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Evidence for the Coupling of ATP Hydrolysis to the Final (Extension) Phase of RecA Protein-mediated DNA Strand Exchange*

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RecA protein promotes a limited DNA strand exchange reaction, without ATP hydrolysis, that typically results in formation of short (1–2 kilobase pairs) regions of hybrid DNA. This nascent hybrid DNA is extended in a reaction that can be coupled to ATP hydrolysis. When ATP is hydrolyzed, the extension phase is progressive and its rate is 380 ± 20 bp min^{-1} at 37 °C. A single RecA nucleoprotein filament can participate in multiple DNA strand exchange reactions concurrently (involving duplex DNA fragments that are homologous to different segments of the DNA within a nucleoprotein filament), with no effect on the observed rate of ATP hydrolysis. The ATP hydrolytic and hybrid DNA extension activities exhibit a dependence on temperature between 25 and 45 °C that is, within experimental error, identical. This provides new evidence that the two processes are coupled. Arrhenius activation energies derived from the work are 13.3 ± 1.1 kcal $mole^{-1}$ for DNA strand exchange, and 14.4 ± 1.4 kcal $mole^{-1}$ for ATP hydrolysis during strand exchange. The rate of branch movement in the extension phase (base pair min^{-1}) is related to the k_{cat} for ATP hydrolysis during strand exchange (min^{-1}) by a factor equivalent to 18 bp throughout the temperature range examined. The 18-base pair factor conforms to a quantitative prediction derived from a model in which ATP hydrolysis is coupled to a facilitated rotation of the DNA substrates. RecA filaments possess an intrinsic capacity for DNA strand exchange, mediated by binding energy rather than ATP hydrolysis, that is augmented by an ATP-dependent molecular motor.

The RecA protein of *Escherichia coli* promotes a DNA strand exchange reaction that mimics key steps in recombinational DNA repair and homologous recombination. A RecA nucleoprotein filament forms on a single-stranded DNA circle. The bound single strand is aligned with a homologous linear duplex DNA, and strand exchange leads to the formation of a hybrid circular duplex DNA with a nick in one strand (Cox, 1993, 1994; Kowalczykowski and Eggleston, 1994; Roca and Cox, 1990; West, 1992). The RecA nucleoprotein filament exhibits an ATPase activity with a monomer k_{cat} of about 30 min^{-1} when bound to ssDNA[1] (Cox, 1994). The RecA protein is found in essentially all bacteria (Roca and Cox, 1990). The paradigm extends to eukaryotes, with the RAD51 protein of yeast now proving to be a true RecA homologue in both structure and function (Ogawa *et al.*, 1993; Sung, 1994).

NTPases find cellular employment as motors, timing devices, or recycling functions (Alberts and Miake-Lye, 1992). The ATPase activity of RecA protein has come to be viewed largely as a recycling function, involved primarily in the disassembly of the RecA filament or the recycling of monomers within a filament (Menetski *et al.*, 1990; Alberts and Miake-Lye, 1992; West, 1992; Rehrauer and Kowalczykowski, 1993; Kowalczykowski and Eggleston, 1994; Kowalczykowski and Krupp, 1995). RecA nucleoprotein filaments have an intrinsic capacity to promote DNA strand exchange without hydrolyzing ATP, as shown by the limited DNA strand exchange reactions observed with ATPγS (Menetski *et al.*, 1990), with a RecA mutant protein which binds but does not hydrolyze ATP (RecA K72R) (Rehrauer and Kowalczykowski, 1993), and in the presence of ADP-AlF_4^- (Kowalczykowski and Krupp, 1995).

The recycling function is readily demonstrable, but provides an incomplete explanation for the RecA ATPase activity. Reported filament disassembly reactions are filament end-dependent and rarely account for more than a minute fraction of the ATP hydrolyzed in a RecA nucleoprotein filament (Lindsley and Cox, 1989, 1990a). When homologous duplex DNA is added to RecA nucleoprotein filaments to initiate strand exchange, the monomer k_{cat} for ATP hydrolysis drops abruptly to about 20 min^{-1}, and then is invariant during the subsequent strand exchange reaction (Schutte and Cox, 1987). This ATP hydrolysis has not been accounted for by any model which assigns the primary role of the ATPase to dissociation of RecA monomers.

A more complete view arises from a closer examination of the limitations RecA-mediated DNA strand exchange reaction in the absence of ATP hydrolysis. ATP hydrolysis alters the DNA strand exchange reaction fundamentally, conferring properties important to the function of RecA protein in recombinational DNA repair (Clark and Sandler, 1994; Cox, 1993, 1994). When RecA hydrolyzes ATP, the DNA strand exchange reaction becomes unidirectional (Jain *et al.*, 1994; Shan *et al.*, 1996), generates longer hybrid DNA products much more efficiently (Jain *et al.*, 1994), bypasses a range of structural barriers in either DNA substrate (Rosselli and Stasiak, 1991; Kim *et al.*, 1992a; Shan *et al.*, 1996), and accommodates 4 DNA strands (Kim *et al.*, 1992b; Shan *et al.*, 1996). We have suggested that the undirected DNA strand exchange occurring in the absence of ATP hydrolysis constitutes a distinct phase of the normal reaction (Shan *et al.*, 1996). The resulting nascent hybrid DNA, typically 1–2 kilobase pairs in length, is extended in a subsequent reaction phase that is greatly facilitated by ATP hydrolysis.

If the two processes are coupled, the rates of hybrid DNA extension and ATP hydrolysis during strand exchange should be correlated in predictable ways. In particular, the two processes should exhibit a similar dependence on temperature. The

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; bp, base pair(s); ATPγS, adenosine 5′-*O*-(3-thio)-triphosphate; SSB, the single-stranded DNA binding protein of *E. coli*.

effects of temperature have provided evidence for the coupling of NTP hydrolysis to a number of motor functions. An interesting example was reported by Koshland and co-workers, who used coincident Arrhenius plots to relate the myosin ATPase activity to the walking rate of ants (Levy *et al.*, 1959).

To date, there has been no systematic attempt to measure the rate of DNA strand exchange with different substrates or examine the temperature dependence of RecA reactions. The existing data set is probably insufficient even to establish rigorously that there is an intrinsic and characteristic rate of branch movement during DNA strand exchange that can be associated with ATP hydrolysis. In this report, we develop an improved method for estimating the rate of branch movement during DNA strand exchange and use it to establish a closer experimental correlation between RecA-mediated DNA strand exchange and ATP hydrolysis.

MATERIALS AND METHODS

Enzymes and Reagents—*E. coli* RecA protein was purified to homogeneity and stored as described previously (Cox *et al.*, 1981). *E. coli* single-stranded DNA binding protein (SSB) was purified as described by Lohman *et al.* (1986), except that an additional step utilizing DEAE-Sepharose chromatography was included to ensure removal of single-stranded exonucleases. The RecA protein and SSB concentrations were determined by absorbance at 280 nm, using extinction coefficients of $\epsilon_{280} = 0.59\ A_{280}$ mg^{-1} ml (Craig and Roberts, 1981), and $\epsilon_{280} = 1.5\ A_{280}$ mg^{-1} ml (Lohman and Overman, 1985), respectively. Restriction endonucleases and β-agarase were purchased from New England Biolabs. Tris buffer was purchased from Boehringer Mannheim. DEAE-Sepharose was purchased from Pharmacia Biotech Inc. Pyruvate kinase, phosphoenolpyruvate, lactic dehydrogenase, ATP, creatine phosphokinase, phosphocreatine, proteinase K, and low melting agarose were purchased from Sigma. Thin layer polyethyleneimine cellulose chromatography sheets were purchased from Brinkmann. The concentration of ATP was determined by absorbance at 259 nm, using the extinction coefficient $\epsilon_{259} = 1.54 \times 10^4$ M^{-1} cm^{-1}. The purity of the ATP was assessed by thin layer chromatography and was shown to be at least 99% pure, with the major contaminant being ADP. ATP labeled at position 2 with ^{3}H (24 Ci mmol^{-1}) was purchased from Amersham Corp. (Amersham TRK.336).

DNA—Circular single-stranded and supercoiled circular duplex DNAs from bacteriophage M13mp8.1037 (8,266 bp) were prepared using methods described previously (Davis *et al.*, 1980; Messing, 1983; Neuendorf and Cox, 1986). The bacteriophage M13mp8.1037 is the bacteriophage M13mp8 with a 1037-bp sequence (*Eco*RV fragment) from the *E. coli gal T* gene inserted into the *Sma*I site (Lindsley and Cox, 1990b). The concentrations of ssDNA and dsDNA stock solutions were determined by absorbance at 260 nm, using 36 and 50 mg ml^{-1} $A_{260}{}^{-1}$, respectively as conversion factors. DNA concentrations are reported in terms of total nucleotides. Full-length linear duplex DNA was derived from M13mp8.1037 supercoiled closed-circular form of plasmid or bacteriophage DNA as isolated from *E. coli* cells by complete digestion with *Alw*NI endonuclease, using conditions suggested by the enzyme supplier. Endonucleolytic digestion of M13mp8.1037 with both *Alw*NI and *Ava*II yielded DNA fragments of 4539 and 3727 bp, while digestion with *Alw*NI, *Ava*II, and *Bgl*II generated DNA fragments of 2058, 2481, and 3727 bp. Finally, when the four endonucleases *Alw*NI, *Ava*II, *Bgl*II, and *Pac*I were used to digest M13mp8.1037, the four DNA fragments produced were 1782, 1945, 2058, and 2481 bp in length. For use in experiments where multiple dsDNA fragments were used in a single reaction, the DNA fragments in the restriction digest were not gel-purified but simply extracted 1:1 with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), followed by ethanol precipitation. In reactions where individual dsDNA fragments were used, the dsDNA fragments were isolated from low-melting agarose gels using β-agarase according to the manufacturer's directions. The fragments were then extracted 1:1 with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), followed by ethanol precipitation.

Strand Exchange Reaction Conditions—The formation of nicked circular heteroduplex product of strand exchange was monitored by the agarose gel electrophoresis assay of Cox and Lehman (1981). Reactions were carried out 37 °C or at the indicated temperature in a standard reaction buffer containing 25 mM Tris acetate (80% cation, pH 7.5), 10 mM magnesium acetate, 3 mM potassium glutamate, 1 mM dithiothreitol, 5% glycerol, and an ATP regeneration system (10 units ml^{-1} creatine phosphokinase, 12 mM phosphocreatine). Reactions were carried out in a Brinkmann Instruments Lauda K-2/RD refrigerated water bath to ensure constant temperature. Reaction volumes ranged from 40 to 300 μl, and the concentrations of DNA and proteins reported below are the final concentrations after addition of all components. Duplex DNA (15 μM in terms of total nucleotides) and ssDNA (5 μM) were preincubated with 2.5 μM RecA protein for 10 min before ATP (3 mM) and SSB protein (0.5 μM) were added to start the reaction. Aliquots (15 μl) of reactions were withdrawn at various times, mixed with 10 μl of gel loading buffer (0.125% bromphenol blue, 0.125% xylene cyanol FF, 15% glycerol, and 5% SDS), and subjected to electrophoresis on a 0.8% agarose gel in Tris acetate-EDTA buffer at 2–2.5 V cm^{-1}. The agarose gel was stained with ethidium bromide and photographed with UV light. The intensities of DNA bands were quantified by scanning the photographic negatives using an Apple One Scanner and analyzing the scanned image with NIH Image 1.44 software. In reactions involving multiple dsDNA fragments, the total dsDNA concentration was kept constant at 15 μM in each reaction. In reactions where individual shorter dsDNA fragments were used, the dsDNA concentration was varied downward so that the ratio of duplex DNA molecules to ssDNA molecules remained constant.

Electron Microscopy—Visualization of reactions by electron microscopy was carried out by spreading the entire strand exchange reaction mixture after deproteinization and dialysis. Aliquots of strand exchange reactions were removed at early time points during the reaction (9 or 11 min). These samples were incubated with Proteinase K (0.5 mg ml^{-1} final) and SDS (0.5% final) at 37 °C for 30 min to remove the RecA and SSB proteins. The samples were then dialyzed into 20 mM NaCl and 5 mM EDTA for 4 h at 25 °C before spreading as described previously (Inman and Schnös, 1970).

Coupled Spectrophotometric ATPase Assay—The coupled spectrophotometric assay used to measure ATP hydrolysis was described previously (Iype *et al.*, 1994; Morrical *et al.*, 1986). The regeneration of ATP from ADP and phosphoenolpyruvate coupled to the oxidation of NADH can be followed by the decrease in absorbance at 380 nm. An NADH extinction coefficient of $\epsilon_{380} = 1.21$ mM^{-1} cm^{-1} was used to calculate the rate of ATP hydrolysis. The reaction conditions during the ATPase assay were identical to DNA strand exchange conditions, except that pyruvate kinase (5 units/ml) and phosphoenolpyruvate (2.3 mM) were used as the ATP regeneration system; NADH (3 mM) and lactic dehydrogenase (4.5 units/ml) were also included. These modifications had no effect on the rate of DNA strand exchange at temperatures above 33 °C; however, they did result in a decrease in the rate of strand exchange at lower temperatures. An alternative ATPase assay was employed at these lower temperatures as described below. Absorbance measurements were obtained in a Perkin-Elmer Lambda 7 double-beam recording spectrophotometer equipped with two six-position thermojacketed cuvette holders attached to a Brinkmann Instruments Lauda K-2/RD refrigerated water bath and circulator. ATP hydrolysis during DNA strand exchange was measured as described previously (Schutte and Cox, 1987). Control experiments varying the amount of the pyruvate kinase and lactic dehydrogenase at different temperatures showed that the activity of these enzymes did not limit the overall rate of ATP hydrolysis within the temperature ranges employed in these experiments.

Thin-layer Chromatography (TLC) ATPase Assay—To measure ATP hydrolysis at temperatures below 33 °C, a TLC assay was employed (Kornberg *et al.*, 1978). TLC plates were washed by elution with water and dried before use. A mixture of nonradioactive ATP and ADP (1 μl of 16 mM ATP and 16 mM ADP) was spotted at the origin of each lane on the plates and dried (these were carriers that permitted the ready detection of the ATP and ADP spots in the developed plates). Strand exchange reactions were carried out as described above except that the ATP regeneration system was omitted, and [^{3}H]ATP was added (4 mCi in a 50-ml reaction). For some of the lower temperatures (below 30 °C), the DNA and protein concentrations were doubled to increase the observed signal. This modification had no effect on the observed k_{cat} for RecA-mediated ATP hydrolysis, nor on the rate of DNA strand exchange (data not shown). Aliquots (1.5 μl) of reactions at various time points were then spotted on the prepared TLC plates. After drying, the plates were developed in 1 M formic acid, 0.5 M LiCl. The positions of the ADP and the ATP spots were determined by their UV fluorescence. The spots were excised and the radioactivity in each quantified by scintillation counting.

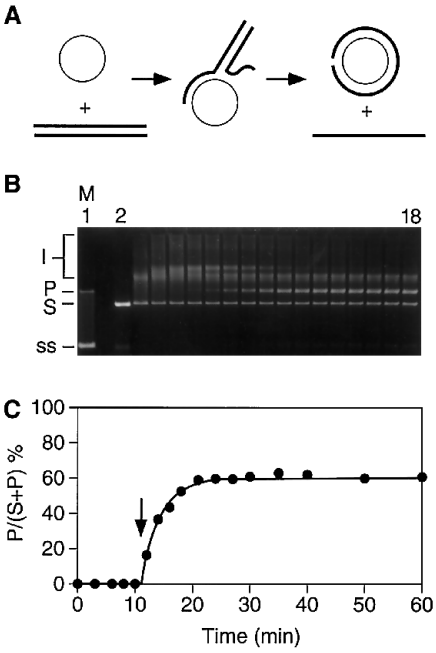

FIG. 1. **Measuring the rate of DNA strand exchange.** Reactions were carried out as described under "Materials and Methods." *A*, the three-strand exchange reaction used in these experiments. The substrates are a single-stranded circle and a linear duplex DNA derived from M13mp8.1037 (8266 bp). The linear duplex DNA was obtained by cleaving supercoiled M13mp8.1037 DNA with *Alw*NI. *B*, a typical reaction at 43.5 °C; *P*, circular duplex DNA products; *S*, linear duplex DNA substrate; *ss*, circular single-stranded DNA; *I*, reaction intermediates. The first lane contains supercoiled and nicked circular duplex M13 mp8.1037 markers. *Lanes 2–18* show the reaction at 0, 3, 6, 8, 10, 12, 14, 16, 18, 21, 24, 27, 30, 35, 40, 50, and 60 min, respectively. *C*, quantitation of the P and S bands. The *arrow* represents the extrapolated point at which the first products appeared (11 min).

RESULTS

Experimental Design—The purpose of this study was to examine the rate of DNA strand exchange in more detail, examining correlations that should exist if DNA strand exchange is coupled to ATP hydrolysis. This requires accurate methods with which to monitor the rate of both ATP hydrolysis and branch movement during DNA strand exchange.

Whereas accurate methods are available to monitor rates of ATP hydrolysis, obtaining a reliable estimate of the rate of branch movement in DNA strand exchange is problematic. Published estimates, which vary over at least a 5-fold range, were obtained from average rates of hybrid DNA formation in a large population of molecules that initiate the reaction asynchronously (Roca and Cox, 1990). Once initiated, secondary interactions with additional DNA molecules in solution might interfere with branch movement in some complexes (coaggregation) (Tsang *et al.*, 1985), exaggerating the apparent asynchrony. We dealt with these uncertainties by focusing only on the formation of the circular duplex DNA product (Fig. 1*A*). In a reaction involving our 8266-bp DNA substrates, this product first appears after a significant lag and thereafter accumulates until most of the substrate DNA has been converted (Fig. 1*B*). We quantified the bands in experiments such as that in Fig. 1*B*, and extrapolated to the time point when the first product appeared (Fig. 1*C*, *arrow*). The time required to generate the first product should provide the most reliable estimate of the intrinsic rate of branch movement in a reacting complex, reflecting an early initiation and a minimization of any subsequent inhibition from interactions with other DNA molecules. The apparent rate of DNA branch movement (in bp min^{-1}) is defined as the length of the duplex DNA substrate divided by the lag (in minutes) before the first appearance of products.

In practice, experiments with relatively few time points were used to roughly characterize the product generation curve, followed by a more detailed time course with time points concentrated near the anticipated lag time to define it as accurately as possible. The error in most lag measurements is about ±1 min. This translates into an error of about ±10% for a typical experiment (the error increases for shorter duplex substrates). Experiments were discarded when excessive scatter in the product generation curve precluded a reliable extrapolation. All of the strand exchange experiments used in this study generated products at levels greater than half of the predicted maximum of 67%.

Two sets of correlations were pursued. In the first, we examined the effect of dividing the duplex DNA substrate into fragments and the capacity of a single RecA filament to carry out multiple strand exchange reactions concurrently. This is done in part to help establish that there is an intrinsic and constant rate of branch movement during DNA strand exchange. In the second, we compare the temperature dependencies of the DNA strand exchange reaction and ATP hydrolysis during strand exchange.

A RecA Protein Nucleoprotein Filament Can Promote Multiple Strand Exchange Reactions Concurrently—ATP is hydrolyzed uniformly throughout a RecA nucleoprotein filament (Brenner *et al.*, 1987). In postulating a separate phase of DNA strand exchange that is coupled to ATP hydrolysis, one assumption is that the rate of branch movement is constant and that all parts of a nucleoprotein filament are equally competent to promote branch movement. If the linear duplex DNA substrate is cleaved into two pieces of equal size, they should react concurrently and produce a fully duplex circular product in half the time required for the reaction shown in Fig. 1*A*. This assumes that both branches move unidirectionally at the same rate. Since ATP is hydrolyzed by each RecA monomer at the same rate regardless of whether it is near a branch (Brenner *et al.*, 1987; Schutte and Cox, 1987), and we assume the rate of ATP hydrolysis has some relationship to the rate of branch movement, no change in the rate of ATP hydrolysis should be observed. Satisfying these conditions does not in itself provide evidence for the coupling of ATP hydrolysis to DNA strand exchange, but results not adhering to the predictions would provide evidence against coupling by some proposed mechanisms (Cox, 1994). Results inconsistent with these predictions would also cast doubt on the assumption that branch movement in strand exchange proceeds at a constant rate.

The linear duplex substrate in these experiments, M13mp8.1037 (8266 bp), was cleaved to produce one, two, three, or four fragments as shown in Fig. 2. The cleaved substrates were then used in DNA strand exchange reactions with RecA nucleoprotein filaments bound to single-stranded M13 mp8.1037 circles. All reactions were done at 37 °C. The results in Fig. 3 demonstrate that the appearance of fully duplex product circles is accelerated as the number of fragments increases. This experiment was carried out three times with similar results. Estimates for the rate of branch movement obtained as outlined in Fig. 1 are summarized in Table I. The rates are fairly uniform, especially when it is assumed that the rate of product appearance is limited by the length of the longest DNA fragment present. The average rate of branch movement obtained by this method is 378 bp min^{-1}.

The results suggest that multiple DNA fragments can react with a single nucleoprotein filament concurrently. To confirm this, some of the reactions were spread and examined by electron microscopy. The molecules shown in Fig. 4, involving two dsDNA fragments, were observed 11 min after the initiation of

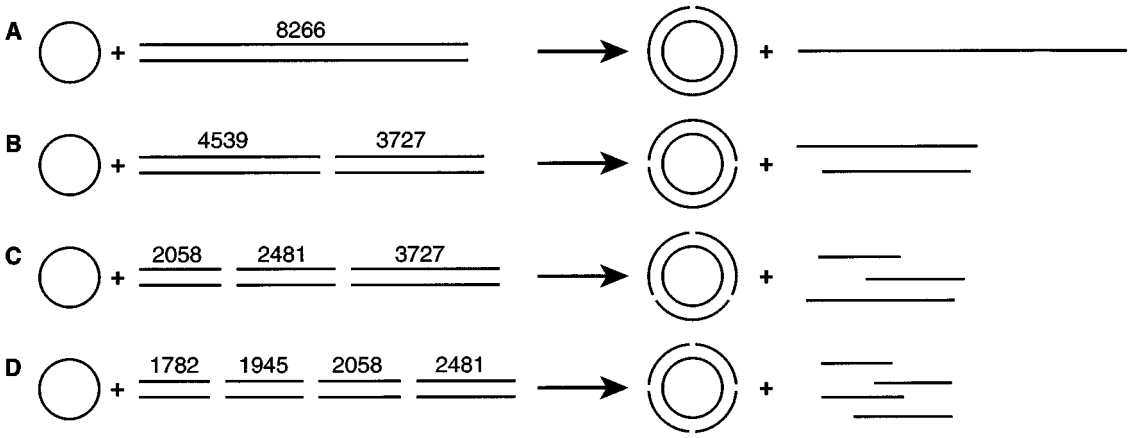

FIG. 2. **DNA strand exchange with multiple branch points.** All DNA substrates are derived from M13mp8.1037. Where the linear duplex DNA substrate is divided into fragments, the fragments are nonoverlapping and have the sizes indicated (in bp). Restriction enzymes used to generate the fragments are listed under "Materials and Methods." The fully duplex circular products have a number of nicks equivalent to the number of fragments required to generate them.

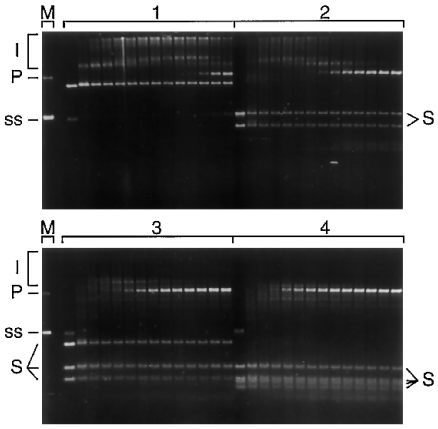

FIG. 3. **Assay for DNA strand exchange with multiple branch points.** Reactions were carried out at 37 °C as described under "Materials and Methods" with the substrates illustrated in Fig. 2. The lanes marked *M* contain supercoiled and nicked circular M13mp8.1037 DNA markers. Labels are: *P*, circular duplex reaction product; *I*, reaction intermediates; ss, single-stranded circular DNA substrate; *S*, linear duplex substrates, with brackets where the duplex is divided into fragments. The numbers above each reaction indicate the number of duplex DNA fragments used. Time points for each reaction are, from left to right, 0, 3, 5, 7, 9, 11, 13, 15, 18, 21, 25, 30, 40, and 60 min, respectively.

TABLE I
DNA strand exchange with multiple branch points

The reactions are those described in Figs. 2–4. The reaction with 0 branch points corresponds to a reaction to which no duplex DNA substrate has been added.

No. of branch points	Time[a]	Rate 1[b]	Rate 2[c]	ATPase k_{cat}[d]
	min	*bp min*$^{-1}$		*min*$^{-1}$
0	NA[e]	NA	NA	28.9 ± 0.4
1	21	394	394	20.6 ± 0.6
2	13	318	349	19.2 ± 1.1
3	9	306	414	19.3 ± 0.2
4	7	295	354	19.4 ± 0.4

[a] Time to first appearance of fully duplex DNA products, using the method shown in Fig. 1.

[b] Rate of branch movement when calculated on the basis of average size of the DNA fragments in an experiment (8266, 4133, 2755, and 2067 for 1, 2, 3, and 4 branch points, respectively. Rate = fragment size divided by the time to first product appearance.

[c] Rate of branch movement when calculated on the basis of the longest DNA fragment in each experiment (8266, 4539, 3727, and 2481 bp for 1, 2, 3, or 4 branch points, respectively).

[d] Linear rates of ATP hydrolysis followed for 30 min, beginning 5 min after the reactions were initiated. The ATP concentration is 3 mM. The k_{cat} assumes 1 bound RecA monomer per 3 nucleotides of ssDNA (1.67 μM bound RecA) in the starting reaction.

[e] NA, not applicable.

the reaction. In both cases, the ssDNA is participating in a strand exchange reaction with two fragments, and the strand exchange is progressing in the same direction with both fragments. Although a detailed characterization of the spreads was not undertaken, the results suggested a significant degree of asynchrony in the initiation of strand exchange by the two fragments on a given nucleoprotein filament, since only one-third to one-half of the intermediates observed were reacting with two duplex fragments. Samples were not cross-linked in these trials, and we do not know to what extent the results are affected by loss of DNA joints due to the spontaneous branch migration during sample preparation observed previously (Jain *et al.*, 1994).

Rates of ATP hydrolysis were also monitored during these experiments (Table I). As observed previously (Schutte and Cox, 1987), addition of a homologous linear duplex resulted in a decrease in the rate of ATP hydrolysis of about 30%. This decrease is complete in about 2 min, and its extent is dependent on the length of available homology (with the 30% maximum observed when the homologous duplex substrate was as long as the ssDNA) (Schutte and Cox, 1987). A linear rate was observed during the 30-min span over which data was taken in the present experiments (between 5 and 35 min into the reaction). Rates observed during DNA strand exchange were not affected by dividing the duplex substrate into as many as four fragments (Table I).

The Temperature Dependence of RecA Reactions—The idea behind these experiments, to compare rates of DNA strand exchange and ATP hydrolysis at different temperatures, is simple. However, a number of complexities were encountered in their execution, some of them unexpected. A useful temperature range of 25 to 45 °C was established empirically for this work. RecA protein activity increases with temperature up to at least 45 °C. We avoided higher temperatures to minimize the chance of rate changes due to RecA inactivation. Below 25 °C, RecA activities become too slow for accurate rate measurements. The rate of ATP hydrolysis reported is the invariant rate observed during strand exchange after the abrupt drop in monomer k_{cat} that accompanies the initiation of the reaction. This drop was approximately 30% throughout the temperature range. Apparent rates of DNA branch movement are reported for the 8266 bp duplex substrate as in Fig. 1*C*.

A series of control experiments were carried out to ensure that any changes observed reflected temperature-dependent

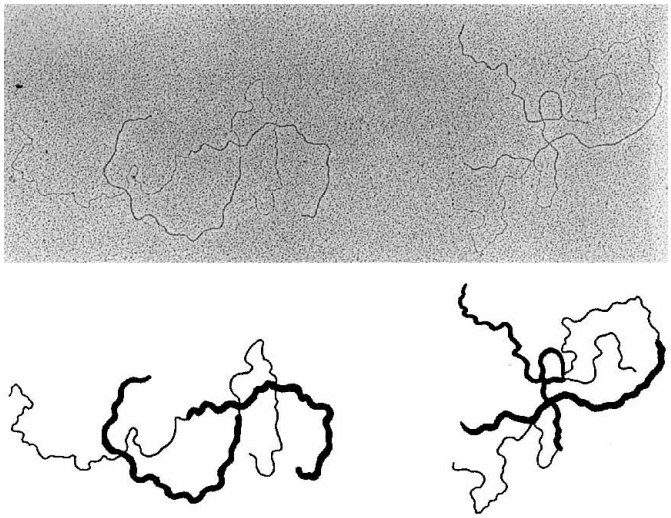

FIG. 4. **Electron microscopy of reactions with multiple duplex DNA fragments.** Molecules derived from a reaction with the duplex substrate divided into two fragments (Fig. 2) are shown along with interpretive drawings. These molecules were found 11 min into the reaction.

changes in the k_{cat} for ATP hydrolysis or the rate of branch movement, as opposed to changes in the amount of bound RecA or the K_m for ATP. We established that the K_m for ATP was below 300 μM throughout the temperature range. In RecA protein titrations carried out at five different temperatures spanning the chosen temperature range, the rate of ATP hydrolysis saturated in each case at a RecA concentration corresponding to 3 $\pm$ 0.5 nucleotides of DNA per RecA monomer.

The final conditions and methods used to measure rates were affected by our unexpected discovery that ATP regeneration systems based on pyruvate kinase and phosphoenolpyruvate inhibit both DNA strand exchange and ATP hydrolysis in a temperature-dependent fashion. Direct comparisons of DNA strand exchange reactions carried out with the pyruvate kinase ATP regenerating system and the alternative system based on creatine phosphokinase revealed a substantial inhibition of the strand exchange reaction by the former below 33 °C, an effect that increased as the temperature was lowered to 25 °C. A parallel inhibition of the ATPase activity was also observed when experiments using the spectrophotometric coupled assay (which includes pyruvate kinase and phosphoenolpyruvate) were compared to initial rates of ATP hydrolysis measured with the alternative TLC assay (which contains no regenerating system). The basis of the inhibitory effect of the pyruvate kinase/phosphoenolpyruvate system at low temperatures was unclear. At 35 °C and above, the two ATP regeneration systems gave comparable results in strand exchange reactions, and the two methods for monitoring ATP hydrolysis also gave identical results. We do not know which component of the pyruvate kinase/phosphoenolpyruvate system is responsible for the low temperature inhibition. The rates of ATP hydrolysis during strand exchange were monitored with the more accurate spectrophotometric assay above 35 °C, and with the thin layer chromatographic assay at 33 °C and below. Measurements with the latter assay reflect initial rates determined between 2 and 10 min after initiation of DNA strand exchange. Although the two assays for ATP hydrolysis were in good agreement in experiments done at the higher temperatures, the error in the TLC assay is inherently greater. We compensated by increasing the number of experiments done at the lower temperatures. All of the DNA strand exchange experiments were done in the presence of the creatine phosphokinase/phosphocreatine ATP regenerating system.

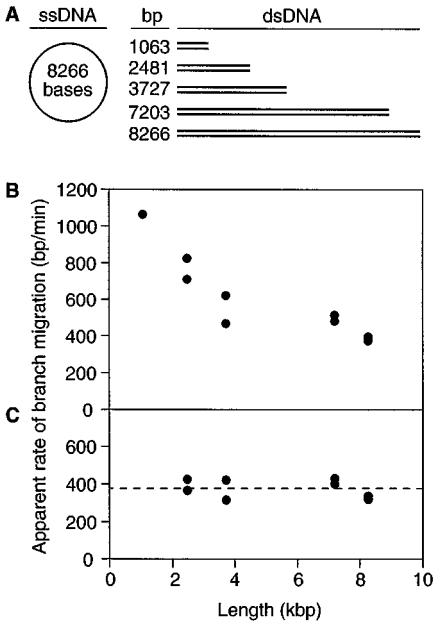

FIG. 5. **The apparent rate of DNA strand exchange as a function of the length of the duplex DNA substrate.** *A*, substrates used in the reactions. The numbers give the lengths of each substrate in nucleotides or base pairs. *B*, apparent rates of branch movement during DNA strand exchange. Two sets of experiments, done on different days, are shown. Rates (bp min^{-1}) = the length of the duplex DNA substrate in bp, divided by the time (min) required for the appearance of the first product (see Fig. 1). *C*, apparent rates of branch movement if it is assumed that 1200 bp of hybrid DNA is created in a rapid phase independent of ATP hydrolysis. Rates (bp min^{-1}) = length of the duplex substrate (minus 1200 bp), divided by the time (min) required for the appearance of the first product. The 1200-bp figure was determined by trial and error as the optimal correction factor to produce the result in *Panel C*.

A final factor that could affect the interpretation of the observed rates of DNA strand exchange is the possibility that exchange occurs in more than one kinetic phase. As described in the Introduction, a phase not requiring ATP hydrolysis may contribute a significant amount of hybrid DNA near the beginning of the reaction. If this phase is as rapid as some studies suggest (Menetski *et al.*, 1990), it could result in a burst of hybrid DNA formation preceding the putative ATP hydrolysis-dependent process we are interested in measuring. This could, in turn, affect the outcome of the temperature dependence study, particularly if the size of the burst phase varied with temperature.

To evaluate the effects of a possible rapid phase in strand exchange on our overall measurements, we carried out a series of strand exchange reactions with individual truncated linear duplex DNA substrates. If a significant rapid phase exists, the apparent rate of branch movement should increase with shorter DNA substrates, where the rapid phase would contribute a correspondingly larger fraction of the hybrid DNA product. Although shorter duplex substrates were employed in the experiment of Fig. 2, a rapid phase may have been obscured by any asynchrony in the reaction of the multiple DNA fragments. The results are shown in Fig. 5. An increase in the apparent rate of branch movement during DNA strand exchange is observed as the length of the duplex substrate decreases (Fig. 5*B*). If we assume that there is a rapid phase that contributes 1200 bp of hybrid DNA to the reaction, the apparent rate of branch movement for the remainder of each substrate becomes independent of length (Fig. 5*C*). Similar experiments were carried out at 25, 30, and 42 °C. The effect in Fig. 5*B* was the largest effect observed. The data in Fig. 5*C* give an average rate of branch movement of 377 bp min^{-1}. This compares with a

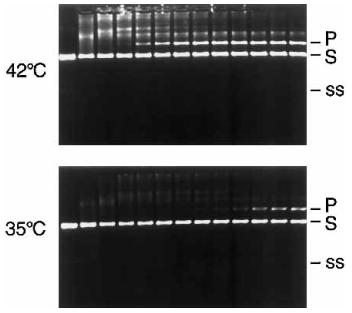

FIG. 6. **The temperature dependence of DNA strand exchange.** Reactions were carried out as described under "Materials and Methods" at the two temperatures indicated. Labels are described in the legend to Fig. 1*B*. Time points are, from left to right: 0, 5, 10, 15, 20, 23, 26, 29, 33, 37, 45, 60, and 90 min, respectively.

series of uncorrected rate measurements with the 8266-bp duplex substrate ranging from 360 to 394 bp min^{-1}. Since the postulated burst phase appears to have a minimal effect on the results when long duplex substrates are used, and there was no evidence that the effects increased in any part of the temperature range examined, we chose not to apply any corrections to our rate measurements.

As shown in Fig. 6, there is a substantial effect of temperature on the rate of DNA strand exchange. An Arrhenius plot for RecA-mediated ATP hydrolysis when bound to ssDNA is shown in Fig. 7*A*. The plot is linear in the range of 25 to 45 °C, with no breaks that might signal a change in rate-limiting step, and yields an Arrhenius activation energy of 11.8 ± 0.3 kcal $mole^{-1}$. When heterologous dsDNA is added to this system, there are no measurable changes in the rates of ATP hydrolysis at 37 or 42 °C (Schutte and Cox, 1987).[2] The rates of ATP hydrolysis decrease when homologous DNA is added to initiate DNA strand exchange (by 30% at 37 °C), as reported previously (Schutte and Cox, 1987). Arrhenius plots for RecA-mediated DNA strand exchange and the ATP hydrolysis that accompanies it are presented in Fig. 7*B*, with the data obtained for ATP hydrolysis included for comparison. The Arrhenius plots are again linear over this temperature range, and there appears to be a small increase in Arrhenius activation energy for ATP hydrolysis. Within experimental error, the slopes of the lines fit to the data for ATP hydrolysis and DNA branch movement during strand exchange by unweighted linear regression are identical. The rates of both processes change by a factor of about 6 over the 25 to 45 °C temperature range. The slopes of the respective lines yield Arrhenius activation barriers of 13.3 ± 1.1 kcal $mole^{-1}$ for DNA branch movement, and 14.4 ± 1.4 kcal $mole^{-1}$ for ATP hydrolysis during strand exchange. In Fig. 7*C*, the data for DNA branch movement and ATP hydrolysis during strand exchange are broken out and compared directly, with parallel lines drawn through the data replacing the best fit lines of Fig. 7*B*.

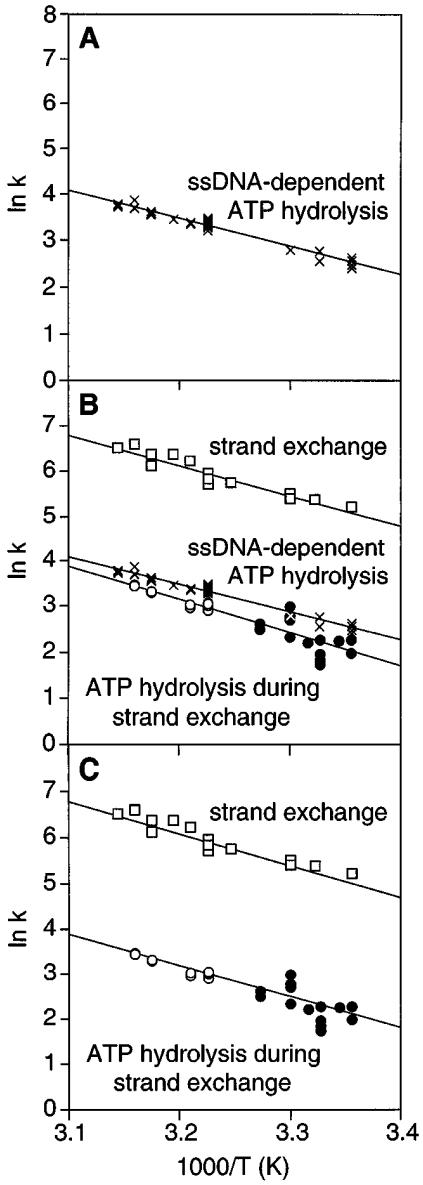

FIG. 7. **Arrhenius plots.** The rate of DNA strand exchange (in bp min^{-1}, *square symbols*), the k_{cat} for ATP hydrolysis in the presence of ssDNA alone (min^{-1}, ×'s), and the k_{cat} for ATP hydrolysis during strand exchange (min^{-1}, *circles*) are plotted as a function of temperature. Reactions were carried out under standard conditions described under "Materials and Methods." In the case of ssDNA-dependent ATP hydrolysis, data above 37 °C were obtained with the coupled spectrophotometric assay for ATP hydrolysis, and data below this temperature were obtained with the thin layer chromatography assay. Data shown at 37 °C represent multiple experiments carried out with both assays. In the case of ATP hydrolysis during strand exchange, the *open circles* reflect data obtained with the coupled spectrophotometric assay for ATP hydrolysis (at least two measurements at each temperature shown). The *closed circles* reflect data obtained with the thin layer chromatography assay. For a few of the ATPase measurements carried out below 30 °C, the RecA, SSB, and DNA concentrations were doubled. This had no discernible effect on the k_{cat} values obtained. *A*, rates of RecA protein-mediated ATP hydrolysis in the presence of ssDNA alone. *B*, the data of *Panel A* combined with the data for both ATP hydrolysis and DNA branch movement during DNA strand exchange. Each data set in *Panels A* and *B* was fitted separately by unweighted linear regression of ln *k* on 1/*T*, using the Minitab statistical software package. *C*, unfitted parallel lines are drawn through the data for both ATP hydrolysis and DNA branch movement during strand exchange, separated by 2.89 units on the *y* axis. See "Discussion" for details.

DISCUSSION

We have two primary conclusions. First, there is an intrinsic and constant rate of branch movement in the final phase of DNA strand exchange. This rate is 380 ± 20 bp min^{-1} at 37 °C. Second, the rate of branch movement exhibits a dependence on temperature that parallels the temperature dependence of ATP hydrolysis during DNA strand exchange. The close correspondence provides a new piece of evidence that ATP hydrolysis is coupled to the extension phase of the DNA strand exchange reaction. While the Arrhenius plots cannot provide rigorous proof for coupling, the correlation fulfills the most fundamental requirement for a motor protein that a unit of ATP hydrolysis should provide a definable unit of work under coupled conditions.

[2] Q. Shan and M. Cox, unpublished data.

These observations can be placed in the context of an emerging view encompassing aspects of many different mechanistic proposals. RecA nucleoprotein filaments formed on single-stranded DNA have an intrinsic capacity to take up a homologous duplex DNA, using binding energy to promote a strand exchange reaction independent of ATP hydrolysis (Menetski *et al.*, 1990; Kim *et al.*, 1992a; Rehrauer and Kowalczykowski, 1993; Kowalczykowski and Eggleston, 1994; Shan *et al.*, 1996). This process can account for the limited exchange seen with ATPγS or with the RecA K72R mutant, and the small burst of exchange observed under some conditions with ATP. There is no obvious reason why this process should not produce an efficient and complete DNA strand exchange between long homologous DNA substrates, but it usually halts or slows greatly before strand exchange is completed. The extent of the rapid strand exchange observed without ATP hydrolysis is presumably limited by discontinuities either in the RecA filament (Menetski *et al.*, 1990) or in a DNA pairing intermediate (Shan *et al.*, 1996). Extension of this nascent hybrid DNA can occur without ATP hydrolysis under some conditions (Shan *et al.*, 1996), but is very slow and undirected. Under conditions optimal for RecA-mediated DNA strand exchange *in vitro*, extension of the hybrid DNA makes use of a built-in protein machine. This use of ATP can be rationalized in part by the special requirements of recombinational DNA repair (Clark and Sandler, 1994; Cox, 1993). An additional rationale for catalyzing a unidirectional strand exchange can be found in the relatively slow rates of uncatalyzed branch migration (Panyutin and Hsieh, 1994).

We use the term "coupling" in the simplest sense. When ATP is hydrolyzed, the rate of DNA branch movement is determined and limited by the rate of ATP hydrolysis. It might be expected that a coupling between ATP hydrolysis and DNA branch movement could lead to a change in the measured activation energy for ATP hydrolysis when homologous duplex DNA was added. ATP hydrolysis is fully activated when RecA protein is bound to ssDNA, where it is clearly not coupled to work. Addition of homologous duplex DNA results in a measurable and abrupt decrease in the rate of ATP hydrolysis (Schutte and Cox, 1987), but generates only a small apparent change in the measured Arrhenius activation energy for ATP hydrolysis. While changes in the Arrhenius activation energy might reflect a coupling to work, such changes need not occur nor are they an experimental criterion for coupling. For example, myosin heads hydrolyze ATP in the presence of actin filaments under conditions where no work is accomplished, and Arrhenius activation energies for this process have been reported (Levy *et al.*, 1959; Anson, 1992). The Arrhenius activation energy (above 20 °C) for myosin-mediated ATP hydrolysis does not change appreciably when the same reaction is measured under conditions where work is produced, in intact muscle or with tethered filaments *in vitro* (Levy *et al.*, 1959; Anson, 1992). The Arrhenius activation energies we report for RecA-mediated ATP hydrolysis reflect whatever steps in the ATP hydrolytic cycle are rate-limiting, and cannot be interpreted (beyond the correlation we present here) without a more refined understanding of the cycle. There seems little doubt that the chemical energy (ATP hydrolysis) utilized by a RecA filament is in substantial excess to that required to move a DNA branch between homologous DNA molecules. We presume that this is coupled to some set of conformation changes in individual RecA monomers. When a DNA branch is present, its unidirectional movement is a byproduct of these same conformational changes. A major load may not be placed on the system until the branch encounters a DNA lesion or structural barrier in the course of recombinational DNA repair (Clark and Sandler, 1994; Cox, 1993).

The focus of this work is on the correlations illustrated by the Arrhenius plots rather than the activation energies derived from them. There is no reason why the observed temperature dependence of DNA branch movement should be similar to that for the completely distinct chemical process of ATP hydrolysis unless they are linked in some way. Our interpretation of the strong correlation seen in Fig. 7, that ATP hydrolysis is coupled to the extension of hybrid DNA during strand exchange, does not presuppose any particular coupling mechanism. There are at least two coupling mechanisms in the literature that remain viable. Kowalczykowski and colleagues have proposed that discontinuities in the RecA filament must be rectified by ATP hydrolysis-dependent redistribution of the RecA protein (Menetski *et al.*, 1990). This laboratory has proposed an alternative model that couples ATP hydrolysis to a coordinated rotation of the DNA substrates in order to effect branch movement and resolve discontinuities that seem likely to arise in a key DNA pairing intermediate (Cox, 1994; Shan *et al.*, 1996; Roca and Cox, 1990). Another version of the DNA rotation idea has recently been proposed by Radding and colleagues (Burnett, *et al.*, 1994).

The Arrhenius plots in Fig. 7*C* are parallel rather than coincident because the rate of DNA strand exchange and the k_{cat} for ATP hydrolysis are reported in different units (bp min^{-1} *versus* min^{-1}). The facilitated DNA rotation model makes a specific prediction about how these two rates should be related (Cox, 1994). DNA bound by RecA protein has a helical periodicity of about 18 bp per turn, so that rotating the DNA by 360° should move a branch point by 18 bp. The model holds that each RecA monomer hydrolyzes one ATP for each 360° rotation of the DNA. The DNA branch should therefore advance at a rate (in bp min^{-1}) equivalent to the ATPase turnover rate (min^{-1}), multiplied by a factor equivalent to the expected 18 bp advance per coupled rotation (Cox, 1994). In Fig. 7*C*, the Arrhenius plots for these processes in Fig. 7*B* are presented with parallel lines drawn through the data separated by 2.89 natural log units. The lines are well within the experimental error of the experiment, and their separation corresponds to a factor of 18 bp.

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