RuvAB-directed branch migration of individual Holliday junctions is impeded by sequence heterology

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The Holliday junction, the key intermediate of recombination, is generated by strand exchange resulting in a covalent connection between two recombining DNA molecules. Translocation of a Holliday junction along DNA, or branch migration, progressively exchanges one DNA strand for another and determines the amount of information that is transferred between two recombining partners. In *Escherichia coli*, the RuvAB protein complex promotes rapid and unidirectional branch migration of Holliday junctions. We have studied translocation of Holliday junctions using a quantitative biochemical system together with a 'single-molecule' branch migration assay. We demonstrate that RuvAB translocates the junctions through identical DNA sequences in a processive manner with a broad distribution of individual branch migration rates. However, when the complex encounters short heterologous sequences, translocation of the Holliday junctions is impeded. We conclude that translocation of the junctions through a sequence heterology occurs with a probability of bypass being determined both by the length of the heterologous region and the lifetime of the stalled RuvAB complex.

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Introduction

Homologous recombination, an essential process in virtually all organisms, describes exchanges between DNA sequences that are very similar but not necessarily identical. These exchanges are crucial for the generation of genetic diversity and the proper segregation of chromosome homologs during meiosis (Kowalczykowski *et al*, 1994; Camerini-Otero and Hsieh, 1995). Homologous recombination is the most con-

servative mechanism for the repair of DNA double-strand breaks caused by ionizing irradiation, mechanical stress, endonuclease cleavage or the replication of a nicked chromosome (Paques and Haber, 1999). In addition, it provides a pathway for the rescue of stalled replication forks (Seigneur *et al*, 1998).

Homologous recombination initiates at the site of double-strand breaks. The broken DNA ends are processed to generate 3' single-stranded protruding ends. Homologous pairing and strand exchange between the processed 3' ends and intact duplex DNA lead to the formation of the major intermediate of recombination, the Holliday junction, which marks the exchange point between two recombining DNAs. Branch migration of a Holliday junction, in which one DNA strand is progressively exchanged for another, extends the length of heteroduplex DNA and affects the amount of genetic information transferred between two DNA molecules. Finally, isomerization and resolution of the Holliday junction complete recombination. The fundamental mechanisms of homology recognition, heteroduplex formation and resolution of recombination intermediates are conserved throughout evolution (Cromie *et al*, 2001).

In *Escherichia coli*, the RuvA, RuvB and RuvC proteins process the Holliday junctions into mature recombination products (Shinagawa and Iwasaki, 1996; West, 1997). Genetic, biochemical, electron microscopic and structural studies established that these proteins act in concert to promote both branch migration by action of RuvAB and resolution of the junctions by the RuvC endonuclease. RuvA binds the Holliday junctions as a tetramer (Rafferty *et al*, 1996; Hargreaves *et al*, 1998; Ariyoshi *et al*, 2000) or double tetramer (Roe *et al*, 1998; Yamada *et al*, 2002) with high affinity and unfolds the junctions from the stacked X-structure into a square-planar conformation. Targeted to the Holliday junction by interactions with the RuvA–junction intermediate, two hexameric rings of RuvB encircle opposing DNA duplex arms of the junction and act as ATP-dependent DNA motors extruding heteroduplex DNA (Parsons and West, 1993; Shiba *et al*, 1993; Stasiak *et al*, 1994). The RuvC dimer resolves the Holliday junctions into duplex products by cleavage of the opposite DNA strands. The rapid, unidirectional translocation of Holliday junctions through the coordinate action of the RuvA and RuvB proteins, which extends heteroduplex DNA, is essential for homologous recombination and recombinational repair.

Homologous recombination can occur between similar but not identical DNA molecules. As a result, branch migration must somehow bypass sequence heterologies, generating mismatches, insertions or deletions in the heteroduplex DNA products. Thus, the passage of Holliday junctions through sequence heterology is an important aspect of RuvAB activity. Biochemical studies indicate that RuvAB is able to bypass long tracks of heterology, albeit at a reduced

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efficiency (Parsons *et al*, 1995; Adams and West, 1996). However, the precise mechanisms whereby RuvAB-directed translocation of Holliday junctions is affected by sequence heterology still remain obscure.

Although the biochemical properties of the RuvAB protein complex have been extensively studied, a quantitative experimental system to study translocation of Holliday junctions by RuvAB is currently lacking. A 'single-molecule' approach, such as the tethered-particle motion (TPM) technique, provides an opportunity for the direct observation of interactions between single DNA and protein molecules in the absence of applied forces (Yin *et al*, 1994; Finzi and Gelles, 1995; Dohoney and Gelles, 2001; Tolic-Norrelykke *et al*, 2004). Since RuvAB acts as a DNA motor by transforming the chemical energy generated during ATP hydrolysis into mechanical work required for translocation of Holliday junctions, RuvAB-mediated branch migration can be measured using similar experimental strategies.

In the present work, we have studied RuvAB-directed translocation of Holliday junctions using a quantitative biochemical system as well as TPM. These approaches, combined with computer simulations, allow for the precise determination of the translocation rate. In addition, we show that this experimental system can also be applied to the study of the translocation of Holliday junctions through heterologous sequences. We show that sequence heterologies much shorter than previously described (Parsons *et al*, 1995; Adams and West, 1996) impede further translocation of the complex, resulting in two possible outcomes: disassembly and reassembly of the RuvAB complex permitting backward translocation, or bypass of the noncomplementary sequence, which allows passage through this reflecting barrier. Thus, branch migration through the heterology occurs with a probability that depends on the length of the heterologous sequences and the lifetime of the stalled RuvAB complex.

Results

Population-averaged RuvAB-directed branch migration

The S1 and S2 DNA branch migration substrates (Figure 1A) were constructed as described (Panyutin and Hsieh, 1994; Grigoriev and Hsieh, 1998). Annealing of two substrates, via their single-strand tails, generates a Holliday junction at a defined origin at one end of the homologous duplex regions. A 3-bp heterologous sequence was introduced in the S2 substrate, adjacent to the *AvaI* site (Figure 1B). This sequence heterology blocks spontaneous branch migration, which might interfere with bulk and 'single-molecule' measurements, by constraining the junctions to remain in the region spanning 27 bp between the origin and the sequence heterology, as documented by T7 endonuclease I footprinting experiments (data not shown). Therefore, the Holliday junctions were trapped in a short, defined stretch of DNA before the addition of RuvAB, and did not dissociate spontaneously under our experimental conditions. As expected, the 3-bp heterologous sequence did not affect the overall rate of RuvAB-mediated branch migration (data not shown).

We analyzed conversion of Holliday junctions (HJ 20/0; Figure 1B), formed by 4411-bp pBR322-derived substrates, to the monomer products of branch migration (Figure 2A). Radiolabeled Holliday junctions were incubated in the presence of RuvAB and ATP between 0 and 15 min, deprotei-

Figure 1 Experimental design. (A) Holliday junctions are performed by annealing of two branch migration substrates, S1* and S2. Branch migration to the distal end results in irreversible dissociation of the Holliday junction intermediate and formation of two heteroduplex products (P1* and P2). (B) pBR322-derived Holliday junctions used in this study (not to scale). The boxed gray regions represent heterologous sequences. The arrow indicates the location of the 3-bp mutation in the vicinity of the *AvaI* site. The asterisk denotes a ^{32}P -label.

nized and analyzed by gel electrophoresis. The appearance of the monomer products of branch migration was quantified and plotted as a function of time. We first compared the RuvAB-mediated conversion of Holliday junctions to the monomer products at 37 and 20°C (Figure 2A). The gels revealed the dissociation of the bands corresponding to Holliday junctions and the appearance of a monomer band that co-migrates with the radiolabeled S1 (S1*; Figure 2A, first lane). The multiple bands labeled as HJ* in Figure 2A are the Holliday junctions with different positions of the cross-over points, an observation consistent with previously published results (Panyutin and Hsieh, 1994). In our experimental system, RuvAB-directed branch migration of Holliday junctions can result in two possible outcomes. If the RuvB rings are assembled around the long arms of the junction, the reaction regenerates the branch migration substrates S1* and S2. If the RuvB rings encircle the short arms of the junction, branch migration leads to the formation of heteroduplex products P1* and P2 (Figure 1A). Therefore, for proper identification of the monomer band generated during branch migration, we applied restriction enzyme mapping of the molecular species generated within 900 s of reaction (Figures 2B and C).

The products of branch migration generated at 37 and 20°C were digested with *AvaI* and *EcoRV* and analyzed by electrophoresis on 6% polyacrylamide gels. In the case of branch migration substrate S1*, *AvaI* digestion results in the appearance of the partial duplex used to generate the branch migration substrates (Figure 2C, lanes 2 and 8). In the product P1*, *AvaI* digestion generates a 50-bp duplex that migrates faster than the partial duplex in polyacrylamide gels (Figure 2C, compare lanes 2 and 5). Taking into account that, after 900 s of branch migration at 37°C, only one band is

Figure 2 Population-averaged branch migration assay. (A) Branch migration of HJ 20/0 at 37 and 20°C. The first lane in both gels contained 32 P-labeled S1 in the absence of S2. (B) Restriction map of S1* and P1*. *Ava*I and *Eco*RV restriction sites are located at 50 and 22 bp, respectively. (C) Control of the ratio between S1* and P1* generated in 900 s of branch migration at 37°C (left panel) and 20°C (right panel). S1* (lanes 1–3, 7 and 8) was digested with *Ava*I (lanes 2 and 8) or with *Eco*RV (lane 3). Branch migration products obtained after 900 s of incubation at 37°C (lanes 4–6) or 20°C (lanes 9 and 10) were digested with *Ava*I (lanes 5 and 10) or *Eco*RV (lane 6), electrophoresed on 6% polyacrylamide gels and quantified. AI, *Ava*I; RV, *Eco*RV.

present in the branch migration assay (Figure 2A, 37°C), this result indicates that at this time of RuvAB-mediated branch migration, the Holliday junctions were fully converted into the products of branch migration (P1* and P2). This was further confirmed by digestion of the products by *Eco*RV, whose recognition site is generated by annealing of the single-strand tails (Figure 2C, lane 6). In contrast, at the same time point of branch migration at 20°C, *Ava*I digestion revealed the presence of a mixture of S1* and P1* in the band corresponding to the monomer products of branch migration (Figure 2C, lane 10). The relative amount of the partial duplex generated after *Ava*I digestion, approximately 32%, reflects the portion of the Holliday junctions that underwent branch migration toward the substrates S1* and S2 at 20°C.

Computer simulation of 'bulk' RuvAB-mediated branch migration

Since the conversion of Holliday junctions into products in the presence of RuvAB and ATP is a multistage process, we used a computer simulation to separate the kinetics of branch migration into individual reaction steps. This allowed us to estimate the relative contributions of the rate-limiting steps to

Figure 3 Computer simulations of RuvAB-directed branch migration. Simulations are shown for experiments performed in the absence (A) or in the presence (B) of a medially located sequence heterology. The different parameters used for computer simulations are shown (see text for details).

the process under our experimental conditions. Computer simulations were carried out according to the scheme shown in Figure 3. The initial state (state 1) was defined as a semimobile Holliday junction randomly positioned in the 27-bp region located between the heterologous sequences formed by the single-strand tails and the mutation in the vicinity of the *Ava*I site. After assembly of the RuvAB complex on a Holliday junction (state 2), translocation can proceed in two opposite directions with equal probabilities (states 3 and 6). Branch migration to the 'left' brings the complex to the sequence heterology formed by the single-strand tails. This causes the translocation of the complex to stop (state 4), and the complex can either return to the initial state 1 or go through the heterology (state 5). Bypass through the sequence heterology formed by the single-strand tails generates unpaired branch migration substrates (S1* + S2, state 5) that are capable of reannealing and re-forming the Holliday junctions (return to state 1). Translocation of the complex to the 'right' (state 6) for a number of base pairs

corresponding to maximal processivity results in the dissociation of the complex (state 7), which can be recycled through states 3 and 6. Branch migration to the distal end results in the irreversible formation of two heteroduplex products ($P1^* + P2$) and can be simulated as a runoff (state 8).

All transitions between the states of branch migration were simulated as random Poisson processes with characteristic times: the time of assembly of RuvAB on the junctions (τ_{AB}), the time of reannealing of the unpaired substrates (τ_{S1+S2}), the time of bypass through the sequence heterology (τ_{het}) and the lifetime of the complex stalled at a sequence heterology (τ_{life}). Translocation of the junction within states 3 and 6 was simulated as a Poisson process with $1/V$ characteristic time of translocation for 1 bp, where V is the mean velocity of branch migration and processivity is expressed as number of base pairs (N_{bp}). As RuvAB generates unpaired branch migration substrates as well as heteroduplex products that have identical electrophoretic mobility on agarose gels (Figure 2), the time courses of appearance of $S1^*$, $P1^*$ and $S1^* + P1^*$ were plotted as a function of time.

The time courses of branch migration of Holliday junctions HJ 20/0 at 37 and 20°C, averaged from three and four experiments, respectively, are shown in Figures 4A and B (filled circles). At 37°C, RuvAB-directed branch migration occurs rapidly, reaching saturation after 180 s (Figure 4A). The time course at 20°C is consistent with a decrease in the overall reaction rate and shows two distinct phases of accrual of the products of branch migration (Figure 4B). We detected a slow phase followed by a rapid accumulation of the monomer products with an inflection point at approximately 460 s. A similar biphasic shape of the branch migration time course was also observed when the assays were performed at 25°C (Supplementary data 1). Thus, lowering the overall rate of the reaction allows us to distinguish between different steps of the branch migration process.

The simplest explanation for the biphasic shape of the branch migration curve observed at 20°C can be derived from computer simulations (Figure 4B). The time course before the inflection point manifests a fraction of the Holliday junctions that revert to the unpaired branch migration substrates ($S1^* + S2$). This portion of the curve is rate-limited by the time of assembly of the complex on Holliday junctions (τ_{AB}), branch migration through the 20-bp sequence heterology (τ_{het} and τ_{life}) and the time required for reannealing of unpaired branch migration substrates (τ_{S1+S2}). The inflection point corresponds to the beginning of appearance of the heteroduplex products ($P1^* + P2$). The slope of the curve after the inflection point reflects both the translocation of Holliday junctions toward the distal ends, or runoff (τ_{AB} , V and N_{bp}) and reannealing of $S1^*$ and $S2$ substrates (τ_{S1+S2}).

To estimate the values of the 'best fit' parameters, we used a sectioning method for each parameter as described (Shoup, 1979). Given that the parameter values could be covariant, which might result in a systematic error of unknown magnitude, the sectioning method was followed by a global minimum localization by calculation of the deviation value between the simulations and the experimental points for each pair of simulated parameters. The combination of the two approaches for the fitting procedure allowed us to confirm that the observed 'best fits' are located within the boundaries of the global minimum (data not shown). The

Figure 4 Kinetic modeling of branch migration. Results are shown for experiments carried out at 37°C (A) and 20°C (B). Filled circles, experimental points of monomer-product appearance during branch migration assays; lines, computer-generated simulations. Branch migration is expressed as the fraction of the monomer band relative to the total amount of radioactivity for each time point. (C) Minimization analysis for the 'best fit' translocation rate for branch migration at 20°C. Deviation is the normalized χ^2 (see Materials and methods).

minimal deviation between the simulation curves ($S1^* + P1^*$) and actual measurements is illustrated as a cross-section of the global minimum in Figure 4C for the RuvAB translocation rate at 20°C (see Materials and methods for details). The best agreement between the simulations and the data for branch migrations at 20°C was obtained with a translocation rate of 9 ± 0.1 bp/s and a processivity of the complex exceeding the total length of the Holliday junctions

Table 1 Key parameters for branch migration corresponding to the values determined by the best fit between the simulations and experimental data (see text for details)

Parameters	'Best fit' values
Translocation rate (bp/s) at	
20°C	9 ± 0.1
25°C	17 ± 0.2
37°C	50 ± 0.6
Processivity (bp)	> 4411
Time of assembly, τ_{AB} (s)	400 ± 8
Reannealing time, τ_{S1+S2} (s)	> 2400
Bypass time, τ_{het} (s), through	
20-bp heterology	60 ± 0.3
40-bp heterology	155 ± 0.5
Lifetime, τ_{life} (s), at	
20-bp heterology	96 ± 1.4
40-bp heterology	83 ± 1.2

The uncertainty of the simulations was estimated as described in Materials and methods.

(the values of the 'best fit' parameters are given in Table 1; also see Supplementary data 1). Lowering the reaction temperature to 20°C allowed us to slow down the overall reaction rate by a factor of approximately 5.5 with respect to that at 37°C (the rate of translocation at 37°C was estimated to be 50 ± 0.6 bp/s). The ratio between S1* and P1* generated at 900 s of the branch migration time course at 37 and 20°C, as estimated by computer simulations, was in full agreement with restriction enzyme cleavage analysis (Figure 2C), as well as with the time course of reannealing of S1* and S2, which was found to be slow (>2400 s, data not shown) as previously described for nanomolar DNA concentrations under similar experimental conditions (Bjornson *et al*, 1994).

Passage of Holliday junctions is impeded by a sequence heterology

The slopes of the time course before and after the inflection point are different, suggesting the existence of a bias in branch migration toward the appearance of heteroduplex products (P1* + P2; Figure 4B). The Holliday junctions (HJ 20/0; Figure 1B) used in this experiment were flanked on the left side by the 20-bp sequence heterology, formed by the single-strand tails, and on the right side by the 3-bp mutation in the vicinity of the *AvaI* site followed by fully homologous sequences. As mentioned above, the 3-bp mutation did not have any effect on RuvAB-directed branch migration. Therefore, we reasoned that the effective rate of branch migration to the 'left', generating the unpaired substrates, and to the 'right', toward the products, might not be the same due to the 20-bp heterology.

To address this question, we simulated the effect of changes of the bypass time, τ_{het} , and the lifetime of the stalled complex (τ_{life}). The best agreement between the simulations and the experimental data for branch migration of HJ 20/0 was obtained with $\tau_{het} = 60 \pm 0.3$ s and $\tau_{life} = 96 \pm 1.4$ s (Figures 5A and C and Table 1). This indicates that a heterologous sequence as short as 20 bp may affect the RuvAB-directed translocation of Holliday junctions and thus create a bias in the time course of appearance of the monomer products.

To further investigate the bypass of heterology by RuvAB, we constructed pBR322-derived Holliday junctions in which we changed both the length and position of the sequence

Figure 5 RuvAB-directed branch migration is impeded by sequence heterology. (A) Time course of monomer-product appearance during branch migration of HJ 20/0 (filled circles). The lines represent computer-generated simulations of the appearance of S1* + P1* during branch migration. For comparison, the simulations of branch migration generated with $\tau_{het} = 20$ and 200 s are also shown. (B) Branch migration in the presence of a medially located 40-bp heterology. Open circles, HJ 40/0; open triangles, HJ 40/40. In (A, B), the ordinate axis is the same as in Figure 4. (C, D) Minimization analysis for the 'best fit' bypass time (τ_{het}) through 20-bp (filled circles) or 40-bp (open circles) heterologies and for the 'best fit' lifetime (τ_{life}) of the complex stalled at 20- or 40-bp heterologies, respectively.

heterology to be encountered by RuvAB. For these experiments, the junctions were constrained between a 40-bp sequence heterology on the left and the 3-bp *AvaI* mutation on the right (HJ 40/0; Figure 1B), and contained 40-bp heterologous inserts medially located approximately 0.3 kb

from the origin (HJ 40/40; Figure 1B). Based on the results described above, one prediction for this experiment is that the presence of a medially located 40-bp insert should affect the rate of RuvAB translocation through the insert and thus decrease the slope of the curve after the inflection point corresponding to the appearance of the heteroduplex products ($P1^* + P2$). Indeed, the time courses of branch migration of the junctions HJ 40/0 and HJ 40/40 (Figure 5B) showed the expected results. In the absence of a medially located sequence heterology (open circles, HJ 40/0), the shape of the branch migration curve was very similar to that obtained with the HJ 20/0 junction (Figure 5A). In contrast, in the presence of the medially located 40-bp heterology (open triangles, HJ 40/40), the rate of appearance of the heteroduplex products ($P1^* + P2$) significantly decreased. The best agreement between the data and the computer simulations, in which the bypass through a medially located sequence heterology was included (Figure 3B, state 9), was obtained with $\tau_{\text{het}} = 155 \pm 0.5$ s and $\tau_{\text{life}} = 83 \pm 1.2$ s (Figures 5C and D and Table I). As expected, the lifetime of the complex stalled at the 40-bp heterology was similar to that obtained on the 20-bp heterology (Figure 5D). However, the bypass time was 2.6 higher. Thus, the apparent velocity of the branch migration through the heterology can be estimated as approximately 0.3 bp/s at 20°C under our experimental conditions, a 30-fold decrease relative to the rate of branch migration through identical sequences.

Thus, results from bulk measurements of RuvAB-directed branch migration indicate that the translocation of Holliday junctions can be impeded by a sequence heterology as short as 20 bp. Increasing the extent of heterology exaggerates this impediment, in a manner that can be successfully modeled by computer simulations. These results suggest that translocation of Holliday junctions through a heterology might occur randomly, its possible outcomes being determined by the length of the noncomplementary sequences. Furthermore, our results also indicate that the overall probability to move Holliday junctions through the heterology might be determined by the lifetime of the stalled RuvAB complex.

Branch migration of individual Holliday junctions through identical DNA sequences

While results from the bulk experiments can be simulated to yield a plausible model for the translocation of Holliday junctions through identical or heterologous sequences, it is important to study these events in real time at the molecular level. Therefore, we next applied the TPM technique to analyze the effect of heterology in more detail. TPM uses a DNA molecule attached at one end to a glass surface while its other end is labeled with a bead. The Brownian motion of the bead, recorded by video microscopy, varies as a function of the length of the DNA tether (Yin *et al*, 1994). Thus, branch migration can be visualized as a shortening of the opposite arms of a Holliday junction tethering the bead to the glass surface (Figure 6A). We designed 2- and 3-kb Holliday junctions, derived from HJ 20/0 and formed by the 997- and 1513-bp branch migration substrates, respectively (Figure 6B). Holliday junctions were immobilized on the slide coated with anti-digoxigenin antibodies via the digoxigenin moiety covalently attached to one of the long arms.

Figure 6 'Single-molecule' branch migration assays. (A) A Holliday junction is attached to the glass surface via its long arms. RuvAB loading on the short arms of the junction results in a shortening of the tether, leading to a decrease in the amplitude of bead Brownian motion. (B) The 2- and 3-kb Holliday junctions used for TPM. The Holliday junctions were modified with digoxigenin (solid circles) and biotin (solid squares) at the 5' ends of the long duplex arms. (C) Positions of the bead (given in pixels), tethered with a 2-kb Holliday junction, after the addition of RuvAB and ATP as a function of time. (D) Time course of Brownian motion of the bead in (C) calculated at 4-s intervals. (E) Five examples of time courses of RuvAB-directed branch migration of 2-kb (filled circles) and 3-kb Holliday junctions (filled triangles). The numbers shown correspond to the observed rates of branch migration determined from the slopes of the linear fits (bp/s). (F) Distribution of individual branch migration rates (bin width 8 bp/s).

The other long arm contained biotin to which we attached a bead, allowing us to follow changes in the length of the tether during RuvAB-directed branch migration.

In the presence of RuvAB and ATP, a fraction of beads tethered by 2-kb Holliday junctions displayed the behavior predicted for RuvAB-directed branch migration (see Materials and methods). As shown in Figures 6C and D, a progressive decrease in the amplitude of bead Brownian motion was observed over a period of less than 1 min, after which the bead became immobile due to nonspecific binding to the glass surface (also see Supplementary data 2). These observations are consistent with binding of RuvB to the short arms of the junction and branch migration toward the distal ends of the homologous duplex region. Similar results were obtained on 3-kb Holliday junctions. In control experiments, no tether shortening was detected in the absence of RuvA, RuvB or ATP, indicating that the changes in the bead Brownian motion we observed were caused by bona fide RuvAB-mediated branch migration of individual Holliday junctions.

Representative individual branch migrations are shown in Figure 6E. The individual rates of branch migration were measured as the slope of a line fit to the tether length versus time. In 30 observed translocations of Holliday junctions, the rates of tether shortening were found to be constant within the detection limit for variation of the individual rates, or 4.5 bp/s (see Materials and methods). This result suggests a uniform movement of the Holliday junctions during RuvAB-mediated branch migration through identical DNA sequences within the resolution of TPM applied to branch migration. The values of the individual branch migration rates were found to be spread over a large range, from 7 to 37 bp/s (15.9 ± 8.7 bp/s, mean $\pm$ s.d., $N = 30$; Figure 6F). The distribution of the individual branch migration rates was found to be unimodal but asymmetrical with a median value of 12.5 bp/s. This asymmetry is most probably due to underestimation of individual velocities that are equal or less than the rate detection limit. Nevertheless, the majority of observed individual branch migration rates was found comprised between the values from bulk measurements carried out at 20 and 25°C (9 ± 0.1 and 17 ± 0.2 bp/s, respectively; Figure 4 and Supplementary data 1). A similar spread of the apparent rates was observed for the TPM-measured elongation rates of single molecules of *E. coli* RNA polymerase (Yin *et al*, 1994; Tolic-Norrelykke *et al*, 2004) or for the translocation rates of RecBCD measured in a 'single-molecule' helicase assay (Bianco *et al*, 2001). It is possible that the broad distribution of individual branch migration rates might reflect the formation of distinct RuvAB–Holliday junction complexes. It could also reflect an influence of temperature on the rate of branch migration. Note that these possibilities are not mutually exclusive.

Branch migration of individual Holliday junctions in the presence of a medially located sequence heterology

Can the impediment to RuvAB translocation by a sequence heterology predicted by computer simulations be detected using the 'single-molecule' approach? We next used 2-kb Holliday junctions derived from HJ 40/40, and formed by 1057-bp branch migration substrates to monitor the effect of medially located 40-bp heterologous inserts on RuvAB-directed branch migration (Figure 7A). In all observed individual branch migrations, translocation of Holliday junctions was impeded by the sequence heterology (see Materials and methods). The distributions of several hundred amplitudes of Brownian motion collected from 30 beads that started

Figure 7 RuvAB-directed branch migration of individual Holliday junctions in the presence of a medially located 40-bp sequence heterology. (A) The 2-kb Holliday junction containing a 40-bp heterologous insert (2-kb HJ 40/40). (B) Histograms for several hundred amplitudes of Brownian motion before (filled circles) and after (open circles) branch migration collected from 30 individual branch migrations. The lines correspond to best fits for a Gaussian distribution with 143 ± 33 and 112 ± 19 nm (mean $\pm$ s.d.) for each population, respectively. (C) Individual branch migration time course showing oscillations between forward and backward translocations of a Holliday junction constrained between two 40-bp blocks of heterology. (D) Bypass through a medially located 40-bp heterology.

branch migration and, presumably, ended it at the medially located heterology are shown in Figure 7B. The average value for the amplitudes at the end of branch migrations was found to be 112 ± 19 nm (mean $\pm$ s.d.). This value is in good agreement with the position of the 40-bp heterologous inserts that, in the case of stalled branch migration, should generate a 1434-bp tether.

Two examples of individual branch migrations are shown in Figures 7C and D. In Figure 7C, the amplitude of Brownian motion of the bead progressively changed several times from 154 ± 13 nm (mean $\pm$ s.d., $N = 67$) to 114 ± 8 nm ($N = 170$), within the range expected for Holliday junctions of 2034 bp (full-length tether) and 1434 bp (stalling at the heterologous insert), respectively. The rates of branch migration through

300-bp identical sequences located between the heterologous sequences, formed by annealing of the single-strand tails on the left and the medially located 40-bp inserts on the right, were similar to those observed in the absence of a medially located heterology. This result indicates that the 40-bp heterologous sequences flanking the junction on both sides act as reflecting barriers for RuvAB-directed translocation of the Holliday junction. The changes in the directionality of RuvAB-directed branch migration suggest partial or complete dissociation of the complex from the junction while stalled at the sequence heterology, followed by reassembly of RuvAB with the RuvB hexameric rings encircling the alternative pair of arms of the Holliday junction. This can ultimately result in oscillations between forward and backward translocations of the Holliday junction constrained between two blocks of sequence heterology, as exemplified by the overall shape of the time course in Figure 7C.

In agreement with the results of bulk measurements of branch migration (Figure 5B), we observed that a fraction of Holliday junctions (four out of 30 beads) could overcome the barrier imposed by the 40-bp heterology and proceed further. The behavior of such a junction is shown in Figure 7D. The first translocation, reflected by the decrease in amplitude of Brownian motion from 148 ± 8 nm (mean $\pm$ s.d., $N=13$) to 94 ± 8 nm ($N=126$), was similar to that in Figure 7C. In this case, initial translocation proceeded to the sequence heterology and stalled, most probably due to dissociation of the complex. After a long pause (>1000 s), branch migration resumed again in the same direction, indicative of bypass through the heterology.

The prediction from our bulk measurements of branch migration is that a sequence heterology should either force a complex to disassemble or slow down its translocation by a factor of 30 to approximately 0.3 bp/s (Figure 5). Since 4.5 bp/s is the rate detection limit in our experimental system, such a decrease in the velocity of translocation through the heterology would be ultimately detected as a pause of branch migration by individual Holliday junctions (Figure 6E).

The mechanism allowing stalled branch migration complex to overcome the impediment imposed by the sequence heterology remains to be elucidated, but such occasional bypasses are likely to play an important role in determining the outcomes of homologous recombination *in vivo*.

Discussion

Using a quantitative biochemical system together with a 'single-molecule' branch migration assay, we have shown that branch migration occurs in a processive manner when Holliday junctions are translocated through identical DNA sequences. When the RuvAB complex encounters a heterologous sequence as short as 20 bp, branch migration is impeded. We conclude from our results and kinetic model that translocation through the heterology occurs with a probability that depends on the extent of noncomplementary sequences and on the lifetime of the complex undergoing branch migration through the heterologous region.

Bypass of sequence heterology by recombination proteins has been of special interest because of its obvious biological significance (Iype *et al*, 1994, 1995; Parsons *et al*, 1995; Adams and West, 1996; West, 1997; Holmes *et al*, 2001).

During the strand-transfer reaction *in vitro*, the *E. coli* RecA protein is capable of progressing through heterologous inserts up to 200 bp, in a process requiring ATP hydrolysis (Iype *et al*, 1994); however, its eucaryotic homolog, Rad51, exhibits less prominent tolerance to heterology (Holmes *et al*, 2001). In the presence of RuvA, RuvB and single-strand DNA-binding (SSB) protein, the efficiency of heterology bypass during RecA-mediated three-strand exchange reaction is increased, resulting in accommodation of 1-kb heterologous inserts into heteroduplex DNA products (Iype *et al*, 1994; Adams and West, 1996). In a four-strand exchange reaction, RuvA and RuvB proteins together with SSB were able to dissociate χ -structures by traversing heterologous sequences up to 1.8 kb (Parsons *et al*, 1995). We note that in the latter case, the reaction was described as 'highly asynchronous', leading to the suggestion that the RuvAB complex has low processivity while translocating junctions through a sequence heterology. Our observations, which show that branch migration complexes that are impeded by short heterologous sequences can eventually progress through the heterology, provide direct evidence for this hypothesis.

It should be noted that our bulk and 'single-molecule' measurements of RuvAB-directed branch migration were carried out at 20°C. This allowed us to slow down the overall reaction rate by a factor of approximately 5.5 with respect to that at 37°C and therefore to emphasize the observed effects of short heterologous sequences on translocation of Holliday junctions by RuvAB. Nevertheless, even at 37°C, the difference between the time courses of branch migration of HJ 40/0 and HJ 40/40 was visible and coherent with computer-generated simulations of branch migration (Supplementary data 1), thus ruling out the possibility that the observed behavior of RuvAB at short heterologous sequences is an artifact of reducing the temperature and, consequently, the rate of the reaction.

The characteristic time for the lifetime of the RuvAB complex stalled at a sequence heterology is an assumption implicit in our kinetic model. The physical significance of this empirical parameter is not clear. One possibility is that it may reflect dissociation of the RuvB hexameric rings. During branch migration through a heterology, unpaired DNA is passing through the hole in the RuvB ring. Since the binding affinity of RuvB for double-stranded DNA is higher than for single-stranded DNA (Muller *et al*, 1993), the presence of unpaired DNA within the RuvB rings might increase the probability of partial or complete dissociation of RuvB. This is likely, given the results shown in Figure 7C: bouncing between forward and backward translocations can only be explained if there is initial disassembly of RuvB rings, followed by reassembly in the opposite conformation. Our observations are consistent with the hypothesis that, during branch migration through a heterology, RuvAB can act either as a facilitator of recombination by driving branch migration through a heterology or as a 'anti-recombinase' by reversing branch migration on long tracks of sequence heterology (West, 1997).

If the impediment to RuvAB-directed translocation of Holliday junctions by short tracks of sequence heterology increases the probability of dissociation of RuvB and/or RuvA from the junction, this may create a favorable situation for further processing of a Holliday junction by RuvC. Therefore, our experimental approaches should be useful for direct

visualization of the coupling between branch migration and resolution of Holliday junctions by RuvC, essential steps in homologous recombination and recombinational repair.

Materials and methods

Materials

RuvA and RuvB were purified as described (Tsaneva *et al*, 1992). Branch migration substrates were prepared by ligation of synthetic partial duplexes containing 20- or 40-nucleotide (nt) single-stranded tails to the unique *AvaI* site of PCR-amplified fragments derived from pBR322 using a nonmodified 20-nt forward primer (position 1381 with respect to the *EcoRI* site as +1) and a reverse primer containing either digoxigenin or biotin at the 5' end (positions 2371 and 2887) to yield the branch migration substrates of 997 and 1513 bp. Biotin or digoxigenin moieties were attached to the PCR primers via 16- or 12-atom spacers, respectively (Eurogentec, Belgium). The 4411-bp branch migration substrates were prepared by ligation of synthetic partial duplexes to the *AvaI* site of pBR322 as described (Grigoriev and Hsieh, 1998). The pBR322-derived plasmids containing 40-bp heterologous inserts were constructed by introducing the *XbaI* site at position 1743 by PCR-directed mutagenesis followed by ligation of 46-bp synthetic duplexes (for S1, CTA GAG CCT GTT TTA CTA GAG CCG CAA TTT GGG TAA CCT TGT ATA G and the complementary strand; for S2, CTA GCC GCG TGG AAC CCG GTA CCT CAT TAT CTC GAC GGG GTG AAT C and the complementary strand).

Branch migration assays

Branch migration reactions were performed at 20, 25 and 37°C as described (Grigoriev and Hsieh, 1998) in 10 µl of reaction mixture containing 25 mM Tris-acetate (pH 8.0), 10 mM Mg(CH₃COO)₂, 50 mM NaCl, 0.5 mM DTT, 10% glycerol and 65 µg/ml casein. The Holliday junctions (0.114 nM) formed by the branch migration substrates S1 and S2, were incubated in the presence of 90 nM RuvA, 280 nM RuvB and 1 mM ATP between 0 and 15 min, deproteinized by the addition of 2 µl of stop solution containing 1 mg/ml proteinase K, 1.25% SDS, 10 mM Tris-HCl, 0.06% bromophenol blue, 0.06% xylene cyanol and 30% glycerol in the presence of an excess of the oligonucleotides complementary to the single-stranded tails of S2 (to prevent the reannealing of S1* dissociated during branch migration). Samples were electrophoresed on native 0.8% agarose gels in Tris-borate buffer containing 2 mM MgCl₂ and 100 µg/ml ethidium bromide at 4°C. Gels were dried and quantified on a Fuji BAS 1000 phosphorimager.

For restriction enzyme mapping, the products of branch migration generated at 37 and 20°C were deproteinized as above (proteinase K was omitted in this case), gel-filtrated on G25 spin-columns pre-equilibrated with the appropriate restriction buffers and digested with *AvaI* or *EcoRV* for 60 min at 37°C. Samples were electrophoresed on native 6% polyacrylamide/Tris-borate/EDTA gels. Gels were dried and quantified on a phosphorimager.

Tethered-particle motion measurements

Experiments were performed using a protocol adapted from Finzi and Gelles (1995). A coverslip flow chamber (30 µl volume) was incubated with the anti-digoxigenin antibody (20 µg/ml; Roche) in PBS for 20 min at 4°C. After washing, the chamber was incubated overnight in branch migration assay buffer at 4°C. The Holliday junctions (30 pM) were incubated in the flow chamber for 1 h, washed and labeled with NeutrAvidin-coated latex beads (0.2 µm; Molecular Probes). After the addition of 30 nM RuvA, 90 nM RuvB and 2 mM ATP, the tethered beads were observed and recorded by video-enhanced differential interference contrast microscopy at a recording rate of 25 frames/s in an air-conditioned room at 22 ± 2°C. The amplitude of Brownian motion (*A*, nm) of the beads was evaluated as the standard deviation over 4 s of 100 successive bead positions determined by image processing. The length of the DNA molecule in the range of 300–3500 bp (*L*, bp) can be calculated from the amplitude of Brownian motion of the tethered bead for linear DNA fragments using the following relationship: $L = 20.5 + 5.648A + 0.0574A^2$ (experimental data from Pouget *et al*, 2004). The standard deviation of the length measurements (σ_L , bp) is $\sigma_L = -1.9 + 0.98A + 0.18A^2$. The individual branch migration rates and the standard errors were determined by weighted least-

square linear fits of the tether length versus time. The average of the velocity error was found to be 4.5 ± 2.5 bp/s (mean ± s.d.). The obtained value was higher than previously reported for individual transcription elongation velocities (Tolic-Norrelykke *et al*, 2004) due to a relatively short time span of branch migrations and therefore to a small number of the fitted points for each individual branch migration (8–15 tether length positions). Note that the translocation of a junction for 1 bp results in the tether shortening for 2 bp. We considered the error value of 4.5 bp/s as the rate detection limit in our experimental system.

During 'single-molecule' branch migration assays of HJ 20/0 or HJ 40/40 junctions, 767 or 173 beads, respectively, were recorded and analyzed. For HJ 20/0, 50% of the beads (390 beads) did not display any changes in the amplitude of Brownian motion during the observation, 46% (356 beads) became detached from the glass surface without a visible decrease of their Brownian motion and 4% (30 beads) displayed progressive decrease of the amplitude of Brownian motion. For HJ 40/40, 35% (60 beads) did not display any changes of the amplitude of Brownian motion during the observation, 48% (83 beads) were detached from the glass surface and 17% (30 beads) displayed decrease of the amplitude of Brownian motion. Among them, four out of 30 beads bypassed the heterology, four out of 30 displayed changes (2–5 times) in the directionality of branch migration and 22 beads showed only one round of branch migration toward the medially located 40-bp heterology. In control experiments, a similar fraction of detached beads was observed when the beads were tethered either by linear DNA fragments or by 2- or 3-kb Holliday junctions in the absence of the proteins, suggesting that this process is most probably determined by the interactions between digoxigenin and anti-digoxigenin antibodies.

Computer simulations

The computer simulation of population-averaged RuvAB-directed branch migrations was based on the Monte-Carlo method, written and compiled using the Interactive Data Language (Research System Inc., USA). The simulation algorithm used the following parameters: the characteristic time of assembly of RuvAB on Holliday junctions (τ_{AB} , s); the mean rate of branch migration (V , bp/s); the processivity of RuvAB with the characteristic number of base pairs (N_{bp}); branch migration through a heterologous sequence with a characteristic time τ_{het} proportional to the length of sequence heterology; the lifetime of the complex stalled at a sequence heterology (τ_{lifc}); and the characteristic time of reannealing of the unpaired branch migration substrates (τ_{S1+S2}). All were simulated as Poisson processes. The entire simulation time was divided into Δt time steps. At each time step and for each trial, the state of the branch migration was changed with the probability $p = \Delta t / \tau_{AB}$ for the assembly of the complex, $p = \Delta t / \tau_{het}$ for branch migration through the sequence heterology (same for all τ and N_{bp}) and $p = \Delta t V$ for the movement of the junction by 1 bp. If Δt is small enough to keep the probability of each event < 0.01 , this algorithm simulates the Poisson process when the probability of a random event (P) inside the time interval T is $P = (T/\tau)e^{-T/\tau}$, where τ is τ_{AB} , τ_{S1+S2} , τ_{het} , τ_{lifc} , $1/V$ or N_{bp} . Typically, 2000–5000 trials were performed, and the results were plotted as a fraction of trials that reached the runoff versus time.

To estimate the values of the 'best fit' parameters, the deviation between the simulation curves ($S1^* + P1^*$) and the actual measurements was determined as follows:

- (1) The standard deviation of the simulated points, σ , is

$$\sigma(t) = \sqrt{\frac{W(t)(1 - W(t))}{N_{trials}}}$$

where W is the value of simulated points, and N_{trials} is the number of trials (shown as 95% confidence interval in Figures 4 and 5 as $\pm 2\sigma$ on the simulation curves).

- (2) The χ^2 value was calculated as

$$\chi^2 = \sum_{i=1}^N \sum_{j=1}^{M_i} \frac{(W_j^i P_i)^2}{\sigma_i^2}$$

where i is the index of experimental points P_i , j is the index of simulation points around the experimental point within the time interval $|T_i - T_{ij}| < 10$ s, N is the number of experimental points, M_i is the number of simulated points $W(t)$ inside the i time interval

around the experimental point and σ_i^j is the standard error of W_i^j point.

(3) The deviation, D , was calculated as χ^2 and normalized to the number of simulated points:

$$D = \sqrt{\frac{\chi^2}{\sum_{i=1}^N \sum_{j=1}^{M_i} 1/\sigma_i^j}}$$

The simulations were performed for each value of each simulated parameter, while other parameters were kept unchanged using a sectioning method (Shoup, 1979) and the position of a global minimum was determined. For illustration purposes, the deviation values were plotted versus given parameter to determine the minimal deviation corresponding to the 'best fit' value of this parameter. The value of the 'best fit' parameter was estimated as the value corresponding to the minimal value of the deviation determined by weighted least square parabola fits. The error for the simulation of the parameter value estimate is shown in Table I as $\pm 2\sigma$ of the uncertainty for the parameters estimation.

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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