Reassociation rate limited displacement of DNA strands by branch migration

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ABSTRACT

    Large branched DNA structures are constructed by two-step reassociation of separated complementary strands from restriction fragments of different lengths. The displacement of DNA strands initially annealed to longer complementary DNA sequences, a process mediated by branch migration, is very rapid and has thus far been followed only under conditions which are second order, DNA reassociation rate limiting. The average lifetime of branched DNA leading to displacement of 1.6 Kb strands is estimated to be less than 10 seconds under conditions of DNA reassociation, $T_m-25°C$.
    Several DNA-binding drugs, including intercalating dyes, have been tested to determine their influence, if any, on the kinetics of DNA strand displacements by branch migration. Only actinomycin D was found to have significant effect under the conditions we have described. The kinetics of the strand displacement in the presence of low concentrations of actinomycin D remain second order and slower rate of strand displacement must be attributed to decreased rate of reassociation of DNA strands to form the branched intermediates.
    Consideration is given to the potential manipulation of DNA structures at site-directed branches and the limitations due to rapid strand displacements. The feasibility of constructing sufficiently large branched DNA regions to approach first order, branch migration rate limiting kinetics is also discussed.

INTRODUCTION

    Displacement of one strand from a double-helical nucleic acid by

concomittant replacement with an equivalent nucleotide sequence is a familiar

and integral aspect of DNA or RNA replication and genetic recombination in

vivo. Branch points result in molecules subject to such strand displacements

where two strands compete for base pairing interactions with complementary

sequences of the third strand. The movement of branch points in these

molecules, branch migration, may proceed with or without the concerted action

of specific enzymes and other proteins. The phenomenon of branch migration in

vitro was first recognized by Lee, Davis, and Davidson (1) in the renatured

molecules of terminally repetitious, circularly permuted bacteriophage DNA.

Broker and Lehman (2) and Broker (3) implicated branch migration in the in vivo recombination of T4 bacteriophage. Thompson et al. (4) suggested branch migration as a driving force in recombination of dimeric RF I species of bacteriophage ØX 174 DNA. The recA gene product of E. coli is active in promoting single-stranded DNA uptake by double-helical DNA to initiate recombination events (5).

During replication of double-stranded nucleic acids the leading 3' end of the growing strand extends by polymerase-mediated nucleotide addition while other proteins may facilitate the unwinding of the parental double helix ahead of the fork (6). Examples of partially single-stranded replicative intermediates undergoing this process are numerous, including linear adenovirus DNA (7), circular plasmid colE$_1$ and animal mitochondrial DNA (8, 9) and phage and animal virus RNA (10, 11). As purified nucleic acids, freed of protein, these intermediates are labile, since the branch point at the replication fork may migrate in the opposite direction from replication. In that case the partially displaced parental strand may completely dislodge the generally shorter segments of newly synthesized DNA or RNA.

Branched nucleic acid structures suitable for the study of strand displacements can be constructed in vitro, in general by taking advantage of conditions for nucleic acid reassociation or hybridization. Formation of the branched products at elevated temperatures may be energetically favored by choosing conditions which preclude or decrease the opportunity for branch migration. Regions of double-stranded DNA can take up complementary RNA sequences to form R-loops under conditions where RNA:DNA hybrids are more stable (12). Positive free energy in underwound closed circular DNA promotes the uptake of complementary sequences of single-stranded DNA to form D-loops (13). Displacement of newly acquired RNA or DNA strands can be initiated by return of R-loop structures to conditions in which the hybrid and duplex regions are of comparable stability or by introduction of single- or double-strand breaks in the closed circular DNA bearing a D-loop (14). Efforts to analyze the kinetics of the branch migration process have revealed that the resulting strand displacements can be exceedingly rapid, but displacement from both ends of branched loop structures can also be impeded by complex topological and hydrodynamic limitations (15).

In this report we describe DNA strand displacements from large branched structures formed during the reassociation of DNA. We reasoned that the elevated temperatures of reassociation conditions would minimize the

intramolecular interactions of single-stranded DNA which can complicate branch migration. Reassociation of DNA would also provide for the formation of branched molecules at well defined, predictable rates of interstrand nucleation (16). The experimental design utilizes labeled DNA single-strands, preannealed to longer DNA sequences. Displacement is initiated by addition of the complementary strand of the longer DNA. The displacement of DNA strands longer than 1 Kb is so rapid under these conditions that the kinetics remain second order reassociation rate limiting. Our estimate for the upper limit lifetime of the branched DNA intermediates is in good accord with the rates of rapid branch migration that have been observed for the displacement of considerably shorter DNA strands from D-loop bearing superhelical DNA (15).

Several DNA-binding drugs were found to have no effect on the kinetics of strand displacement under the conditions that we describe here. Actinomycin D does retard the displacement, albeit by decreasing the rate of DNA reassociation leading to branch formation. We have considered the potential manipulation of DNA structures at site-directed branches and limitations due to rapid strand displacement. The feasibility of constructing sufficiently large branched regions to approach branch migration limited kinetics is also discussed.

MATERIALS AND METHODS

Enzymes: Bacterial alkaline phosphatase was purchased from Worthington. Endonuclease EndoR.Bam HI and polynucleotide kinase were purchased from New England Biolabs. Endonuclease EndoR.Sal I was prepared in this laboratory as previously described (17).

DNA: Adenovirus type 3 (Ad3, strain G.B.) was isolated from infected HeLa cell suspension cultures, purified by density gradient centrifugation, prior to viral DNA extraction and purification as previously described (18). Conditions for restriction endonuclease treatment of viral DNA and maps of restriction sites in Ad 3 DNA are described elsewhere (17).

Agarose gel electrophoresis of restricted DNA: Low melting point agarose (an hydroxyethylated agarose derivative, Bethesda Research Labs) was employed for electrophoresis of restricted DNA samples in horizontal slab gels. Selected fragments were recovered from gel slices by chromatography of the molten gel at 70°C on hydroxyapatite columns followed by removal of the phosphate elution buffer by dialysis or Sephadex G-50 gel filtration as described elsewhere (19). The DNA fragments selected for preparative strand

separation, Sal I-C (6.1 Kbp) and Bam HI-G (1.6 Kbp) were subjected to electrophoresis following denaturation in 0.3 M NaOH, essentially as described by Hayward (20) except for the use of low melting point agarose. Agarose gel slices containing separated DNA strands were melted at 70°C and incubated 12-18 hours at 65°C to permit reassociation of contaminating complementary strands. The samples were adjusted to 0.04 M sodium phosphate and applied to hydroxyapatite columns for removal of agarose and ethidium bromide. Purified single-stranded DNA was then recovered free of reassociated duplex DNA by elution with 0.14 M phosphate. No attempt was made to remove the phosphate acquired in the course of purification as this generally led to losses of the separated strand DNA.

End labeling of probe DNA strands: The 5' ends of the DNA strands of Bam HI-G were labeled with (γ-^{32}P)ATP and polynucleotide kinase using the method of Smith and Birnstiel (21). Labeled DNA was subjected to gel filtration on Sephadex G-50 prior to electrophoretic strand separations in order to remove most of the free nucleotide and estimate specific activity of labeled DNA.

Computer simulations: A Hewlett-Packard 9810A calculator/9862A plotter system was programmed to simulate the equations of Chien (22) describing sequential, irreversible second and first order reaction rates. Extended iterations (69 terms) were employed to ensure convergence of expanded series approximations of the exponential integrals.

Monte-Carlo analyses of random walks corresponding to displacements from terminally branched and D-loop (2 branch) structures were also performed with the calculator. Pseudorandom number generators consistently failed to provide non-repeating sequences (odd/even) of sufficient length for effective determination of left-right transitions. Noise level potentials were successfully utilized by least significant bit input from a digital volt meter to determine the random sequences of transitions.

At least 100 trials were performed for each analysis of displacements from a given starting length of branch or D-loop. Summation distributions were treated to show the fraction of incompletely displaced trials after specified number of total stops. Each system clearly presented first order decay properties following a lag time corresponding to the starting length of the branch or D-loop (steps).

RESULTS AND DISCUSSION

Two fragments of Ad 3 DNA, Sal I-C and Bam HI-G were isolated from

restriction digests by electrophoresis as described in Materials and Methods. The larger Sal I-C fragment represents the left end 6.1 Kbp of the viral genome while Bam HI-G represents the 1.6 Kbp in the interval 1.7 to 3.3 Kbp from the left terminus. The 5'-ends of the complementary strands of Bam HI-G were labeled with ^{32}P as described in Materials and Methods to a specific activity of 4x10^5 cpm/ug of DNA.

Each of the two fragments of DNA were denatured in alkali and strand separated by agarose gel electrophoresis as shown in Figure 1. As we have observed earlier (19) the physical separation of complementary strands is often improved by use of the low melting point, hydroxyethylated agarose. The slow and fast strands of each fragment were found to be of the same viral DNA complementary strand as determined by reassociation of Bam HI-G fast strands (^{32}P-labeled) in the presence of Sal I-C slow strands. The labeled DNA remained single-stranded, as determined by hydroxyapatite chromatography, throughout prolonged incubation alone or with Sal I-C fast strand DNA (results not shown).

Purified single-strands of the labeled Bam HI-G (fast) and Sal I-C (slow) were mixed in approximately equimolar quantities and incubated 12-16 hours at
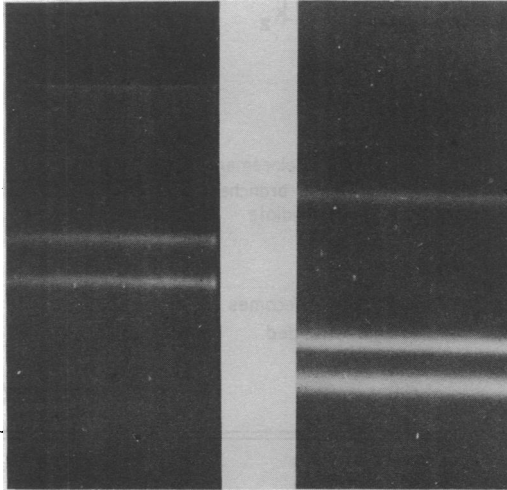

Figure 1. Preparative electrophoretic strand separation of Ad 3 DNA fragments Sal I-C (6.1 Kbp, left panel) and Bam HI-G (1.6 Kbp, right panel). Limited amounts of the more slowly migrating reassociated duplex DNA can also be seen in the gels above the pairs of separated DNA strands. The physical separation of the complementary DNA strands is on the order of 0.5 to 1.0 cm after 10 to 15 cm migration through the agarose gel.

65°C, 1 M NaCl, 0.14 M sodium phosphate to permit formation of the partially duplex species indicated in Figure 2A. The mixture was then applied to hydroxyapatite columns at 65°C, washed with 0.14 M phosphate and the partially duplex product was recovered by elution with 0.40 M phosphate. Concentration of this species was estimated from the specific activity of the annealed Bam HI-G strands.

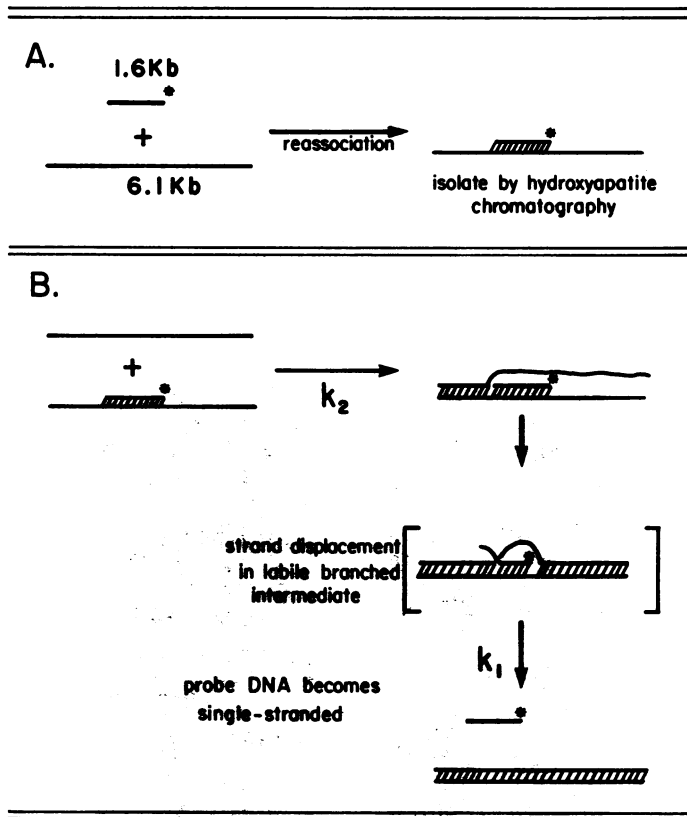The sequence of formation and decay of branched DNA, leading to strand

Figure 2. Two-step DNA reassociation procedure for the construction of branched DNA leading to strand displacements. A. Isolation of partially duplex DNA after reassociation of 5' ^{32}P-labeled Bam HI-G strands with complementary Sal I-C strands. B. Formation and decay of branched DNA by reassociation of Sal I-C strands with the complementary sequences of the partially duplex molecule prepared as above. The displacement of the ^{32}P-labeled Bam HI-G strands is monitored by analysis of aliquots from the reaction mixture by hydroxyapatite chromatography.

displacement, Figure 2B, was initiated by addition of <u>Sal</u> I-C fast strand DNA
to the partially double stranded structure bearing the labeled <u>Bam</u> HI-G fast
strand DNA (probe). Each reactant was adjusted to 0.14 M sodium phosphate,
pH6.8, 0.3M NaCl, 65°C. Aliquots were withdrawn from the mixture in rapid
succession for analysis on hydroxyapatite columns, pre-equilibrated with 0.14M
phosphate at 65°C. In this fashion the probe DNA could be followed from its
initially double-stranded state to completely displaced single strands.

Figure 3 shows the results of displacement reactions, identical in
composition except for the concentrations of the <u>Sal</u> I-C fast strand DNA and
labeled <u>Bam</u> HI-G/<u>Sal</u>I-C complex. The results indicate concerted displacement
of the labeled <u>Bam</u> HI-G strands with second order kinetics. The second order
rate constant, $k_2 = 1.0 \times 10^4$ $M^{-1}sec^{-1}$, is in excellent agreement
with the predicted value based on the paramaters of Wetmur and Davidson (16)
applied to the reassociating <u>Sal</u> I-C DNA strands in this experiment. We
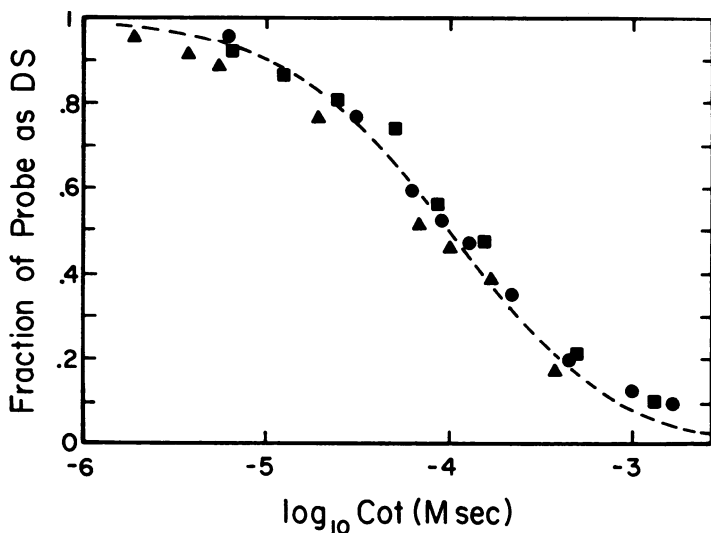conclude that the conditions thus far achieved represent displacement of

Figure 3. Strand displacement kinetics are second order DNA reassociation
rate limiting. Three reactions were followed according to the protocol in
Figure 2, at three different concentrations of <u>Sal</u> I-C DNA: 200 nM (●), 40
nM (■), and 2 nM (▲). The dashed line represents the theoretical second
order reassociation kinetics for the values of $C_0t_{1/2} = 1 \times 10^{-4}$
M(nucleotide) sec. The strand displacement reaction was followed by the
conversion of the ^{32}P-labeled <u>Bam</u> HI-G probe DNA from the partially duplex
(DS) molecules to single-strands.

strands which is second order reassociation rate limited.

It would be feasible to increase the reassociation rate in these experiments about 10 times by increasing the total concentration of reactants by that factor. However the scale of our present apparatus and material rendered this difficult and alternative methods for concentrating the extensively single-stranded reagents compromised precise estimation of concentrations or DNA strand integrity. If successful, the effort might still fail to show the effects of branch migration kinetics accurately since the time interval over which most of the displacement could be followed would be reduced to less than 1-2 minutes.

At this stage we proceeded to consider the available data to estimate a lower limit of the first order of branch migration in the intermediates which the experiment was designed to construct. The integrated rate equations of Chien (22) were used with the observed second order reassociation rate constant, k_2, to simulate the time course of strand displacments at various assumed values of k_1, the first order branch migration rate constant. As shown in the second order linear format of Figure 4, values of k_1 can be reduced to about $k_1 = 0.8$ min^{-1} before the deviations from second order rate limited conditions would be experimentally detectable. This estimate for k_1 (lower limit) can be used to calculate the average lifetime of the branched species with 1.6 Kb strands for displacement:

$$t_{1/2} \text{ (upper limit)} = \ln 2/k_1 = 0.8 \text{ minute.}$$

We have not attained branch migration rate limiting conditions in these experiments and we cannot yet assert that the intermediates undergoing strand displacment are singly branched or double nucleated to form D-loops as shown below:

Monte Carlo analyses of the random walk displacements from either of the above structures reveal the stochastic processes to be first order decay events with rate constants inversely proportional to the square of the starting lengths of the shorter probe DNA strands, Figure 5. The average lifetime expected for intermediates with strands of nucleotide length N to be displaced is approximatley N^2 steps of random walk (or about $0.25 \times N^2$ steps in D-loops treated as two independently migrating branches). This estimate is in accord with the more direct analysis of the single branch case presented by Weesakul (23), in which the average lifetime is shown to be
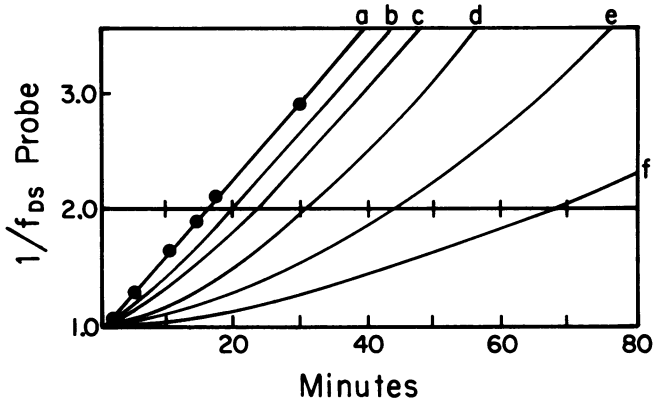
Figure 4. Estimation of the lower limit of the rate constant k_1 for first order branch migration-mediated strand displacement. The most rapid of the second order strand displacement reactions shown in Figure 3 (200 nm DNA, circles) had a $t_{1/2}$ of 16 minutes. The second order linear plot of this data is shown in the graph as the straight line (a). Curves (b) through (f) are computer simulated kinetics based upon the equations of Chien (22), assuming a second order reassociation rate constant $k_2 = 1.0 \times 10^4$ $M^{-1}sec^{-1}$, and assumed first order rate constant: b, $k_1 - 0.8$ min^{-1}; c, $k_1 = 0.2 min^{-1}$; d, $k_1 - 0.08 min^{-1}$; e, $k_1 = 0.04$ min^{-4}; and f, $k_1 = 0.02 min^{-1}$. It is estimated that a first order rate constant as low as $0.8 min^{-1}$ would have led to conditions in which the kinetics would not have been second order reassociation rate limiting and this would have been experimentally detectable by longer $t_{1/2}$ of strand displacements at the higher concentrations of DNA undergoing reassociation.

$N(N+1)$. In the case of our experiments $N = 1.6$ Kb, $N^2 = 2.6 \times 10^6$ steps, and the estimated value (lower limit) for $k_1 = 0.8 min^{-1}$ corresponds to an average step time of about 20 usec.

Using the distribution of fork positions in renatured coliphage 15 DNA, observed by electron microscopy, together with the assumption of an attained equilibrium, Lee et al. (1) estimated an upper limit of 10^{-3} second for the step time in the branch migration process. This estimate was considerably reduced by the experiments of Radding et al. (15), to about 10^{-5} second for the rapid displacement of short strands from D-loops in relaxed superhelical DNA. This value and our own estimated upper limit for the step time suggest that the size of the branch regions in the molecules we have constructed may approach the limits that will be required for straightforward analysis of the kinetics of branch migration. Technical barriers make separation and manipulation of longer complementary strands more difficult. Electrophoretically separated strands from longer restriction fragments (a 15
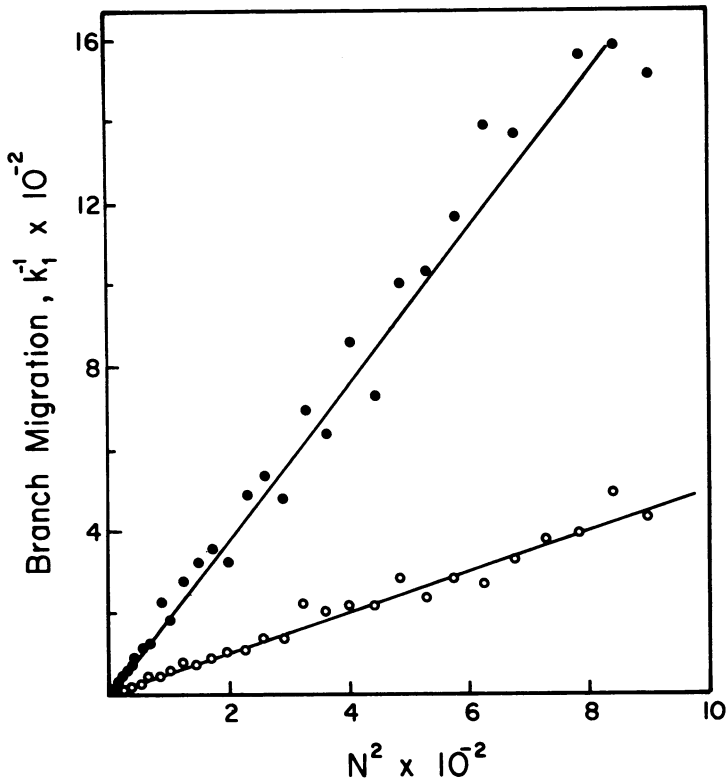
Figure 5. Monte Carlo analysis of random walk models for strand displacements from single branches (●) or D-loop molecules with two independent branches (O). The rate constant for strand displacement, k_1 was estimated in terms of the number of steps of random walk required in the distribution of trials leading to displacements. When the D-loops are treated as two independent branch points, leading to displacement whenever the branch points meet within the interval of the D-loop, the expected rate of displacement is about 4 times greater than for displacement of the same length strand from a single branch. In each case the rate of displacement is inversely proportional to the square of the length of the strand to be displaced (N).

Kbp fragment of Ad 3 DNA has been strand separated but with low recovery) or even the full length complementary strands of the viral DNA (24) may eventually be employed. The experiments, while difficult, now appear to be feasible. First order decay by displacement of Sal I-C probe strands from full length Ad 3 complements should be at least 16 times slower than that of the Bam HI-G fragment probe which we have employed.

We were interested in the possibility that certain DNA binding drugs may

selectively retard the branch migration process. This could lead to strand
displacement reactions having first order, branch migration-rate limiting
kinetics in the presence of such drugs. We first examined the following
drugs, at concentrations from 1 to 100 ug/ml, to determine if the extent of
strand displacement was influenced:

ethidium bromide	proflavine	chloroquine
propidium iodide	actinomycin D	chlorpromazine
acridine orange	chromomycin A_3	daunomycin.

The only measureable effects, under conditions as shown in Figure 3, were with
actinomycin D. At 2 ug/ml, this drug reduces the rate of displacement of the
BamHI-G strands from the SalI-C strands by a factor of 3.0, Figure 6. We
observed, however, that these slower kinetics of strand displacement remain
second order. The effect of actinomycin D on straightforward DNA
reassociation kinetics was evaluated under the same reaction conditions,
Figure 7. The results clearly indicate that the second order reassociation
rate constant is reduced by a factor of 3.0 in the presence of 2 ug/ml of the
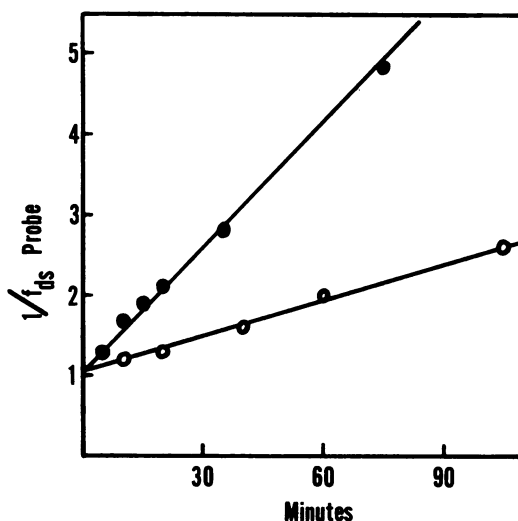

Figure 6. Diminished rate of strand displacement in the presence of
actinomycin D. The closed circles represent the second order displacement of
^{32}P-labeled BamHI-G DNA strands from SalI-C strands during DNA
reassociation as in Figure 3. The open circles show the decreased second
order displacement of the BamHI-G probe DNA strands under the same conditions
except for the presence of 2 ug/ml actinomycin D. The slopes, and thus the
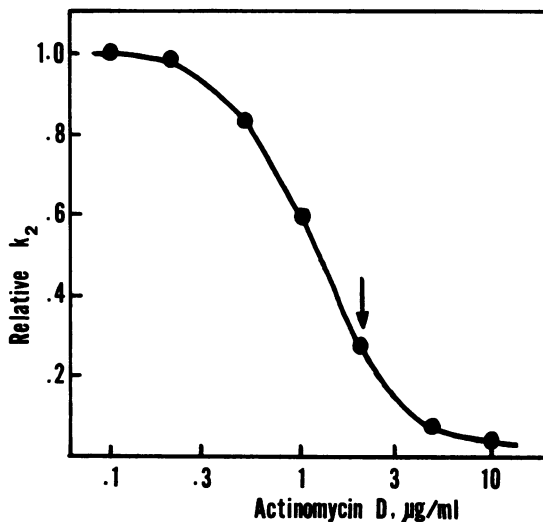second order rate constants differ by a factor of 3.0.

Figure 7. Effect of actinomycin D on the second order rate constant k_2 for DNA reassociation. ^{32}P-labeled Sal I fragments of Ad 3 DNA were denatured in alkali (0.3 M NaOH), neutralized with HCl, adjusted to 0.3 M NaCl, 0.14 M sodium phosphate, pH 6.8 and incubated at 65°C. Aliquots were withdrawn from the reactions and analyzed by hydroxyapatite chromatography to determine the fraction of double-stranded DNA reassociation product. The observed second order rate constant is shown here as it varies with the concentration of actinomycin D present during the DNA reassociation. The arrow indicates decrease of k_2 by a factor of 3.0 at 2 ug/ml actinomycin D.

drug. We therefore conclude that any effect of actinomycin D on the rate of branch migration is still obscured by the rate limitation of the formation of the branched structures by DNA reassociation.

Our failure to detect significant reduction in the rate of branch migration, in the presence of the above reagents attests to the very substantial difference in the rates of the formation and the decay of the branched structures. Branch migration may in fact be significantly retarded by these drugs yet fail to limit the reaction to first order kinetics of strand displacement. Our method of analysis can be applied to other types of DNA ligands in further attempts to specifically distinguish effects on DNA reassociation and branch migration. As efforts proceed to utilize larger substrates for branch migration kinetics, as described above, these and other DNA-binding agents can be evaluated.

Part of our original interest in branch migration experiments was directed towards the construction of branched DNA molecules for site-directed

manipulation in vitro. We recently exploited stable D-loops in superhelical DNA in a general procedure for targeted deletion mutagenesis (19). Application of this approach for uses with linear DNA molecules would extend its usefulness and generality, but at present seems to be impractical, given the minimal life expectancy of even very large branched intermediate substrates. As studies focused on the kinetics of branch migration continue along the lines which we and others have pursued, it may be possible to identify and exploit drugs or protein reagents to selectively interfere with the branch migration process that leads to the rapid displacements of target DNA strands.

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