2:0.8 acrylamide:methylene(bis)acrylamide; stock is filtered through a 0.45-μm cellulose acetate membrane] is poured in 1/2× TBE, allowed to polymerize for about 1 h, and brought to 4° in a cold room. Binding reactions are assembled similarly to cleavage reactions, except that potassium acetate is substituted for potassium glutamate and $MgCl_2$ (or $MnCl_2$) is replaced by $CaCl_2$, which supports RAG-mediated RSS binding, but not cleavage. In addition, DMSO (20%, v/v) is routinely included in the reaction. To assemble complexes on an isolated RSS substrate, reactions are incubated for 10 min at 25°. To form paired RSS complexes, the RAG complex is incubated with HMGB1 (300 mg) and the radiolabeled RSS for 1 min at

FIG. 2. *In vitro* cleavage assay using purified coexpressed core MBP-RAG proteins and purified full-length HMGB1. Intact (lanes 3–7 and 15–19) or nicked (lanes 8–12 and 20–24) 23-RSS substrates were incubated with a catalytically inactive or a wild-type RAG complex (D600A cMR1/cMR2 or WT cMR1/cMR2, respectively) in standard *in vitro* cleavage reactions containing Mg^{2+} in the absence (lanes 3–12) or presence (lanes 15–24) of 20% DMSO, with or without added HMGB1 (300 ng) as indicated above the gel. Samples were fractionated on a 15% polyacrylamide sequencing gel containing 7 *M* urea along with oligonucleotides representing the predicted hairpin (lanes 1 and 13) and aberrantly nicked (lanes 2 and 14) reaction products to serve as markers (positions indicated at left). Reaction products were quantified using a phosphorimager running the ImageQuant software and shown as a percentage of total DNA below the gel. N, 16-mer nicked product; HP, 32 nucleotide hairpin product; Abnick, 27-mer aberrant nicked product.

25°, after which a 50-fold excess of cold partner RSS is added (either intact or nicked, as appropriate). Binding reactions are then incubated at 25° for an additional 10 min. Next, samples are placed on ice for 5 min, and then 4 μl of cold loading solution (25% glycerol, 0.001% bromphenol blue) is added to the side of the tube above the sample. The loading solution is mixed into the sample by gentle flicking and the sample is immediately loaded onto the gel. The mixture is subjected to electrophoresis at 250 V for 1.5 h at 4°. Gels are dried onto Whatman cellulose chromatography paper (0.35 mm; Whatman Inc., Florham Park, NJ) and protein–DNA complexes are visualized using a phosphorimager.

In-Gel Cleavage Assays

We and others have shown that when the RAG proteins are incubated with standard oligonucleotide RSS substrates *in vitro* (either an isolated RSS or RSS pairs), multiple RAG–RSS complexes are detected by EMSA (Hiom and Gellert, 1998; Mundy *et al.*, 2002; Swanson, 2002b). To compare the activity of these discrete protein–DNA complexes on a single nondenaturing polyacrylamide gel directly, an in-gel cleavage assay was developed (Swanson, 2001). In this assay, binding reactions (scaled up fivefold) are assembled in the presence of Ca^{2+} as described in the previous section and fractionated using an EMSA. After electrophoresis is complete, the glass plates are separated so that the gel adheres to one of the plates. Next, a piece of Whatman paper is prewet in cleavage buffer (25 m*M* MOPS-KOH, 60 m*M* potassium glutamate, and 5 m*M* $MgCl_2$ or $MnCl_2$) and placed on top of the gel. The gel is then gently peeled onto the Whatman paper, and both are then submerged in 200 ml cleavage buffer prewarmed to 37° in a Pyrex dish. After incubation for 1 h without shaking, the gel is lifted from the cleavage buffer (using the Whatman paper as a support) and the DNA is transferred to DEAE cellulose chromatography paper (DE81, Whatman) in 1/2× TBE (45 m*M* Tris-borate, 1 m*M* EDTA) using a Bio-Rad trans blot apparatus running at 20 V for 18 h at 4°. After transfer, the DEAE cellulose paper is peeled off the gel together with the Whatman paper to avoid tearing it. The DEAE paper is then placed face down on Saran wrap, and the Whatman paper is separated from the DEAE paper. Next, the DEAE paper is wrapped in Saran wrap, taped onto a piece of Whatman paper, and exposed to film (BioMAX MR, Kodak, New Haven, CT) for several hours at −80°.

The desired bands on the film are cut out and the film is used as a template to cut the DEAE paper (which is easier if the paper remains frozen). The cut strips are placed into 1.5-ml microcentrifuge tubes and 300 μl of TES buffer [10 m*M* Tris–HCl (pH 8.0), 1 *M* NaCl, 1 m*M* EDTA]

is added. After incubation for 30 min at 65°, supernatant is transferred to a centrifuge tube filter (Spin-X 0.22-μm cellulose acetate filter, Costar) and the DEAE strip is placed on the bottom of the filter insert. The tubes are pulsed for 15 s at high speed in an Eppendorf centrifuge, collecting the eluate in the bottom tube. The DEAE strip is then washed with an additional 100 μl of TES buffer. The pooled eluate is transferred to a 1.5-ml microcentrifuge tube and the DNA is precipitated by adding 3 *M* sodium acetate (pH 5.0) (40 μl), 10 μg tRNA as a carrier, and, after vortexing briefly, 100% ethanol (1 ml). After at least 30 min at −80°, the sample is centrifuged at 16,000*g* for 20 min at 25° using an Eppendorf microcentrifuge. The supernatant is decanted, and the inside of the tube is wiped dry with a rolled-up half of a Kimwipe, making sure to avoid the pellet. The pellet and side of the tube are washed with 0.5 ml cold 95% ethanol, taking care to retain the pellet, and the inside of the tube is wiped dry. The pellet is dried in a Savant Speed-Vac for at least several minutes to remove residual ethanol, and the pellet is dissolved in 7 μl of 80% formamide containing 0.001% bromphenol blue. The radioactivity in the samples is measured using the Cerenkov method, normalized, loaded onto a 15% polyacrylamide sequencing gel as described earlier, and the reaction products analyzed using a phosphorimager.

Discussion

RAG proteins are essential for the initiation of V(D)J recombination. However, until relatively recently, little was known about the assembly, organization, composition, and activity of the RAG–RSS complexes involved in this process. In addition, how the RAG proteins recognize DNA and what the relative roles of RAG-1 and RAG-2 are in this process remained unclear. With the development of methods to purify the RAG and HMG-box proteins, and the establishment of assays to examine the binding and cleavage activity of the RAG complexes, significant progress has been made in addressing many of these issues. The interested reader will find a fuller discussion of these advances in Swanson (2004). With purified full-length RAG proteins now available for biochemical analysis, the future promises new advances in our understanding of how noncore portions of RAG-1 and RAG-2 regulate aspects of RAG function, including the activity and fidelity of the RAG complex (Steen *et al.*, 1999; Talukder *et al.*, 2004), the ordering of antigen receptor gene rearrangement (Akamatsu *et al.*, 2003; Dudley *et al.*, 2003; Liang *et al.*, 2002), the suppression of alternative reaction outcomes mediated by the RAG complex (Elkin *et al.*, 2003; Sekiguchi *et al.*, 2001; Swanson *et al.*, 2004; Tsai and Schatz, 2003), the sequestration and degradation of RAG-2 (Corneo *et al.*, 2002;

Jiang *et al*., 2005; Li *et al*., 1996; Mizuta *et al*., 2002; Ross *et al*., 2003), and the ubiquitin ligase activity of RAG-1 (Jones and Gellert, 2003; Yurchenko *et al*., 2003).

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