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Wayne P. Wahls
University of Arkansas for Medical Sciences, wahlswayne@uams.edu

K. Mark DeWall
University of Arkansas for Medical Sciences

Mari K. Davidson
University of Arkansas for Medical Sciences

Mapping of ssDNA Nicks Within dsDNA Genomes by Two-dimensional Gel Electrophoresis

WAYNE P. WAHLS[1,2], K. MARK DEWALL[1], AND MARI K. DAVIDSON[1]

[1]Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA

[2]Correspondent: WahlsWayne@UAMS.edu

Abstract

DNA molecules within chromosomes undergo constant, dynamic changes yet maintain the integrity of the primary DNA sequence. DNA replication, adjustment of helical density, resolution of catenenes, repair of DNA damage, and homologous recombination each involve breakage and religation of the phosphate backbone of the double helix. Although the analysis of dsDNA breaks is facile, the analysis of ssDNA nicks is not. The principal impediment is that conventional, one-dimensional electrophoresis methods cannot readily detect ssDNA nicks in the context of dsDNA breaks. We therefore developed a two-dimensional (native/denaturing) gel electrophoresis approach to map the positions of ssDNA nicks. Analysis of cohesive ends of lambda phage DNA, UV-nicked DNA molecules, and DNA treated with ssDNA nicking endonuclease N-BbvcIB revealed that the method can detect and map with precision the positions of ssDNA nicks. Titration experiments revealed the ability to detect and quantitate nicked DNA molecules present at a frequency of 1% of total DNA molecules. This method can be used both to scan rapidly through large regions of the genome of interest and to map with high-resolution the location of ssDNA nicks in populations of dsDNA molecules. It is of utility for the analysis of ssDNA nicks involved in a variety of chromosomal processes.

Introduction

Meiosis produces 4 haploid cells from a diploid premeiotic cell (Roeder, 1997; Wahls, 1998; Hassold and Hunt, 2001; Lichten, 2001; Hunt and Hassold, 2002). This is achieved by coupling 1 round of DNA replication with 2 rounds of chromosome segregation. After DNA replication, homologous chromosomes pair and undergo a high rate of homologous recombination. Proper segregation of homologous chromosomes in the first (reductional) division requires that the paired homologs be held together and aligned on the metaphase plate of meiosis I in opposition to spindle tension (Page and Hawley, 2003). In most organisms, a combination of crossover recombination structures (chiasmata) and sister chromatid cohesion distal to chiasmata provide this glue.

Extensive genetic studies have led to the development of models for meiotic recombination (Holliday, 1964; Meselson and Radding, 1975; Szostak et al., 1983). These models each posit that cleavage of DNA strand(s) on one chromatid is followed by recombinational repair using a homologous chromatid as a template. Notably, both ssDNA nicks and dsDNA breaks are implicated. Confirmation of one of these models came from studies of budding yeast mutants (such as rad50S) that accumulate unrepaired, meiotically-induced, dsDNA breaks (Sun et al., 1989; Cao et al., 1990). The distribution and density of the dsDNA breaks in budding yeast correlates well with the distribution and density of recombination (Baudat and Nicolas, 1997; Gerton et al., 2000). However, the types of DNA breaks in vivo have not been adequately characterized for any other organism and artificially-introduced ssDNA nicks can be potent inducers of recombination (Strathern et al., 1991; Lee et al., 2004).

Using 2 independent criteria (one genetic and one biochemical) we obtained evidence for the presence of meiotically-induced, recombinase-dependent, ssDNA nicks in the fission yeast genome (our unpublished observations). This prompted us to develop a powerful, yet simple electrophoresis method for the detection and mapping of ssDNA nicks within dsDNA molecules spanning large distances (up to 1,000 kbp) along chromosomes.

Materials and Methods

Reagents.–Lambda bacteriophage DNA, 1 kbp ladder DNA, and restriction endonucleases were obtained from New England Biolabs. DNA modification enzymes were used according to the instructions of the manufacturer.

Two-dimensional Agarose Gel Electrophoresis.–All electrophoresis was conducted using 10 cm mini gel rigs (Ellard Scientific). DNA samples were fractionated for 200 Vh (40 V for 5 hr) in the first native dimension in 1% agarose gels containing Tris/Acetate/EDTA (TAE) buffer and TAE running buffer (Sambrook et al., 1989). Gels were stained for 15 min in 0.5-µg/ml EtBr and destained 3 times for 15 min each in H_2O prior to being photographed on a UV light box. In cases where exposure to UV light was undesirable the staining and visualization steps were omitted. Gel lanes were excised with a clean razor blade,

nd each lane strip was cast across the top (origin) of a gel ontaining 1% agarose, 50-mM NaCl, 1-mM EDTA. The els were soaked for 60 min in denaturing electrophoresis uffer (30-mM NaOH; 1mM-EDTA) and then subjected to lectrophoresis for 200 Vh (40 V for 5 hr) in denaturing lectrophoresis buffer. Gels were then stained for 15 min in .5-µg/ml EtBr and destained 3 times 15 min each in H_2O rior to being photographed on a long-wave UV light box.

Southern Blotting.–Procedures for Southern blotting were as described (Sambrook et al., 1989; Kon et al., 1997). The lambda phage DNA hybridization probe was prepared using α32P-dCTP (Perkin Elmer) and the RediPrime II prime labeling system (Amersham Biosciences) (Davidson et al., 2004).

Results

Conceptual Development of a Two-dimensional Gel Electrophoresis Method to Detect ssDNA Nicks Within dsDNA Molecules.–Although the analysis and mapping of dsDNA breaks is facile, the analysis and mapping of ssDNA nicks is not. The principal impediment is that conventional, one-dimensional electrophoresis approaches cannot readily detect ssDNA nicks in the context of dsDNA breaks. One must compare in parallel native and denaturing gels using hybridization with strand-specific probes, and such comparisons are restricted to analysis of one restriction fragment in each experiment (lane/hybridization/DNA strand). Because we wish to study the broad distribution of ssDNA nicks in chromosomes, and those nicks may not occur uniformly through the genome, one-dimensional analyses were inadequate.

We developed a 2-dimensional (native/denaturing) agarose gel electrophoresis method to discriminate between ssDNA nicks and dsDNA breaks simultaneously on a single gel for multiple restriction fragments spanning a genomic region. A schematic representation of the method is provided in Fig. 1. For the sake of illustration, we consider the fates of 3 dsDNA molecules–1 that is intact, 1 that contains a ssDNA nick, and 1 that has been bisected into 2 fragments by cleavage of both DNA strands (Fig. 1A). The DNA molecules are fractionated first under native conditions. Because the individual DNA strands of dsDNA are held together by hydrogen-bonding, base-pairing interactions, the dsDNA molecules will migrate proportional (inversely) to their mass, regardless of whether or not ssDNA nicks are present (Fig. 1B). However, if the dsDNA molecules are denatured into their ssDNA strands prior to electrophoresis, then the individual ssDNA strands derived from dsDNA molecules that contain nicks will migrate more rapidly than the ssDNA strands derived from intact dsDNA molecules. Thus, by fractionating a population of DNA molecules on one dimension under native conditions, then in a second dimension under denaturing conditions, it should be possible to resolve nicked dsDNA molecules from intact dsDNA molecules (Fig. 1C).

Detection of ssDNA Nicks Introduced by Ultraviolet Light.–For the initial characterization of the assay, we analyzed the migration pattern of a commercial, 1 kbp ladder typically used as a molecular weight marker for native electrophoresis. The samples were fractionated in the first dimension using conventional agarose gel electrophoresis in native (TAE) gel running buffer. The gels were stained with EtBr and subjected to short-wavelength UV light in order to visualize (and photograph) the DNA bands and to guide the excision of the portion of the gel (lane) containing DNA. The excised lane was cast into a second gel then denatured and subjected to electrophoresis in the second dimension in the presence of denaturant (NaOH). As expected, most of the DNA molecules were distributed along a diagonal arc of the gel (Fig. 2). However, a significant portion of the DNA migrated in a smear below the arc of intact dsDNA molecules (Fig. 2).

Material migrating as a smear in the ssDNA nick area could be due to the presence of pre-existing ssDNA nicks in the dsDNA molecules applied to the gel, or due to the introduction of ssDNA nicks by UV light used to visualize bands after running the first dimension, or due to nicking of DNA molecules by the denaturing conditions used to run the second dimension. To distinguish between these possibilities, we loaded on the gel, just prior to running the second dimension under denaturing conditions, an additional DNA sample that had not been exposed to UV light. DNA molecules in that control sample migrated to discrete (i.e., not smeared) positions in the second dimension (Fig. 2). Thus, ssDNA nicks were not present in the initial DNA molecules and they were not induced by the denaturing conditions. We conclude that the material migrating in the ssDNA nick area was due to exposure of the DNA to UV light. The UV light either introduced ssDNA nicks or some other type of lesion (e.g., pyrimidine dimers) that caused some of the DNA molecules to migrate below the intact dsDNA arc.

Detection of Staggered ssDNA Nicks Held Together by Compatible, Cohesive Ends.–As an independent test of the assay, we examined the fractionation of DNA molecules produced by digestion of lambda bacteriophage DNA with restriction endonuclease HindIII. There are 7 lambda/HindIII DNA fragments (Fig. 3A), two of which (4 kbp and 23 kbp) contain protruding, complementary, ssDNA tails 12 nucleotides (nt) in length (cos sites). These tails can form hydrogen-bonding, base-pairing interactions with one another that are stable enough to be maintained during electrophoresis under native conditions (Fig. 3B). As

a consequence, the abundance of the 4 kbp band in the "expected" position is often reduced–those dsDNA molecules co-migrate with the 23 kbp molecules near the exclusion point of the gel. This provided an excellent way to determine whether two staggered ssDNA nicks (located 12 nt away from each other) could be resolved by two-dimensional (native/denaturing) electrophoresis.

In the first (native) dimension, the 4 kbp band was reduced in abundance, as expected (Fig. 3C). In the second (denaturing) dimension, a band of 4 knt appeared in the ssDNA nick area below the position at which the 23 kbp band migrated in the first dimension (Fig. 3C). We make two conclusions from these data: First, base pairing between the 12 nt long, complementary, ssDNA tails of the cos sites on the 23 kbp and 4 kbp restriction fragments is sufficiently strong to be maintained under native electrophoresis conditions. Second, the two-dimensional approach can melt cohesive ends with 12 nt of base pairing and resolve the products as ssDNA molecules.

Mapping of ssDNA Nicks Within Multiple dsDNA Fragments Treated with Nicking Endonuclease.–Analysis of UV-treated DNA molecules (Fig. 2) and the DNA molecules held together by their cos sites (Fig. 3) indicated that the two-dimensional electrophoresis method can identify ssDNA nicks in dsDNA molecules. However, for the method to be practical it would have to be able to identify and map the positions of ssDNA nicks located long distances from the ends of dsDNA restriction fragments. We therefore compared the migration pattern of lambda/HindIII DNA fragments to the migration pattern of lambda/HindIII DNA fragments that had been treated with nicking restriction endonuclease N.BbvCIB (New England Biolabs). This enzyme has an asymmetric recognition site and nicks specifically one of the two DNA strands at that site. Based upon the DNA sequence of bacteriophage lambda, we would expect N.BbvCIB to introduce nicks at specific locations on three of the seven lambda/HindIII DNA fragments (Fig. 4A).

The lambda/HindIII DNA fragments migrated predominantly along the linear, intact dsDNA arc, except for the 4 kbp fragment, which migrated with the 23 kbp fragment in the first dimension due to its cos site (Fig. 4B). Lambda/HindIII DNA fragments that had been treated with nicking restriction endonuclease N.BbvCIB produced a distinct pattern: All of the fragments that lacked a recognition site for N.BbvCIB (except the 4 kbp fragment with cos) migrated on the linear, intact dsDNA arc (Fig. 4B). All of the fragments that contained a recognition site for N.BbvCIB migrated to their expected positions in the ssDNA nick area below the dsDNA arc (Fig. 4C). We conclude that two-dimensional (native/denaturing) electrophoresis analysis can detect and map the positions of ssDNA nicks located long distances (≥10 knt) away from the ends of dsDNA fragments.

Detection and Quantitation of Low-abundance ssDNA Nicks Within a Large Excess of Intact dsDNA Molecules.–The preceding experiments demonstrated that the positions of several ssDNA nicks can be mapped simultaneously within a population of dsDNA molecules. To determine the resolution of the assay, we mixed varying amounts of nicked and intact DNA fragments, ran the two-dimensional (native/denaturing) gels, and conducted Southern blotting. We were able to detect the nicked DNA molecules present at a frequency of about 1% of total DNA molecules (Fig. 4D and data not shown). By using phosphorimage analysis or similar methods, the amount of hybridization signal from nicked DNA molecules can be standardized relative to the signal intensity from intact dsDNA molecules. Thus, the assay can detect the presence of low abundance ssDNA nicks, map their positions, and determine their frequencies relative to those of intact dsDNA molecules (internal controls).

Discussion

Genetic models suggest that ssDNA nicks may initiate meiotic recombination, but molecular evidence for such lesions is lacking. Formal demonstration that ssDNA nicks initiate recombination will require, among other things, high-resolution mapping to determine whether the distribution of ssDNA nicks is coincident with the local frequencies of recombination. Unfortunately, available methods will not permit large-scale mapping of ssDNA nicks. We therefore developed a simple, inexpensive approach for the identification and mapping of ssDNA nicks. By fractionating a population of dsDNA molecules on one dimension under native conditions, then in a second dimension under denaturing conditions, it is possible to resolve ssDNA molecules from dsDNA molecules. Importantly, this method can detect ssDNA nicks located at least 10 kbp from the ends of dsDNA molecules (Fig. 4). Furthermore, it can identify specifically which dsDNA restriction fragment harbors ssDNA nick(s) (Fig. 4).

The finding that 12 nt of cohesive, ssDNA tails is sufficient to render a dsDNA break invisible in native electrophoresis (Fig. 3) has implications for data and conclusions published previously. The 501 kbp NotI-NotI restriction fragment "J" of fission yeast is located on the left arm of chromosome I (Fan et al., 1989). On this restriction fragment, the density of recombination (per unit distance) for numerous subintervals and the distribution of dsDNA breaks have been mapped (Young et al., 2002). Recombination density varies about 2.5-fold for different subintervals, but dsDNA breaks are reportedly only detectable at two major break sites, mbs1 and mbs2, which are located 105 kbp from one another. Thus, the frequency

nd distribution of dsDNA breaks is inadequate to account or the frequency of recombination. We suggest that ecombinogenic dsDNA breaks in fission yeast may be nore abundant than previously thought, but they may have ohesive ends. In other words, they would be "invisible" to he 1-dimensional, native gel electrophoresis approaches pplied to date. Alternatively, ssDNA nicks may initiate ecombination in fission yeast. Our 2-dimensional assay llows us to test these hypotheses.

Finally, we note that the method allows for elegantly simple scanning of large genomic regions for the presence of invisible dsDNA breaks (i.e., those with cohesive ends) and ssDNA nicks. One can fractionate total genomic DNA that has been digested with a restriction endonuclease and use a series of overlapping cosmids as probes. Each cosmid probe will therefore detect all restriction fragments in a region of approximately 50 kbp, and any ssDNA nicks or invisible dsDNA breaks above the limit of detection will be apparent as signals in the ssDNA nick area of the gels. Similarly, 1 could analyze regions spanning about 1,000 kbp by using hybridization probes derived from bacterial artificial chromosomes (BACs). Any dsDNA restriction fragment that contains a ssDNA nick could then be examined using fragment-specific and strand-specific hybridization probes.

In summary, the method described here can be used both to scan rapidly through large regions of the genome of interest and to map with high-resolution the location of ssDNA nicks in populations of dsDNA molecules. It will likely be of utility for the analysis of ssDNA nicks involved in a variety of chromosomal processes including meiotic recombination.

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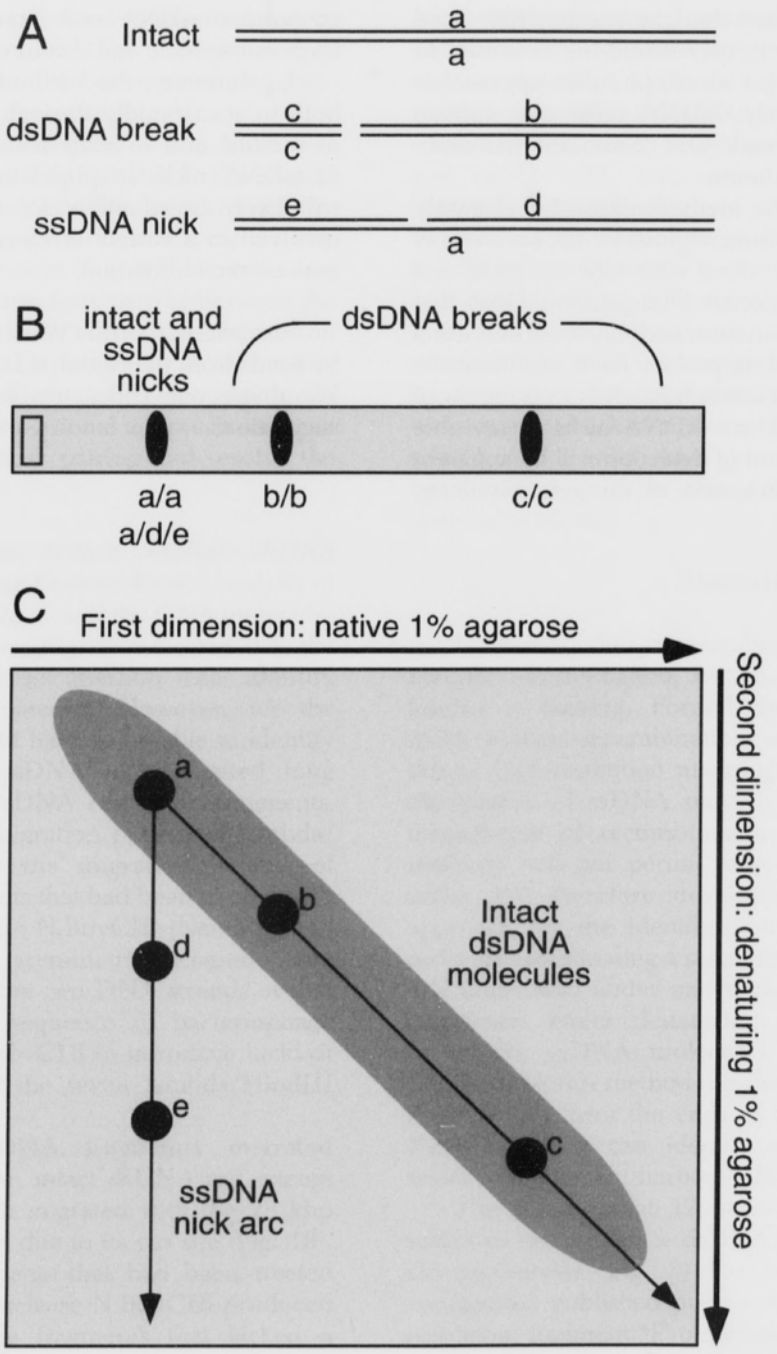

Fig. 1. A 2-dimensional assay for mapping ssDNA nicks in dsDNA molecules. (A) Schematic diagram of intact, broken, and nicked dsDNA molecules with individual strand molecules labeled (a-e). (B) In the first native dimension, nicked and intact molecules are not resolved from one another. (C) In the second denaturing dimension, nicked molecules migrate away from intact molecules.

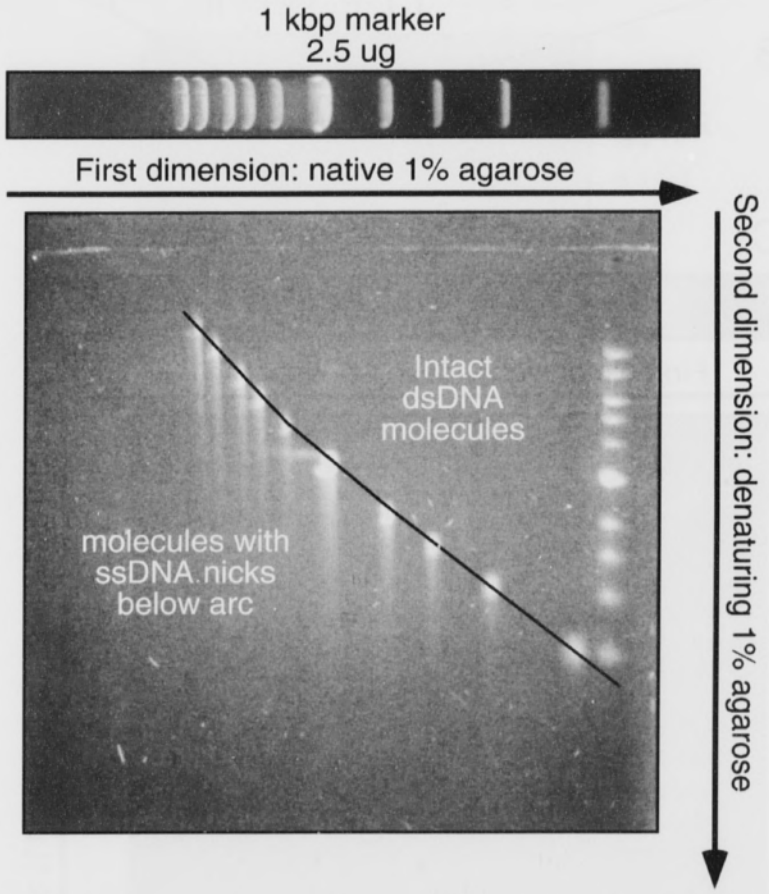

Fig. 2. Effects of UV-induced ssDNA nicks upon migration of dsDNA molecules in 2-dimensional gels. dsDNA molecules were fractionated in the native dimension, then subjected to intense UV light to visualize DNA bands (EtBr fluorescence) and to guide excision of the lane. The lane was cast into a second, denaturing gel, and additional DNA molecules (not exposed to UV) were loaded in a well on the right-hand side of the gel. Following electrophoresis in the second (denaturing) dimension, the gel was stained with EtBr and photographed again. Intact dsDNA molecules migrate to positions along a linear arc (line). Note that the DNA molecules exposed to UV light contain nicks (smearing below dsDNA arc), whereas the marker molecules not exposed to UV light do not contain nicks (rightmost lane).

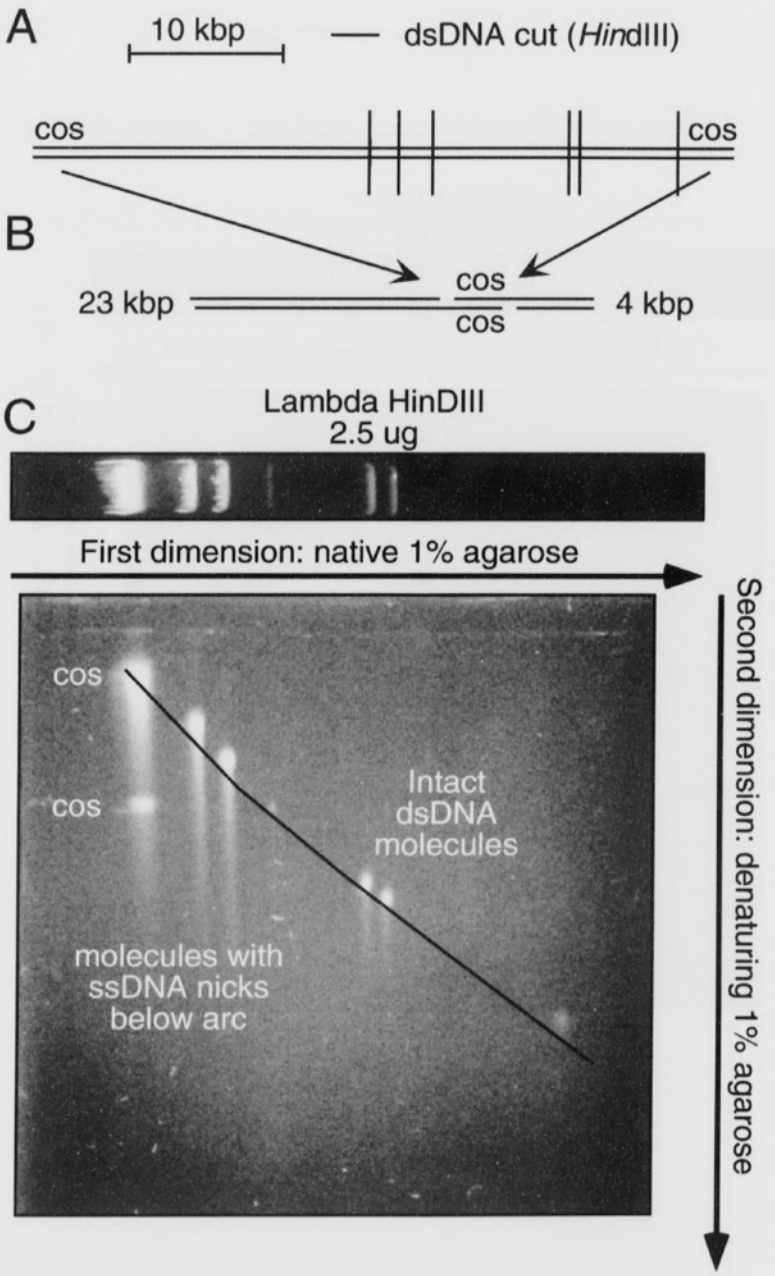

Fig. 3. Effect of compatible, cohesive ends of dsDNA upon the migration of DNA molecules in 2-dimensional gels. (A) Map of bacteriophage lambda chromosome showing positions at which restriction endonuclease HindIII cleaves. (B) The 23 kbp and 4 kbp restriction fragments contain 12 nt long, overhanging, complementary, ssDNA tails from the cos sites on the ends of the lambda chromosome. These can form hydrogen-bond, base-pairing interactions stable enough to hold the two fragments together in native gel electrophoresis. (C) Lambda HindIII fragments were subject to 2-dimensional analysis. Note that the bulk of the 4 kbp restriction fragment migrates with the 23 kbp restriction fragment in the first dimension, but the 4 knt ssDNA fragments migrate away from the 23 knt fragments in the second dimension (cos).

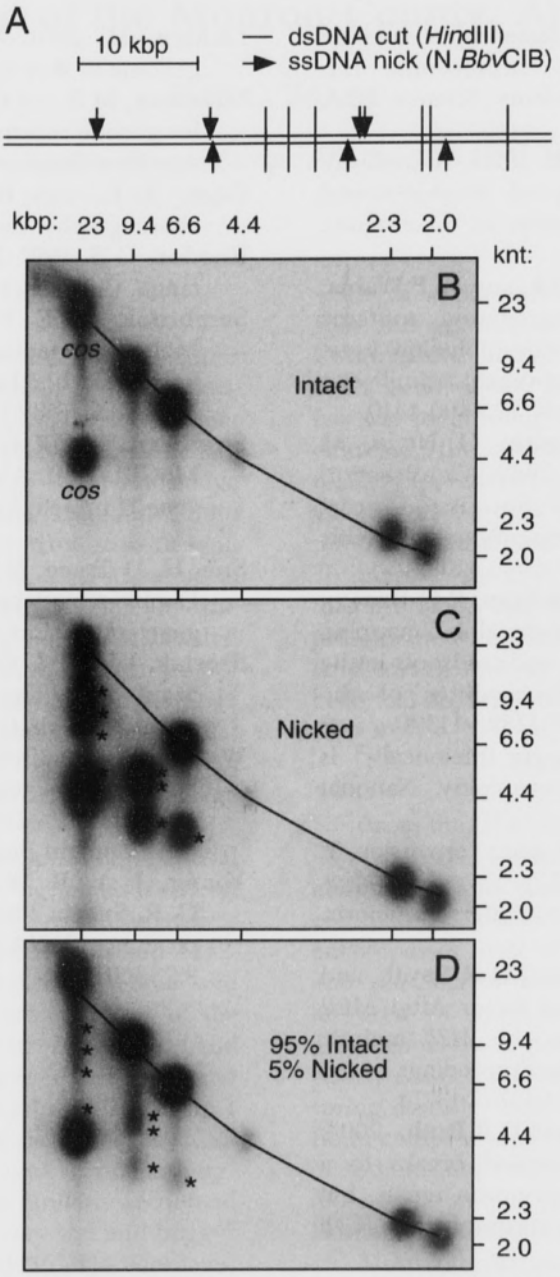

Fig. 4. Mapping the positions and relative abundance of multiple, ssDNA nicks within a population of dsDNA molecules. (A) Map of bacteriophage lambda chromosome showing positions at which restriction endonucleases HindIII and nicking restriction endonuclease N.BbvCIB cleave and nick DNA, respectively. Note that only 3 of 7 HindIII fragments will be nicked. (B-D) Two-dimensional gels of lambda phage genome were subject to Southern blotting using radiolabeled lambda phage DNA as a probe. For the sake of clarity, only a portion (~1/4) of each gel is shown. (B) Analysis of intact dsDNA molecules. With the exception of dsDNA molecules that harbor cohesive termini (cos), all dsDNA molecules migrate on a linear arc. (C) After treatment with nicking endonuclease N.BbvCIB, some of the individual DNA strands become nicked and thus migrate below the intact dsDNA arc (*). One can map the relative positions of ssDNA nicks. (D) DNA molecules as used in panels "B" and "C" were mixed in varying proportions prior to analysis in order to determine the resolution of the assay. The 5% example is shown in order to withstand reproduction, but ssDNA nicks can be detected at a frequency of 1% or less of total DNA molecules (not shown).

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