Adenosine nucleotides affect the ability of RecA single-stranded DNA (ssDNA) nucleoprotein filaments to cooperatively assume and maintain an extended structure that facilitates DNA pairing during recombination. Here we have determined that ADP and ATP/ATPγS affect the DNA binding and aggregation properties of the human RecA homolog human RAD51 protein (hRAD51). These studies have revealed significant differences between hRAD51 and RecA. In the presence of ATPγS, RecA forms a stable complex with ssDNA, while the hRAD51 ssDNA complex is destabilized. Conversely, in the presence of ADP and ATP, the RecA ssDNA complex is unstable, while the hRAD51 ssDNA complex is stabilized. We identified two hRAD51 ssDNA binding forms by gel shift analysis, which were distinct from a well defined RecAsssDNA binding form. The available evidence suggests that a low molecular weight hRAD51-ssDNA binding form (hRAD51-ssDNAlow) correlates with active ADP and ATP processing. A high molecular weight hRAD51-ssDNA aggregate (hRAD51-ssDNAhigh) appears to correlate with a form that fails to process ADP and ATP. Our data are consistent with the notion that hRAD51 is unable to appropriately coordinate ssDNA binding with adenosine nucleotide processing. These observations suggest that other factors may assist hRAD51 in order to mirror RecA recombinational function.

Adenosine nucleotide binding and hydrolysis by the bacterial RecA protein is crucial to mediate the formation, stability, and structure of RecAsssDNA1 (nucleoprotein) filament thought to be the key intermediate in the initiation of recombination in vitro and in vivo (1–3). Although hRAD51 is a homolog of RecA, the extent to which adenosine nucleotides may modulate the formation, structure, and stability of hRAD51 nucleoprotein filaments is unknown.

RecA couples the binding of ADP or ATP/ATPγS with the binding to DNA. In the presence of ATPγS, RecA binds to ssDNA in a highly cooperative, largely irreversible manner and extends the helical pitch of the DNA to ~1.5 times its normal length (4–7). Reciprocally, ssDNA increases the affinity of RecA for ATPγS (8). In the presence of ADP, RecA binding to ssDNA is unstable, and the filament is less extended/inactive (4–6). Similarly, ssDNA decreases the affinity of RecA for ADP (9). In contrast, both ssDNA and dsDNA failed to affect ATPγS binding or ADP release by hRAD51 (10). Moreover, hRAD51 appears to lack ATP-induced cooperativity during ATP hydrolysis (11) and/or the ability to form extended/active hRAD51-ssDNA nucleoprotein filaments in the presence of ATPγS (12). These data suggest that hRAD51 differs significantly from RecA in its ability to couple ADP and ATP/ATPγS processing with ssDNA interactions.

The ability of RecA to self-associate in the absence of DNA adds to the complexity of RecA-ssDNA interactions. Several methods, including gel filtration chromatography, electron microscopy, light scattering, and analytical ultracentrifugation, have demonstrated that in the absence of DNA, RecA exists as dimers, trimers, small planar hexameric-to-octameric rings, and short rods (19–15). Larger rods/filaments and bundles of filaments form in the presence of cations (magnesium or spermidine) or concentrated solutions of RecA (16). While the ability to self-associate appears to facilitate cooperative RecA nucleoprotein filament assembly (17), the exact species of RecA that binds to or dissociates from DNA is unknown. Moreover, beyond a certain threshold of RecA self-association, DNA binding is significantly reduced (18). Efficient DNA binding by RecA appears to involve prior disassembly of self-aggregates to a relatively smaller species (18–20). Both ATP and ADP reduce the size of self-aggregates (14, 16), generating polymers ranging from hexamers to dodecamers in size (13). However, only ATP (ATPγS) efficiently activates RecA DNA binding activity (4–6). Little is known about the effect of ADP, ATP, or ATPγS on the conformation of hRAD51, its ability to form self-polymer molecules, or its ability to assume and maintain an active hRAD51-ssDNA nucleoprotein filament.

Predicting the coordinate self-association, nucleotide binding/hydrolysis, and ssDNA binding activity of hRAD51 based on a comparison with the RecA protein structure is not straightforward. The RecA crystal structure indicated that residues involved in self-association, adenosine nucleotide binding/hydrolysis, and DNA binding appeared juxtaposed and coordinated (21, 22). Genetic studies indicated that RecA residues His97, Lys216, Phe217, Arg222, and Lys248 are involved in monomer-monomer contacts and are also proximal to the ADP binding pocket as well as the unresolved loops implicated in DNA binding (23, 24). It has been proposed that RecA residues Gln194 and Arg196 interact with the γ-phosphate of ATP to coordinate DNA binding by loop L2 (residues 195–210) with ATP hydrolysis (25, 26). Moreover, Hörttagel et al. (26) suggested that Arg196 may participate catalytically in ATP hydrolysis in a manner similar to the "arginine finger" of GTPase activator proteins (27). These residues appear critical for biological activity and probably function as allosteric effec-
tors of the ATP-induced active form of the RecA nucleoprotein filament (23, 24).

Although these residues are highly conserved in the eubacterial RecA family, an entirely different set of residues may substitute for their functions in the eukaryotic RAD51 members (28). Only Arg222 and Gln194 appear conserved between RecA and hRAD51. In addition, hRAD51 protein has an extended amino terminus and truncated carboxyl terminus relative to the bacterial RecA (28, 29). The amino terminus of RecA forms extensive contacts between neighboring protomers within a filament and is critical for self-association (30), while the carboxyl terminus of RecA exists as a distinct domain that appears to modulate DNA binding (31, 32). Interestingly, a study that combined NMR and mutagenesis indicated that the extended amino terminus of hRAD51 may functionally replace the carboxyl-terminal domain of RecA (33).

Despite these differences, preliminary electron microscopy data have indicated that hRAD51 forms planar hexameric-to-octameric ring structures in the absence of DNA (34). In addition, nucleoprotein filaments formed in the presence of ATP·SH with ssDNA grossly resemble inactive/compact RecA nucleoprotein filaments (12). In contrast, neutron scattering performed on Xenopus laevis RAD51 suggested that an extended filament may form on ssDNA in the presence of ATP or ADP but less well with ATP·S (35). Interestingly, hRAD51 forms an extended nucleoprotein filament with the transition state mimetic ADP·AlF_4^−, which appears analogous to activated RecA (36). Both RecA and hRAD51 facilitate ATP-dependent DNA strand exchange. However, unlike RecA, hRAD51 could not utilize ATP·S for DNA strand exchange (37).

Here we have examined the effects of ADP, ATP, and ATP·S on hRAD51 ssDNA binding activity. We find significant differences in the effects of these adenosine nucleotides on the ssDNA binding activity of hRAD51 compared with RecA. Unlike RecA, hRAD51 binding of ADP, ATP, or ATP·S is not coupled to modulation of ssDNA binding activity. In addition, hRAD51 appears largely unable to discriminate ADP, ATP, or ATP·S on hRAD51 ssDNA binding activity. We find significant differences in the effects of these adenosine nucleotides on the ssDNA binding activity of hRAD51 compared with RecA. Unlike RecA, hRAD51 binding of ADP, ATP, or ATP·S is not coupled to modulation of ssDNA binding activity. In addition, hRAD51 appears largely unable to discriminate ADP, ATP, or ATP·S. It is likely that the inability of RAD51 to efficiently process adenosine nucleotides underpins the lack of coordination between protomers during ATP hydrolysis. We propose that inefficient ATP processing contributes to a reduced rate of DNA strand exchange and a dramatically reduced ability to bypass heterologous DNA during strand exchange (38–41).

### MATERIALS AND METHODS

**Reagents—**Chemicals of the highest grade were obtained from Amresco or Sigma. ADP and ATP were purchased from Amersham Biosciences and processed as described (11). ATP·S was purchased from Roche Molecular Biochemicals. Sequencing grade endoprotease Lys-C and trypsin were purchased from Promega or Roche Molecular Biochemicals and resuspended in modified H buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 10% glycerol, without EDTA) at a stock concentration of 200 ng/ml just prior to use. Oligo(dT)_12·18 was synthesized at the Kimmel Nucleic Acids Facility. IAsys cuvettes coated with biotin were purchased from Affinity Sensors (Cambridge, UK), and 5' 3' biotinylated oligo(dT)_12·18 was purchased from Glenn Research (Sterling, VA). hRAD51 was purified as described (11). RecA protein was purchased from U.S. Biochemical Corp.

**Partial Proteolysis—**Approximately 10 μg of hRAD51 was incubated in the standard buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 1% glycerol, 1 mM dithiothreitol, 0.1 mM EDTA) in 50 μl with 10 mM magnesium acetate and 1 mM ATP, ADP, or ATP·S at 37 °C for 30 min in an Eppendorf thermal cycler with heated lid to prevent evaporation. Then 6-μl aliquots were added to 6 μl of various protease dilutions. Shown are digests containing either 220 ng of endoprotease Lys-C or 220 ng of trypsin. Protease incubations continued at 37 °C for an additional 60 min. Reactions were stopped by the addition of 4 μl of 4X loading buffer (0.2 μl Tris·HCl (pH 7.0), 8% SDS, 20%  β-mercaptoethanol, 20% sucrose, 2 mg/ml bromophenol blue) and followed by boiling. Proteolytic products of hRAD51 were resolved by 20% SDS-PAGE and silver-stained. Gels were wrapped in clear plastic wrap and scanned directly with an Epson scanner.

**Analytical Gel Filtration—**100–200 μl of 8 μM hRAD51 was preincubated at 37 °C for 60 min in modified H buffer with 2 mM magnesium acetate without nucleotide or with 20 mM ATP·S or 20 μM ADP. A Superose-12 column (10/30; Amersham Biosciences) was equilibrated in the same buffer. hRAD51 was eluted at a flow rate of 24 ml/h, and 0.5-ml fractions were collected. 1 μg of acetylated bovine serum albumin (New England Biolabs) and 0.5 ml of 1% trichloroacetic acid were added to each fraction, followed by overnight precipitation at −20 °C. The precipitated proteins were then pelleted, washed with ice-cold acetone, air-dried, and resuspended in loading buffer (0.05 μl Tris·HCl (pH 7.0), 2% SDS, 5% β-mercaptoethanol, 5% sucrose, 0.5 mg/ml bromophenol blue). Approximately half of the precipitate was resolved by 10% SDS-PAGE and silver-stained.

**IAsys Bioassay Studies—**IAsys bioassay (IAB) studies were performed using an IAsys Auto Reader (Affinity Sensors, Cambridge, UK). A model oligonucleotide (oligo(dT)_12·18) with either the 3'-end or 5'-end biotinylated (Glen Research; Sterling, VA) was attached via streptavidin to the surface of an IAsys SPR cuvette precoated with biotin (Affinity Sensors). The kinetics of hRAD51 DNA binding were measured after the cuvettes were equilibrated in H buffer containing the indicated amount of magnesium. The binding constant, k_b, was obtained directly from the slope of a plot of the k_b determined at multiple hRAD51 protein concentrations. Dividing the k_b determined experimentally via IAB and averaged from several protein concentrations, by the k_b results in the K_b (K_b = k_b/k_h). This method was also used by DeZutter et al. (42) to determine the K_b for hRAD51 binding to an 83-mer random sequence oligonucleotide.

**Gel Shifts—**Oligo(dT)_12·18 binding was performed in H buffer with 250 nM (nucleotide equivalents) of oligo(dT)_12·18 and the indicated amount of hRAD51. Reactions were incubated as indicated in each figure legend and resolved by 4% nondenaturing PAGE (acylamide/bisacrylamide, 37.5:1; Amresco) containing 0.5% glycerol. The system was buffered with 1X TBE, and 30-mM constant current was applied to each gel for ~2.5–3 h. The gels were placed on Whatman No. 3MM paper, exposed directly (without drying) to PhosphorImager screens (Molecular Dynamics, Inc., Sunnyvale, CA), and scanned after overnight exposure. Care was taken not to disrupt hRAD51-DNA aggregates remaining in the wells. We found that if the gel is sufficiently wrapped to avoid contamination of the PhosphorImager screen, it may be exposed without drying, and all of the aggregate species will be retained.

**Sedimentation Assay—**hRAD51 (0.5 μM) was incubated with 5' end-labeled oligo(dT)_12·18 (50 nM nucleotide equivalents) in 20 μl for 60 min at 37 °C in the standard H buffer with 10 mM magnesium acetate in the absence of nucleotide or with either 20 or 500 μM of each adenosine nucleotide added, as indicated in the figure legend. Each reaction was centrifuged in a microcentrifuge at 14,000 rpm (~16,000 × g) for 30 min. The supernatant (19 μl) was removed carefully and mixed with 5 ml of Scintiverse mixture (Fisher) and counted. To the pellet, 10 μl of 10% SDS was added, and the sample was then vortexed, boiled, mixed with 5 ml of Scintiverse, and counted.

### RESULTS

**DNA Binding by hRAD51**

**IAsys Bioassay Studies**—IAB is capable of detecting real time interactions between molecules. We have developed a system in which a model oligonucleotide (oligo(dT)_12·18) is biotinylated at either the 3'- or 5'-end and attached via a streptavidin linkage to an IAB cuvette coated with biotin. We obtained K_b values for hRAD51-oligo(dT)_12·18 binding in the range of 78–176 nM depending upon the magnesium concentration or whether the 5'- or 3'-biotinylated substrate was
These results largely resemble a previous study that measured the interaction of hRAD51 with ssDNA (a random sequence 83-mer oligonucleotide) by IAB (42). Modest differences between these studies may be attributed to the size and/or nature of the secondary structure present in the two substrate DNAs or the different buffer conditions. We observed that 3’-biotinylated oligo(dT)$_{50}$ (3’-end attached to the IAB cuvette surface) consistently displayed a lower $K_D$ that could be largely attributed to an increased $k_a$. These results suggest that hRAD51 can bind ssDNA displaying either a free 5’-end or 3’-end but that it exhibited a modest preference for binding DNA displaying a free 5’-end.

hRAD51 (maintained at 4°C) readily bound the oligo(dT)$_{50}$ IAB DNA substrate in the absence of adenosine nucleotide as well as in the presence of ADP or ATP ($B_{max} \approx 1000$ arc sec for each; Fig. 1A). Moderately less efficient binding was observed in the presence of ATP$_S$ ($B_{max} \approx 500$ arc sec; Fig. 1A). When hRAD51 was preincubated at 37°C for 30 min in the absence of adenosine nucleotide, it failed to bind the oligo(dT)$_{50}$ IAB DNA substrate ($B_{max} < 100$ arc sec; Fig. 1, compare A and C). However, when ADP, ATP, or ATP$_S$ was present during the preincubation, binding to the oligo(dT)$_{50}$ IAB DNA substrate by hRAD51 preincubated at 37°C appeared almost identical to the binding displayed by hRAD51, which was maintained at 4°C ($B_{max} \approx 1000$ arc sec; Fig. 1, compare A and C). These observations suggest that incubation of hRAD51 at 37°C results in a form that is largely incapable of ssDNA binding.

We compared the binding of the oligo(dT)$_{50}$ IAB DNA substrate by hRAD51 to the binding of this substrate by RecA. ATP$_S$ promoted the most stable interaction of RecA with the oligo(dT)$_{50}$ IAB DNA substrate ($B_{max} \approx 900$ arc sec; Fig. 2B). RecA binding in the absence of adenosine nucleotide was slightly less efficient ($B_{max} \approx 500$ arc sec; Fig. 1B). Under our conditions (150 mM NaCl), RecA failed to bind the oligo(dT)$_{50}$ IAB DNA substrate in the presence of ATP or ADP ($B_{max} < 100$ arc sec; Fig. 1B). These results are qualitatively and quantitatively similar to previously published reports (4–7, 42). Moreover, RecA binding appeared identical regardless of the preincubation conditions (Fig. 1, compare B with D). These observations underline the inherent differences in ssDNA binding between RecA and hRAD51.

We determined the amount of ADP (IC$_{50} \approx 1 \mu M$) or ATP (IC$_{50} \approx 0.2 \mu M$) required to preserve half-maximal hRAD51:oligo(dT)$_{50}$ IAB DNA binding activity (Fig. 2). The IC$_{50}$ values appear significantly less than the binding constant determined...
for ADP or ATPγS to hRAD51 (K_D ≈ 5 μM) (10). These data appear to suggest that only a subset of hRAD51 protomers must be bound by ADP or ATP to preserve DNA binding activity. AMP did not significantly preserve DNA binding (data not shown), suggesting that the β-phosphate of ADP is minimally required to preserve oligo(dT)_50 IAB DNA binding activity. These observations are consistent with a significantly reduced affinity of hRAD51 for AMP (10).

### Gel Shift Studies

The effect of ADP, ATP, or ATPγS on the interaction of hRAD51 with oligo(dT)_50 was also examined by gel shift analysis. hRAD51 appears to form at least two qualitatively different forms when bound to the model oligo(dT)_50 DNA.

![Gel shift analysis of hRAD51 and RecA binding to oligo(dT)_50](image)

**FIG. 3.** Gel shift analysis of hRAD51 and RecA binding to oligo(dT)_50. A, the indicated amount of hRAD51 (μM) was incubated with oligo(dT)_50 (250 nM) for 60 min at 37 °C in the absence or presence of 1 mM ADP, ATP, or ATPγS and then resolved by 4% non-denaturing PAGE. B, reactions are identical to A except that hRAD51 was preincubated in the absence or presence of adenosine nucleotide for 30 min at 37 °C and subsequently mixed with oligo(dT)_50. These were incubated at 37 °C for an additional 20 min and then resolved by 4% non-denaturing PAGE. C, reactions were identical to A except that the indicated amount of RecA (μM) was substituted for hRAD51. Two different hRAD51-oligo(dT)_50 complexes are indicated by low and high; an asterisk indicates free oligo(dT)_50 DNA.

**FIG. 4.** Saturation of the hRAD51 high affinity adenosine nucleotide binding site (K_D ≈ 5 μM) enhances the formation of the hRAD51-DNA species. hRAD51 (0.3 μM) was mixed with the indicated amount of each adenosine nucleotide and 250 nM oligo(dT)_50 (nucleotides), incubated at 37 °C for 30 min, and subsequently resolved by 4% non-denaturing PAGE. An asterisk indicates the position of free oligo(dT)_50 DNA.
ssDNA substrate, which we denote hRAD51-DA_{low} and hRAD51-DA_{high} (Fig. 3, A and B). Moreover, the migration of the hRAD51 forms appears distinct from RecA-oligo(dT)_{50} gel shift complexes produced under identical conditions (compare Fig. 3, A and B, with Fig. 3C). The structure of these forms is unknown. The hRAD51-DA_{low} form appears to be a low molecular weight complex that migrates slightly more slowly than free oligo(dT)_{50} (denoted by an asterisk; Fig. 3, A and B). The hRAD51-DA_{high} form is a significantly larger complex (Fig. 3, A and B). The relative amount of these forms appears to be modulated by ADP, ATP, and ATP*S. In the absence of ADP, ATP, and ATP*S, hRAD51-DA_{low} is the singular gel shift form (Fig. 3A, lanes 1–5). In contrast, the addition of 1 mM ADP, ATP, and ATP*S induces the formation of hRAD51-DA_{high} (Fig. 3A, lanes 6–10). The relative amount of the hRAD51-DA_{high} form depends on the type of adenosine nucleotide, where ADP > ATP*S > ATP (Fig. 3A, lanes 6–10 > lanes 16–20 >> lanes 11–15). By quantitating the ratio of free DNA to bound DNA (where bound represents both forms), we calculate a $K_{D_{hRAD51}} \approx 0.3 \mu M$ for binding to the oligo(dT)_{50} ssDNA substrate in the presence of ADP or ATP or in the absence of adenosine nucleotide (Fig. 3A, lanes 6–10, lanes 11–15, or lanes 1–5). In the presence of ATP*S, hRAD51 displayed a reduced affinity for the oligo(dT)_{50} ssDNA substrate ($K_{D_{hRAD51}} \approx 0.5 \mu M$; Fig. 3A, lanes 16–20).

Gel shift analysis following preincubation of hRAD51 at 37°C in the absence or presence of ADP, ATP, or ATP*S also resulted in a DNA binding pattern qualitatively similar to IAB (compare Fig. 1, A and C, with Fig. 3, A and B). In the absence of adenosine nucleotide, hRAD51 appeared to be largely inactivated, since DNA binding was only observed at very high concentrations of hRAD51 (Fig. 3B, lanes 1–5). Under these conditions, the $K_{D_{hRAD51}}$ did not appear to change when nucleotide was included (similar to IAB). However, a significant increase in the hRAD51-DA_{high} form and coincident decrease in the hRAD51-DA_{low} form occurred in the presence of ADP (Fig. 3B, lanes 6–10). A similar but less striking effect occurred in the presence of ATP*S (Fig. 3B, lanes 16–20). In the presence of ATP, the distribution between forms appeared to remain unchanged (Fig. 3B, lanes 11–15).

We examined the effect of ADP, ATP, and ATP*S on hRAD51 binding to the oligo(dT)_{50} ssDNA substrate by gel shift analysis. These studies were performed at a concentration of hRAD51 (0.3 \mu M) that largely generated the hRAD51-DA_{low} form and minimized the production of aggregates (hRAD51-DA_{high}). We found that ADP, ATP, or ATP*S enhanced the formation of hRAD51-DA_{low} ($K_{D_{NNUC}} \approx 1 \mu M$; Fig. 4, A–C). Above 250 \mu M ADP, ATP, or ATP*S, the effect of these nucleotides appears more complex (Fig. 4). ADP promotes the production of the hRAD51-DA_{high} form (Fig. 4A). ATP (Fig. 4B) and ATP*S (Fig. 4C) promote the dissociation of a fraction of the hRAD51-DA_{low} form. Both gel shift analysis and IAB confirm the apparent equivalent high affinity of adenosine nucleotides for hRAD51. These results also suggest that the type of hRAD51-ssDNA form generated is influenced by adenosine nucleotides. Interestingly, dsDNA does not appear to form hRAD51-DA_{high} structures similar to ssDNA (data not shown).

Our previous studies have demonstrated that binding of ADP to hRAD51 displayed two distinct modes: one mode with a high affinity for ADP ($K_{app1} \approx 5 \mu M$), which appeared competitive with ATP, and a second low affinity mode ($K_{app2} \approx 125 \mu M$) (10). The first mode ($K_{app1} \approx 5 \mu M$) appeared to be competent for ADP → ATP exchange with a $t_{1/2} \approx 30$ s, whereas the second mode ($K_{app2} \approx 125 \mu M$) appeared refractory for ADP → ATP exchange (10). We found that the formation of hRAD51-DA_{high} correlated well with the expected $K_{D_{APP2}} \approx 125 \mu M$ (Fig. 5A). Furthermore, there appears to be a transition from hRAD51-DA_{low} to hRAD51-DA_{high} in our gel shift analysis with increasing ADP saturation (Fig. 5A). When 5 mM ATP*S was added subsequent to ADP in these DNA-binding reactions, the hRAD51-DA_{low} form dissociated, while the hRAD51-DA_{high} form remained unchanged (Fig. 5, compare A and B). Moreover, once formed, hRAD51-DA_{high} aggregate appears stable and irreversible (data not shown). Taken together, these results demonstrate that ADP, ATP, and ATP*S modulate the hRAD51-DA_{low} form. However, the hRAD51-DA_{high} form is likely to be a “dead end” complex that may only be significant for in vitro assays.

The apparent replacement of ADP by ATP*S that resulted in the destabilization of hRAD51-DA_{low} occurred with a $K_{ADP/ATP} \approx 100 \mu M$ and translated to a ratio of 1 ADP/4–5 ATP*S (Fig. 5C). ATP appeared significantly less effective in destabilizing the hRAD51-DA_{low} form ($K_{ATP} \approx 250 \mu M$; Fig. 5D). The amount of ATP (ATP*S) required to destabilize the hRAD51-DA_{low} form generated in the presence of ADP is similar to that required for the destabilization of hRAD51-DA_{high} in the absence of ADP (Fig. 4, B and C). These results suggest that a specific ratio of ADP/ATP (ATP*S) may not be required for efficient dissociation.

**Sedimentation Assay**—The RecA protein has been found to form aggregates and co-aggregates with ssDNA and dsDNA. The RecA/DNA co-aggregates appeared to be intermediates in the homologous pairing process and could be easily pelleted (43). Although a comparison between these structures and the hRAD51-DA_{high} aggregates would be premature, we reasoned that a similar co-sedimentation assay in the presence of ADP, ATP, or ATP*S would be useful for the analysis of the hRAD51-DA_{high} form. The addition of hRAD51 resulted in co-sedimentation of the $^{32}$P-labeled oligo(dT)_{50} ssDNA substrate in the presence of 500 \mu M ADP (Fig. 6B). No co-sedimentation was observed in the absence of adenosine nucleotide (Fig. 6A), in the presence of 20 \mu M ADP (Fig. 6B), or in the presence of either 20 or 500 \mu M ATP/ATP*S (Fig. 6, C and D). These results are consistent with the hRAD51 oligo(dT)_{50} ssDNA gel shift analysis and suggest that hRAD51 forms a hRAD51-ssDNA aggregate at supersaturating concentrations of ADP.

**Adenosine Nucleotides Induce Conformational Transitions in hRAD51**

Partial proteolysis has been a useful method for determining conformational transitions associated with nucleotide-binding proteins, including RecA (44). hRAD51 was preincubated with ADP, ATP, or ATP*S and subsequently exposed to a limiting amount of either endoprotease Lys-C or trypsin. Partial proteolysis by both endoprotease Lys-C and trypsin suggest that ADP, ATP, and ATP*S induced conformational transitions in hRAD51 that were distinct from the pattern exhibited in the absence of adenosine nucleotide (Fig. 7, A and B). Endoprotease Lys-C generates two distinct peptides (denoted by arrows in Fig. 7A) of 15–20 kDa that were evident when hRAD51 was incubated with buffer only but not detectable in the presence of ADP, ATP, or ATP*S. Partial trypsin digestion generated two distinct peptides of 25–30 kDa (see arrows in Fig. 7B) that were prominent when ADP, ATP, or ATP*S was added but not detectable in the presence of buffer alone. The specific location of these peptides within the hRAD51 protein is currently under investigation. The appearance of these partial proteolysis peptides corresponded with the ADP, ATP, and ATP*S binding affinity ($K_{D} \approx 5 \mu M$; data not shown), and the peptide banding pattern did not appear to differ between adenosine nucleotides. In the absence of protease only full-length hRAD51 was ob-
served, and in the absence of hRAD51 there were no peptide products (data not shown). These results indicate that ADP, ATP, or ATPγS affects the susceptibility of hRAD51 to protease, suggesting alternate conformations.

**Adenosine Nucleotides Affect hRAD51 Self-association**

Analytical gel filtration chromatography has been used to determine higher order protomer complexes of the bacterial RecA protein (15). In the absence of adenosine nucleotides, analytical gel filtration at 4°C suggests that hRAD51 self-associates to form a protein species that appears to migrate with the relative molecular weight of hexamers to dodecamers (data not shown). These protein species may resemble the hRAD51 hexameric/octameric rings observed previously by electron microscopy (34). In contrast, incubation of hRAD51 at 37°C in the absence of adenosine nucleotide results in an apparent high molecular weight species of hRAD51 (Fig. 7C). A fraction of these complexes are found in the void volume of the gel filtration column that was determined to exclude proteins of ~1 MDa. This result suggests that at 37°C in the absence of adenosine nucleotide, hRAD51 forms higher order self-association complexes similar to RecA. The detailed structure of these complexes is unknown. It is interesting to note that our chromatography buffer contained 150 mM NaCl, which is inhibitory to the self-association/polymerization of RecA (15). The ability of hRAD51 to aggregate in the presence of physiological salt concentration appears similar to C-terminally truncated mutants of RecA (45) and *X. laevis* RAD51 (35). These results suggest that hRAD51 may possess an increased intrinsic potential to aggregate.

Chromatography of hRAD51 in the presence of either 20 μM ADP or ATPγS results in protein species that appear to elute with an average molecular mass of 100–250 kDa, corresponding to 3–8 hRAD51 monomers (Fig. 7C). These results suggest that the addition of ADP or ATPγS significantly reduced the size of the nucleotide-free hRAD51 aggregates. The amount of ADP or ATPγS required for this reduction correlated with the previously identified hRAD51 high affinity binding mode (KD ~ 5 μM; Fig.
Adenosine nucleotides affect hRAD51 sensitivity to limited proteolysis and the ability to self-associate.

Limited proteolysis (A and B) and analytical gel filtration (C) were performed to examine the effects of adenosine nucleotides on hRAD51 structure. For proteolysis, ~10 µg of hRAD51 was preincubated at 37 °C for 30 min in the absence or presence of a 1 mM concentration of the indicated adenosine nucleotide. These were subsequently divided into two 5-µg portions and treated with either endoproteinase Lys-C (A) or sequencing grade trypsin (B). Proteolysis products were resolved by 20% SDS-PAGE followed by silver staining. C, hRAD51 (8 µM) was preincubated at 37 °C for 60 min in the absence or presence of 20 µM ADP or ATPγS and subsequently resolved by elution through a Superose 12 column equilibrated with the same buffer. Each fraction was precipitated with trichloroacetic acid and analyzed by 10% SDS-PAGE followed by silver staining. Relative molecular weights were determined with gel filtration standards (Bio-Rad) and are indicated above the fraction numbers.

Fig. 6. hRAD51 and oligo(dT)50 form large complexes in the presence of high ADP concentrations. To further characterize hRAD51-oligo(dT)50 complexes, the ability to co-sediment in the absence or presence of adenosine nucleotides was examined. hRAD51 (5–500 nM) and oligo(dT)50 (50 nM) were incubated at 37 °C for 60 min in the absence of nucleotide (A) or in the presence of 20 or 500 µM ADP (B), ATP (C), or ATPγS (D). After centrifugation at 16,000 × g for 30 min at room temperature, counts retained in the supernatant (sup) and pellet were determined. Each point represents the average of three experiments.
7C and Ref. 10). These results suggest that both ADP and ATP are capable of modulating the size of hRAD51 polymers.

**DISCUSSION**

Bacterial RecA protein cooperatively binds and hydrolyzes ATP in a process that is intrinsically linked to the structure/stability of a RecA-ssDNA nucleoprotein filament (1, 3). These observations have been described in terms of a “coupled cycle.” Several studies have shown that a tight association exists between ATP/ATPγS-bound RecA and ssDNA and a weak association exists between ADP-bound RecA and ssDNA (1, 3). Cooperative interactions between ATP-bound RecA protomers within this nucleoprotein filament appear to facilitate ADP → ATP exchange and drive the RecA ATPase cycle. The hRAD51 ATPase lacks the magnitude of ATP-induced cooperativity displayed by RecA (11). Moreover, ssDNA does not affect the affinity of hRAD51 for ADP or ATP/ATPγS (10). These observations have suggested that hRAD51 may not couple adenosine nucleotide processing and DNA binding similar to RecA.

We have examined the effect of ADP, ATP, or ATPγS on hRAD51 ssDNA binding by IAB as well as gel shift (GS) analysis. In the absence of adenosine nucleotide, hRAD51 exhibited an enhanced affinity for a model oligo(dT)₃₀ ssDNA substrate compared with RecA. The effect of ADP, ATP, and ATPγS on hRAD51 ssDNA binding appeared nearly opposite to RecA. Unlike RecA, and consistent with a previous report, hRAD51 displayed reduced affinity for ssDNA in the presence of ATP/γS (42). We now report that hRAD51 ssDNA binding was unaffected by ADP and ATP. This contrasts with the reduced or absent RecA ssDNA binding activity in the presence of ADP and ATP (see Figs. 1 and 3) (4–7, 42). These results suggest a fundamental difference between RecA and hRAD51 in the effect of ADP, ATP, and ATPγS on ssDNA binding functions.

A temperature-dependent inactivation of hRAD51 ssDNA binding activity was observed using both IAB and GS analysis. *Saccharomyces cerevisiae* RAD51 also exhibits a temperature-dependent inactivation of ssDNA binding activity (46). Maintaining the yeast RAD51 in the presence of ATP (Kₘ₆₇₈ ≈ 20 µM) or ADP (Kₘ₆₇₈ ≈ 250 µM) appeared to preserve the ssDNA binding activity (46). In contrast, hRAD51 required significantly less ATP (Kₘ₆₇₈ ≈ 0.1 µM) or ADP (Kₘ₆₇₈ ≈ 5.0 µM) to preserve ssDNA binding activity. The amount of ATP required to preserve hRAD51 ssDNA binding activity is 20-fold below the Kₘ₆₇₈. These data suggest that preservation of hRAD51 ssDNA binding activity at elevated temperatures does not require saturation of the protein with ATP. The difference between ATP and ADP preservation of hRAD51 ssDNA binding activity is unknown.

The temperature inactivation of hRAD51 ssDNA binding activity in the absence of ADP, ATP, or ATPγS correlates with the appearance of a high molecular mass (≥760 kDa) aggregate by gel filtration (see Fig. 7C). In the presence of ADP and ATPγS, hRAD51 is resolved into smaller complexes (~40–200 kDa) that correlate with ssDNA binding activity (see Fig. 7C). These results are consistent with the notion of self-aggregation of hRAD51 in the absence of adenosine nucleotide. An aggregation effect that is competitive with ssDNA binding has been observed with RecA (18, 19). In the case of hRAD51, the aggregate appears irreversible and is only likely to be relevant to in vitro assays. It is also theoretically possible that the ADP-dependent irreversible self-association/aggregation of hRAD51 may be modulated by other protein factors.

GS analysis of hRAD51-ssDNA complexes identified two qualitatively different hRAD51-oligo(dT)₃₀ complexes: a fast mobility, apparently low molecular weight form (denoted hRAD51-DNAₙₙₙₙ) and a slow mobility, apparently high molecular weight aggregate form (denoted hRAD51-DNAₙₙₙₙ). While the precise nature of these forms is unknown, the mobilities of the hRAD51-ssDNA forms are distinct from the RecA-ssDNA complex. Since the molecular weights of RecA and hRAD51 are equivalent, it is tempting to speculate that there is significantly less hRAD51 protein in the hRAD51-DNAₙₙₙₙ form than RecA protein in the RecA-ssDNA complex.

There does not appear to be a clear correlation between IAB hRAD51-ssDNA complexes and GS hRAD51-ssDNA forms. In general, it appears that the IAB hRAD51-ssDNA complexes are representative of both the GS hRAD51-DNAₙₙₙₙ and hRAD51-DNAₙₙₙₙ forms. The cumulative GS binding of hRAD51-ssDNA in both the low and high mobility forms appears to be equivalent to the results obtained with IAB. However, we have found that only the hRAD51-DNAₙₙₙₙ form appears capable of adenosine nucleotide processing. For example, the subsequent addition of ATPγS dissociates the hRAD51-DNAₙₙₙₙ form but not the hRAD51-DNAₙₙₙₙ form (see Fig. 5). In addition, ADP → ATP exchange can be measured at low ADP concentrations (20 µM) when the hRAD51-DNAₙₙₙₙ form appears to be exclusive (10). However, we were unable to detect ADP → ATP exchange by hRAD51 at high ADP concentrations (200 µM) when it is largely in the hRAD51-DNAₙₙₙₙ form (10). These results are consistent with the notion that the hRAD51-DNAₙₙₙₙ form is active for both adenosine nucleotide processing and ssDNA binding. It is likely that the hRAD51-DNAₙₙₙₙ form represents an aggregate structure. This conclusion is based on its inability to enter the gel matrix and our finding that it can be pelleted by centrifugation. Since this hRAD51-DNAₙₙₙₙ form appears irreversible and correlates with a secondary nonsaturable mode of ADP binding (see Ref. 10), it is likely to be a nonspecific aggregate that is only important for in vitro studies where incubation times exceed 1 h or where the formation of significant levels of ADP occur. Such conditions may be routinely used in hRAD51-mediated strand exchange studies.

RecA efficiently discriminates ADP, ATP, and ATPγS (1). This discrimination is manifest in cooperative ATP hydrolysis and recombinational strand exchange. In contrast, hRAD51 is unable to adequately differentiate ADP, ATP, and ATPγS. IAB and GS studies indicate that hRAD51 forms similar ssDNA complexes in the presence of all three of these adenosine nucleotides (below 150 µM). In addition, the hRAD51 binding constant (Kₘ₆₇₈ or Kₘ₆₇₈) for ATP/ATPγS and ADP is equivalent. Under similar circumstances, RecA is capable of discriminating between ADP and ATP/ATPγS as well as between ATP and ATPγS. The inability to efficiently discriminate between adenosine nucleotides provides a foundation for the idea that hRAD51 function(s) requires additional protein factors to duplicate RecA function(s). Potential candidates for hRAD51 co-factor(s) are one or all of the known mitotic or meiotic human RecA homologs: hRAD51B, hRAD51C, hRAD51D, XRCC2, XRCC3, and hDMC1 (47–49).

Acknowledgments—We thank Hans-Jürg Alder and the Kimmel Nuclear Acids Facility for oligonucleotide synthesis and sequencing, and we thank Chris Schmutte, Samir Acharya, Scott Gradia, and Kristine Yoder for helpful discussions and careful review of the manuscript.

REFERENCES

1. Kowalczykowski, S. C. (1991) Annu. Rev. Biophys. Biophys. Chem. 20, 19–39.
2. Konola, J. T., Logan, K. M., and Knight, K. L. (1994) J. Mol. Biol. 237, 29–34.
3. Roca, A. I., and Cox, M. M. (1997) Prog. Nucleic Acid Res. Mol. Biol. 56, 129–223.
4. McIntee, K., Weinstock, G. M., and Lehman, I. R. (1981) J. Biol. Chem. 256, 8835–8844.
5. Bryant, F. R., Taylor, A. R., and Lehman, I. R. (1985) J. Biol. Chem. 260, 1196–1202.
6. Menetcki, J. P., and Kowalczykowski, S. C. (1985) J. Mol. Biol. 181, 281–295.
7. Stasiak, A., and Egelman, E. H. (1988) in Genetic Recombination (Kucherlapati, R., and Smith, G. R., eds) pp. 265–307, American Society for Microbiology, Washington, D. C.
14442

hRAD51 DNA Binding

8. Weinstock, G. M., McEntee, K., and Lehman, I. R. (1981) J. Biol. Chem. 256, 8850–8855
9. Cotterill, S. M., Satterwait, A. C., and Fersht, A. R. (1982) Biochemistry 21, 4332–4337
10. Tomblin, G., Shim, K. S., and Fishel, R. (2002) J. Biol. Chem. 277, 14426–14433
11. Tomblin, G., and Fishel, R. (2002) J. Biol. Chem. 277, 14417–14425
12. Benson, F. E., Stasiak, A., and West, S. C. (1994) EMBO J. 13, 5764–5771
13. Brenner, S. L., Zlotnick, A., and Stafford, W. F., III (1990) J. Mol. Biol. 216, 949–964
14. Cotterill, S. M., and Fersht, A. R. (1983) Biochemistry 22, 3525–3531
15. Kuramitsu, S., Hamaguchi, K., Ogawa, T., and Ogawa, H. (1981) J. Biochem. (Tokyo) 90, 1033–1045
16. Brenner, S. L., Zlotnick, A., and Griffith, J. D. (1988) J. Mol. Biol. 204, 959–972
17. Takahashi, M. (1989) J. Biol. Chem. 264, 288–295
18. Morrical, S. W., and Cox, M. M. (1985) Biochemistry 24, 760–767
19. Benight, A. S., Wilson, D. H., Budzynski, D. M., and Goldstein, R. F. (1991) Biochimie 73, 143–155
20. Masui, R., Mikawa, T., Kato, R., and Kuramitsu, S. (1998) Biochemistry 37, 14788–14797
21. Story, R. M., Weber, I. T., and Steitz, T. A. (1992) Nature 355, 318–325
22. Story, R. M., and Steitz, T. A. (1992) Nature 355, 374–376
23. Nguyen, T. T., Muench, K. A., and Bryant, F. R. (1993) J. Biol. Chem. 268, 3107–3113
24. Skiba, M. C., and Knight, K. L. (1994) J. Biol. Chem. 269, 3823–3828
25. Kelley, J. A., and Knight, K. L. (1997) J. Biol. Chem. 272, 25778–25782
26. Hertnapel, K., Volkshin, O. N., Kinai, H. H., Ma, N., Schaffer-Judge, C., and Camerini-Otero, R. D. (1999) J. Mol. Biol. 286, 1097–1106
27. Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G., and Sprang, S. R. (1994) Science 265, 1405–1412
28. Brocchieri, L., and Karlin, S. (1998) J. Mol. Biol. 276, 249–264
29. Ogawa, T., Shinozawa, A., Nabetani, A., Ikeya, T., Yu, X., Egelman, E. H., and Ogawa, H. (1993) Cold Spring Harbor Symp. Quant. Biol. 58, 567–576
30. Mikawa, T., Masui, R., Ogawa, T., Ogawa, H., and Kuramitsu, S. (1995) J. Mol. Biol. 250, 471–483
31. Kurumizaka, H., Aihara, H., Ikawa, S., Kashima, T., Bazemore, L. R., Kawasaki, K., Sarai, A., Radding, C. M., and Shibata, T. (1996) J. Biol. Chem. 271, 33515–33524
32. Aihara, H., Ito, Y., Kurumizaka, H., Terada, T., Yokoyama, S., and Shibata, T. (1997) J. Mol. Biol. 274, 213–221
33. Aihara, H., Ito, Y., Kurumizaka, H., Yokoyama, S., and Shibata, T. (1999) J. Mol. Biol. 290, 495–504
34. Baumann, P., Benson, F. E., Hajibagheri, N., and West, S. C. (1997) Mutat. Res. DNA Repair 384, 65–72
35. Ellouze, C., Kim, H. K., Maeshima, K., Tuite, E., Morimatsu, K., Horii, T., Mortensen, K., Norden, B., and Takahashi, M. (1997) Biochemistry 36, 13524–13529
36. Yu, X., Jacobs, S. A., West, S. C., Ogawa, T., and Egelman, E. H. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 8419–8424
37. Baumann, P., Benson, F. E., and West, S. C. (1996) Cell 87, 757–766
38. Holmes, V. F., Benjamin, K. R., Crisona, N. J., and Cozzarelli, N. R. (2001) Nucleic Acids Res. 29, 5052–5057
39. Sung, P., and Robberson, D. L. (1995) Cell 82, 453–461
40. Namasraev, E. A., and Berg, P. (2000) J. Biol. Chem. 275, 3970–3976
41. Rice, K. P., Egler, A. L., Sung, P., and Cox, M. M. (2001) J. Biol. Chem. 276, 38570–38581
42. De Zutter, J. K., and Knight, K. (1999) J. Mol. Biol. 293, 769–780
43. Tsang, S. S., Chow, S. A., and Radding, C. M. (1985) Biochemistry 24, 3226–3232
44. Kobayashi, N., Knight, K., and McEntee, K. (1987) Biochemistry 26, 6801–6810
45. Benedict, R. C., and Kowalczykowski, S. C. (1988) J. Biol. Chem. 263, 15513–15520
46. Namasraev, E. A., and Berg, P. (1998) Biochemistry 37, 11932–11939
47. Schild, D., Lio, Y., Collins, D. W., Tsomondo, T., and Chen, D. J. (2000) J. Biol. Chem. 275, 16443–16449
48. Thacker, J. (1999) Biochimie (Paris) 81, 77–85
49. Thompson, L. H., and Schild, D. (1999) Biochimie (Paris) 81, 87–105