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The kinetics of spontaneous DNA branch migration

IGOR G. PANYUTIN AND PEGGY HSIEH

Genetics and Biochemistry Branch, National Institute of Diabetes and Kidney and Digestive Diseases, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT An important step in genetic recombination is DNA branch migration, the movement of the Holliday junction or exchange point between two homologous duplex DNAs. We have determined kinetic parameters of spontaneous branch migration as a function of temperature and ionic conditions. The branch migration substrates consist of two homologous duplex DNAs each having two single-strand tails at one end that are complementary to the corresponding single-strand tails of the other duplex. Upon rapid annealing of the two duplex DNAs, a four-stranded intermediate is formed that has a Holliday junction at one end of the duplexes. Branch migration to the opposite end of the duplexes results in complete strand exchange and formation of two duplex products. The rate of branch migration is exceedingly sensitive to the type of metal ions present. In magnesium, branch migration is quite slow with a step time, τ, equal to 300 msec at 37°C. Surprisingly, branch migration in the absence of magnesium was 1000 times faster. Despite this difference in rates, apparent activation energies for the branch migration step in the presence and absence of magnesium are similar. Since metal ions have a profound effect on the structure of the Holliday junction, it appears that the structure of the branch point plays a key role in determining the rate of spontaneous DNA branch migration. We discuss the role of proteins in promoting the branch migration step during homologous recombination.

Genetic recombination involving the exchange of genetic information between two DNA molecules has been observed in virtually all organisms. An important intermediate in both homologous and site-specific recombination is the Holliday junction, the branch point connecting two duplex DNAs that are undergoing recombination. If the branch point is flanked by DNA sequence homology, the Holliday junction can spontaneously migrate in either direction by the exchange of hydrogen bonds between the bases in homologous DNA strands. This process is known as branch migration and is an important step in genetic recombination. In homologous recombination, the extent of branch migration affects the amount of genetic information that is transferred between two homologues. In site-specific recombination, such as in the integration of bacteriophage λ into the *Escherichia coli* chromosome, branch migration in the region of homology shared between donor and recipient sites is a prerequisite for subsequent steps in the recombination pathway.

Early estimates of the rate of branch migration yielded a step rate of several thousand bases per sec (1), a rate that was robust enough to be accommodated within the time scale of recombination in bacteriophages. This suggested that branch migration could occur spontaneously during recombination without the need for proteins to facilitate this step. Later, several groups studying cruciform transitions in supercoiled DNA obtained data suggesting that the rate of spontaneous branch migration might be significantly slower (2–4).

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In addition to knowing the inherent rate of branch migration, it is also critical to know whether spontaneous branch migration can traverse sequence heterology such as mismatches, insertions, and deletions since homologous recombination usually involves the exchange of DNA strands between two similar but not identical duplexes. We recently observed that a single base mismatch was sufficient to slow the overall rate of branch migration (5). Moreover, this attenuation by sequence heterology was more pronounced in magnesium than in sodium, suggesting that branch migration is influenced by metal ions.

To clarify questions concerning the intrinsic rate of branch migration, we have developed an improved assay for branch migration to determine kinetic parameters of spontaneous DNA branch migration as a function of temperature and ionic conditions. We observe that branch migration in magnesium is ≈1000 times slower than was previously reported. In the absence of magnesium, branch migration is 3 orders of magnitude faster. We discuss the relationship between the structure of the Holliday junction and rates of branch migration and the need for proteins to promote branch migration during recombination.

MATERIALS AND METHODS

Substrates. The 296-bp *Eco*RI/*Nde* I fragment of pUC18 was generated by PCR according to the manufacturer's directions (Perkin–Elmer) by using a pair of primers mapping to positions 219–240 and 494–514 of pUC18 (6). The PCR was purified by extraction with phenol and chloroform and digested with *Eco*RI (New England BioLabs). The longer fragment was purified from a polyacrylamide gel. Partial duplexes (D1 and D2) were obtained by annealing synthetic oligonucleotides followed by purification of the products from polyacrylamide gels. The sequences of the oligonucleotides were as follows: 5′-ACCATGCTCGAGATTACGAGATATCGATGCATGCG-3′ and 5′-AATTCGCATGCATCGATATAATACGTGAGGCCTAGGATC-3′ for D1; 5′-GATCCTAGGCCTCACGTATTATATCGATGCATGCG-3′ and 5′-AATTCGCATGCATCGATATCTCGTAATCTCGAGCATGGT-3′ for D2; and 5′-ACCATGCTCGAGATTACGAGATATCGATATGCATG-3′ and 5′-AATTCATGCATATCGATATAATACGTGAGGCCTAGGATC-3′ for D1H, which has a heterologous duplex region with respect to D2. The second oligonucleotide in each pair was 5′-phosphorylated during synthesis.

Partial duplexes were ligated to the pUC18 fragments and the substrates for branch migration assays were purified from polyacrylamide gels for the 300-bp fragments or by two rounds of gel filtration through Sephacryl S-300 (Pharmacia) spin columns for the mixture of 956- and 1730-bp fragments. The 160-bp substrate was obtained by digesting the 300-bp substrate with *Pvu* II (New England BioLabs). Substrates were 5′ ^{32}P-end-labeled using T4 polynucleotide kinase (Pharmacia) and purified by extraction with phenol/chloroform followed by gel filtration through a Sephadex G50 spin column (Boehringer Mannheim).

Branch Migration Assay. A ^{32}P-labeled duplex substrate (final concentration, 3 μg/ml) was mixed on ice with a 10-fold

molar excess of a second unlabeled substrate (final concentration, 30 μg/ml) in a 10-μl reaction volume containing TNM buffer (10 mM Tris·HCl, pH 8.0/0.1 mM EDTA/50 mM NaCl/10 mM $MgCl_2$). The rate of branch migration was not altered depending on whether the substrate present in excess had D1 or D2 tails or on whether the substrates were made by PCR or derived from restriction endonuclease digestion. In some cases, after annealing, the TNM buffer was exchanged with TNE buffer (10 mM Tris·HCl, pH 8.0/0.1 mM EDTA/100 mM NaCl) by gel filtration through a Sephadex G-50 spin column equilibrated with TNE. After incubation at the appropriate temperature, the reaction was stopped by adding 10 μl of ice-cold TNM buffer containing 1 μg of ethidium bromide per ml and kept on ice prior to electrophoresis.

Samples were analyzed by electrophoresis in 4% (wt/vol) (160- and 300-bp substrates) or 1% (wt/vol) (956- and 1730-bp substrates) agarose gels at 10°C in TBE buffer (0.89 M Tris borate/1 mM EDTA) containing 3 mM $MgCl_2$ and 0.5 μg of ethidium bromide per ml in order to retard branch migration during separation. Gels were fixed in 10% trichloroacetic acid, dried, and autoradiographed using Kodak XAR 2 film or analyzed on a Phosphorimager (Molecular Dynamics). In some cases, samples were electrophoresed on 10% (wt/vol) native polyacrylamide gels in TBE buffer (NOVEX, San Diego).

Computer simulations of random walks were carried out as described (5). Step times derived from computer simulations were in good agreement with step times derived from models for one-dimensional diffusion (discussed in refs. 7 and 9). Our computer simulation is useful for short duplex lengths and allows one to model barriers to branch migration such as mismatches.

RESULTS

Branch Migration in the Presence of Magnesium. Substrates (S1 and S2) for the branch migration experiments were obtained by ligation of different fragments of pUC18 to one of two synthetic partial duplexes, D1 and D2 (Fig. 1 *A* and *B*). These partial duplexes contain 20-base noncomplementary, single-stranded tails and identical 15-bp duplex regions. The 5′ tail of D1 is complementary to the 3′ tail of D2; similarly, the 3′ tail of D1 is complementary to the 5′ tail of D2. Therefore, upon annealing, the two duplex substrates, S1 and S2, are joined in a four-stranded complex in which the branch point is a Holliday junction that can commence branch migration in only one direction (Fig. 1*C*). When the Holliday junction has traversed the length of the duplex region, strand exchange is complete, resulting in the irreversible dissociation of two product duplexes, P1 and P2. In branch migration assays, one of the substrates is 5′-end-labeled with ^{32}P while the second, unlabeled substrate is present at a 10-fold molar excess to promote rapid annealing. The accumulation of four-stranded intermediates and duplex products is quantitated after electrophoresis on agarose gels.

The branch migration assay was carried out using the 300-bp duplex substrates at 50°C in TNM buffer containing 10 mM $MgCl_2$ (Fig. 2*A*). In the absence of incubation at elevated temperature (0-min lane), there was no appreciable annealing of the two substrates. After incubation at 50°C for 0.5 min, more than half of the labeled substrates were converted to four-stranded complexes that migrated roughly at the position of linear dimer. Annealing of the fragments was completed by 1 min. The initial four-stranded complexes containing asymmetric arm lengths were rapidly converted to more slowly migrating species as evidenced by a broadening of the band corresponding to the early intermediates. The broadening of this band most likely reflects the appearance of a heterogeneous population of four-stranded intermediates

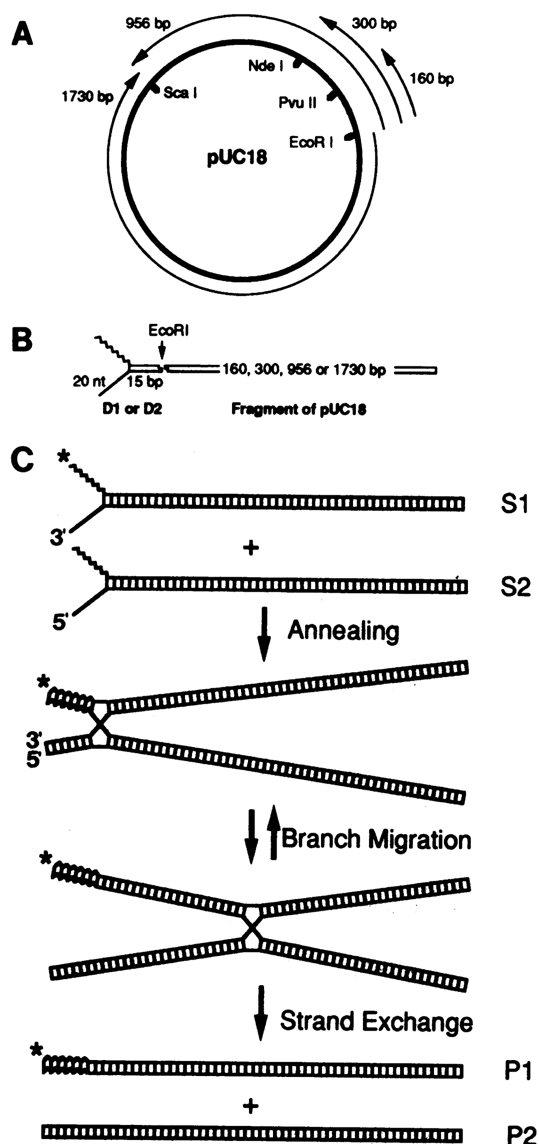

FIG. 1. Scheme for branch migration assay. (*A*) Map of pUC18 duplex fragments. (*B*) Tailed substrate, S1 or S2, containing D1 or D2 partial duplexes ligated to pUC18 fragments of various lengths. (*C*) Branch migration assay using S1 and S2 tailed substrates, which are annealed to form a four-stranded complex containing asymmetric arm lengths and a Holliday junction at one end. Branch migration results in intermediates containing Holliday junctions at various locations along the duplex regions. Complete strand exchange leads to production of two duplex products, P1 and P2. Structures are drawn for illustrative purposes only and do not imply any particular conformation.

whose branch points are located various distances from the ends of the duplexes due to branch migration (see below). After 10 min of incubation, product duplexes begin to appear at the position of the monomer band. The intensity of this band increases up to 100 min of incubation when all four-stranded complexes have completed branch migration to yield product duplexes. Although agarose gels cannot resolve the tailed substrates from the duplex products, analysis of branch migration assays on polyacrylamide gels reveals that tailed substrates migrate more slowly than duplex products (data not shown). DNA duplexes with denatured ends have been shown previously to exhibit retarded mobility (8). Thus, we can confirm that the monomer bands appearing late in the reaction represent bona fide products and do not arise by dissociation of four-stranded complexes to starting substrates.

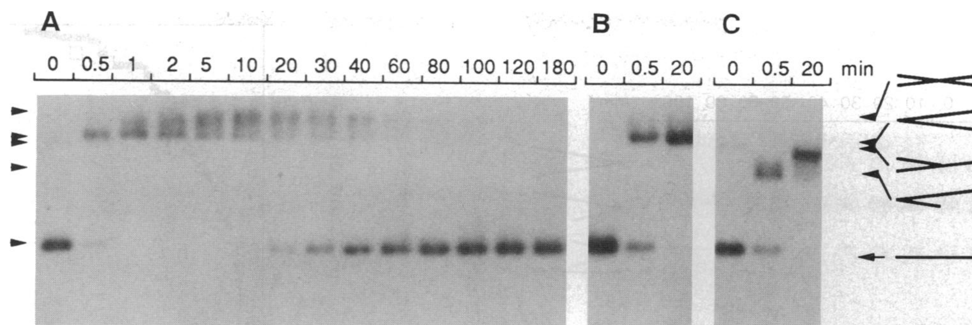

FIG. 2. Branch migration assays in the presence of magnesium. (*A*) Time course of branch migration for the 300-bp fragment at 50°C in TNM buffer. Assays were carried out as described in TNM buffer containing 10 mM $MgCl_2$. (*B*) Electrophoretic mobility of four-stranded complexes containing a Holliday junction constrained to the initiation site by flanking sequence heterology. (*C*) Electrophoretic mobility of complexes containing a branch point trapped near the middle of the 300-bp duplex. Length of incubation at 50°C is indicated at the top of each lane. Arrowheads indicate migration positions of various DNA species.

To further describe the four-stranded intermediates in the branch migration assay, we carried out two control experiments. In the first experiment, we determined the relative electrophoretic mobility of four-stranded complexes having an immobile Holliday junction constrained to the region adjacent to the annealed tails. When the two duplex substrates contain D1H and D2 partial duplexes that have nonidentical 15-bp duplex regions (see Fig. 1*A* and *Materials and Methods*), the four-stranded complex formed after annealing of the two substrates contains an immobile Holliday junction that cannot branch migrate in either direction due to flanking sequence heterology. The electrophoretic mobility of this blocked complex should closely approximate that of the four-stranded intermediates seen at very early time points in branch migration assays (Fig. 2*B*). After 20 min of incubation at 50°C, all such constrained four-stranded complexes comigrate with the intermediate seen at early time points in Fig. 2*A*. As expected, no monomer duplexes are present. Incubation of blocked complexes for 60 min at 50°C gave identical results (data not shown).

In the second experiment, a ^{32}P-labeled 300-bp substrate was incubated with the unlabeled, truncated 160-bp substrate (Fig. 2*C*). The D1 and D2 partial duplex regions in this case are identical; therefore, branch migration commenced after annealing of the substrates. However, when the branch point reached the opposite end of the 160-bp fragment, the Holliday junction was transformed into a three-strand junction. We have previously shown that initiation of branch migration from this state is a slow process (5). Therefore, this point serves to trap complexes having three arms of very similar length. As shown in Fig. 2*C*, complexes with retarded mobility on agarose gels were observed after branch migration.

The experiments presented in Fig. 2 *B* and *C* confirm that the most rapidly migrating intermediates correspond to four-stranded complexes with pairs of arms of vastly different lengths, while the most slowly migrating intermediates are complexes whose branch points have migrated closer to the middle of the duplexes. They also confirm that four-stranded complexes do not dissociate to the starting substrates during incubation at 50°C or during handling or electrophoresis.

The appearance of branch migration intermediates and products was quantitated by Phosphorimager analysis of the agarose gels. The fraction of total radioactivity migrating as four-stranded complexes or monomer duplexes was plotted as a function of time at 50°C for branch migration through 300 bp in 10 mM $MgCl_2$ (Fig. 3). The quantitation was normalized to total cpm migrating as complexes or monomers to take into account nonspecific losses and sample loading differences from lane to lane.

Branch Migration in the Absence of Magnesium. When branch migration assays were repeated in TNE buffer containing no magnesium ions, four-stranded intermediates did not accumulate and the corresponding 300-bp duplex products appeared at the earliest time points, indicating that branch migration is significantly faster in sodium than in magnesium (data not shown). To obtain quantitative data in the absence of magnesium ions, we used substrates with longer duplex regions. D1 and D2 partial duplexes were ligated to *Eco*RI-digested pUC18. After digestion with *Sca* I, we obtained an equimolar mixture of 956- and 1730-bp fragments each with D1 or D2 tails. After annealing of the substrates, three different four-stranded complexes were formed: two containing 956- or 1730-bp fragments annealed to themselves and a chimeric complex containing a 956-bp fragment annealed to a 1730-bp fragment (Fig. 4, annealing lane). The first two complexes contained mobile Holliday junctions, while the third was unable to branch migrate since the 956- and 1730-bp fragments have different sequences (see Fig. 1*A*).

Fig. 4 shows the time course of strand exchange at 25°C in TNE buffer. The general scheme of the experiment is the same as that presented in Fig. 2 with one exception. Since, as we mentioned, branch migration in the absence of magnesium ions is fast, the substrates were annealed in TNM buffer containing magnesium at 50°C for 1 min (Fig. 4, annealing lane). Samples were immediately placed on ice and EDTA was added to a final concentration of 20 mM. The

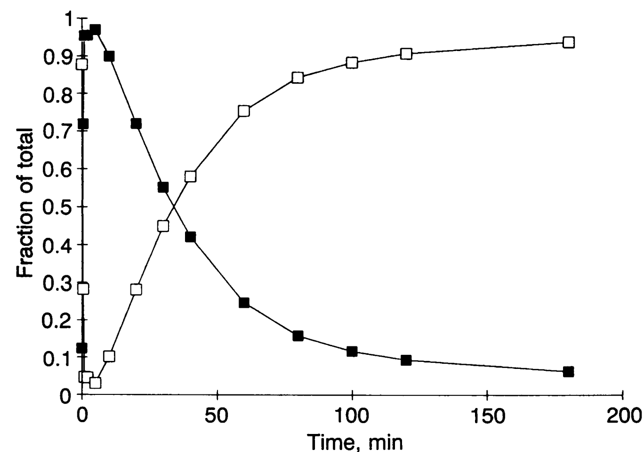

FIG. 3. Quantitation of the branch migration assay for 300 bp at 50°C in TNM buffer. Fraction of total ^{32}P label migrating as four-stranded complexes (■) or monomer duplex (□) was quantitated by Phosphorimager analysis of the data shown in Fig. 2*A*.

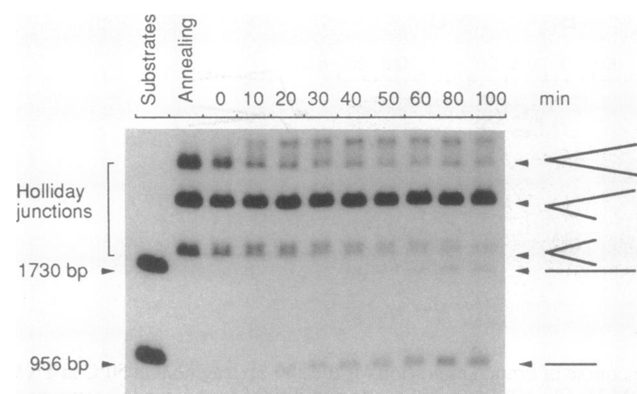

FIG. 4. Branch migration assays in the presence of sodium. Assays using the 956- and 1730-bp substrates were carried out in TNE buffer at 25°C as described.

buffer was then rapidly exchanged by gel filtration through a Sephadex G50 spin column equilibrated with TNE buffer at 4°C followed by incubation at 25°C. As expected, the bands on agarose gels corresponding to the two intermediates containing mobile Holliday junctions broaden with time, reflecting branch migration. Subsequently, linear duplex products appear. In contrast, the chimeric complex containing a nonmobile junction migrates as a discrete band with unchanged intensity during the time course of the reaction. This confirms that the four-strand complex does not dissociate to starting substrates.

Quantitation. The time courses for branch migration were characterized by the value of the half-time ($t_{1/2}$), which was the time required for half of the four-stranded complexes to dissociate to product duplexes by branch migration. For example, from the data in Fig. 3, the $t_{1/2}$ for branch migration through 300 bp in magnesium at 50°C was 32 min. Half-times for strand exchange for different fragments at different temperatures and ionic conditions are summarized in Table 1. These are the results of multiple trials. Errors were determined to be no more than ±10%.

In the simplest case, branch migration can be described as a random walk process in which the direction of movement of the branch point along the DNA is not biased by its previous movement. Modeling branch migration as a random walk allows one to describe the intrinsic process in terms of a step time, τ, the time required for a single step of one base pair (1). To determine the number of steps required in the course of a random walk to reach the end of a fragment of defined length, we carried out computer simulations. Typically, 100 trials were executed, and the results are presented as a plot of the fraction of all trials that reached the end of the fragment by a given number of steps. This representation of the random walk allows comparison of the random walk simulations with the experimentally determined time courses for complete strand exchange in branch migration assays.

The experimental and simulated curves are the functions of different variables—time and number of steps, respectively. To compare the two, we adjusted the scale of the computer

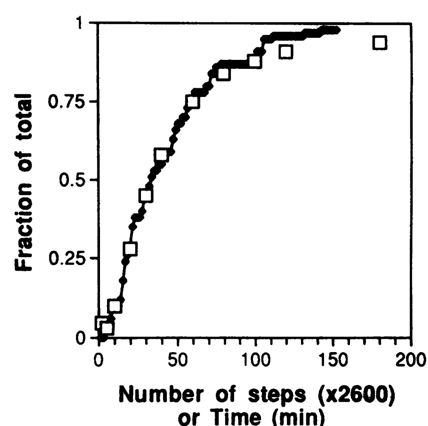

FIG. 5. Comparison of the time course for branch migration with computer simulation of the random walk. A computer simulation of a random walk through 300 bp is graphed as the fraction of trials that have reached the end by a given number of steps (◆). Time course from Fig. 3 for branch migration through 300 bp at 50°C is shown for comparison (□).

simulation to match the two points corresponding to $N_{1/2}$ (the number of steps when half of the simulated trials reach the end of the fragment) and $t_{1/2}$ (the time required for half-maximal product formation in branch migration assays). Fig. 5 shows such a comparison for branch migration through 300 bp at 50°C in magnesium. The fact that the two sets of points coincide indicates that, to a first approximation, the random walk model presented above adequately describes the course of the strand-exchange reaction. Dividing $t_{1/2}$ by $N_{1/2}$, the number of steps when half of the simulated trials reach the end of the fragment, yields an approximation of the step time, τ, for branch migration. Step times for branch migration at different temperatures and ionic conditions are summarized in Table 2. The close agreement of step times obtained for substrates of different lengths is another indication that the simple random walk model is adequate.

An Arrhenius plot of the data in Table 2 yields rough estimates of the apparent activation energies for the branch migration step (data not shown). Surprisingly, despite the 3 orders of magnitude difference in the step time in magnesium compared to sodium, the corresponding activation energies in magnesium and sodium are virtually the same within experimental error—36 and 38 kcal/mol, respectively (1 cal = 4.184 J).

DISCUSSION

The most striking feature of the data presented above is that the rate of uncatalyzed DNA branch migration in the presence of sodium is ≈1000 times faster than in magnesium. This metal ion-dependent difference in the rate of branch migration is sufficiently large to explain our previous observation that branch migration through a single heterologous base pair was retarded in the presence of sodium but essentially blocked in the presence of magnesium (5).

Table 1. Half-times (min) for strand-exchange reactions

t	Mg^{2+}		Na^{+}	
	160-bp fragment	300-bp fragment	956-bp fragment	1730-bp fragment
25°C	—	—	70	—
30°C	—	—	20	100
37°C	100	326	5	20
45°C	—	80	—	—
50°C	—	32	—	—

Table 2. Step times (msec) for branch migration

t	Mg^{2+}		Na^{+}	
	160-bp fragment	300-bp fragment	956-bp fragment	1730-bp fragment
25°C	—	—	3.6	—
30°C	—	—	1.2	1.7
37°C	300	270	0.3	0.4
45°C	—	66	—	—
50°C	—	26	—	—

Our data begin to provide a link between the structure of the Holliday junction and the rate of branch migration. Others have shown that a model four-way DNA junction assumes one of several conformations depending on the metal ion present (reviewed in ref. 10). In the presence of group II metals such as magnesium, the junction is folded and assumes a stacked X structure in which there is presumed to be pairwise interhelical stacking. In the presence of sodium, junctions are partially folded; however, unlike the magnesium-induced stacked X structures, the junctions in the presence of sodium are accessible to osmium tetroxide-mediated cleavage at the crossover point (11).

The conclusion that the structure of the branch point plays a key role in determining the rate of four-stranded branch migration is bolstered by comparison with three-stranded branch migration where the point of exchange between two single strands lacks such defined structure. Three-stranded branch migration is relatively fast with step times on the order of 12 μsec at 37°C in 10 mM NaCl (12). We have observed that the barrier to branch migration of a single base mismatch is much less pronounced in a three-stranded compared to a four-stranded branch. Also, in contrast to the four-stranded case, we observed no difference in the effect of sequence heterology on three-stranded branch migration in magnesium compared to sodium (5).

Our findings indicate that changes in the conformation of the Holliday junction can radically alter the rate of branch migration. The exact nature of the inhibition of branch migration posed by the divalent metal ions is unclear. One possibility is that magnesium restricts the isomerization of Holliday junctions between a stacked structure and a less constrained structure that is poised to undergo the branch migration step. The similarity of the activation enthalpies for the migratory step in magnesium and sodium suggests that the difference in rates is attributable at least in part to a large activation entropy for branch migration in magnesium.

Although our findings dramatically reverse previously published data on branch migration rates, they are consistent with recent data on branch migration in magnesium by using χ structures or cruciform substrates (R. Warner, personal communication). X forms that can dissociate to linear monomers by branch migration were observed to be more stable in magnesium than in sodium, although there was no determination of the extent of the difference or the actual rates (13). Recombination intermediates formed by RecA were unexpectedly stable after removal of RecA protein (14). Our findings indicate that the stability of these four-stranded intermediates may be attributable at least in part to slow branch migration.

The step times presented here are average values. Studies of the effect of sequence context on the structure of four-way junctions (reviewed in ref. 10) make it reasonable to assume that the rate of branch migration is sensitive to the local environment. In addition, we have arbitrarily defined a step as being equivalent to 1 bp. In reality, the Holliday junction could move several bases at each step, although our data on the effect of mismatches would suggest that no more than a few base pairs are melted at each step (5).

Clearly, rates of spontaneous branch migration in magnesium are too slow to account for genetic recombination involving exchange over long regions. Cowart *et al.* (15) proposed that in λ integrative recombination, branch migration through 7 bp of homology shared between phage and bacterial attachment sites occurs spontaneously and is not catalyzed by the phage-encoded Int recombinase. This seems quite reasonable since computer simulations indicate that spontaneous branch migration through 7 bp would occur in less than 1 min in magnesium at 37°C (data not shown). However, in homologous recombination, where strand exchange may involve hundreds or thousands of base pairs and must traverse sequence heterology, proteins must be recruited to promote the branch migration step.

The *E. coli* RecA protein carries out the pairing of homologous DNAs and strand exchange *in vitro* in both three-stranded and four-stranded reactions. While the rate of branch migration in the presence of RecA is modest—3 bp/sec in four-stranded reactions—RecA-mediated branch migration is polar and, when accompanied by the hydrolysis of ATP, can traverse sequence heterology (reviewed in ref. 16).

Several prokaryotic enzymes, all of them DNA helicases, appear to promote branch migration *in vitro*. The rate of branch migration by the bacteriophage T4 uvsX recombinase, a protein with similarities to *E. coli* RecA, was shown to be accelerated ≈4-fold by the T4 dda helicase (17). The *E. coli* RuvA and RuvB proteins when added to four-stranded intermediates made by RecA can promote strand exchange at a rate of 10–20 bp/sec (18, 19). The *E. coli* RecG protein (20) and helicase II (UvrD) protein (21) have also been shown to promote branch migration of Holliday junctions formed by RecA.

The relatively simple system described here for the study of DNA branch migration may yield information on the isomerization events involved in the branch migration step. Additional studies of the effect of metal ions on branch migration may be useful in this regard. This assay may also be useful in identifying and characterizing proteins that affect the rate of branch migration.

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