

Sequence-Directed DNA Curvature in Replication Origins Segments

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1. Introduction

DNA replication is an essential cellular process for the propagation of life. Prokaryotic cells are examples of organisms that display a fast and precise replication process (Robinson & Bell, 2005; Wang & Sugden, 2005). Although the consensus nucleotide sequence in the bacterial replication origin exhibits variation in number and size between different bacterial species (DoriC, a database of the oriC region in bacterial genomes; Gimenes et al., 2008a), it is capable of directing the association of specific proteins in the initiator chromosomal site and thereby the success of the replicative process in eubacteria (reviewed in Mott & Berger, 2007). However, the identification of hundreds to thousands of replication initiation sites in eukaryotes is more complex than locating clearly defined sites with stretches of consensus sequences, as observed in prokaryotes. While complexity exists, some parallels can be drawn between prokaryotic and eukaryotic systems. Less derived eukaryotes, such as the unicellular yeast *Saccharomyces cerevisiae*, exhibit similar replicative processes to prokaryotes. Replication initiation sites have approximately 120-200 base pairs (bp), termed ARS (Autonomously Replicating Sequences). Although there is sequence diversity among the ARS sites, these sites present a short consensus sequence of 10-11 bp that is rich in A/T residues and called the ACS (ARS Consensus Sequence) and a more divergent motif known as domain B (review in Bell & Dutta, 2002). Although not all of ARS sites are efficiently used, it has been shown that they can be initiate replication if necessary (Sharma et al., 2001). However, *S. cerevisiae* and closely related species appear to be the only eukaryotic organisms that present consensus elements in their replication initiation sites. *Schizosaccharomyces pombe* has initiation replication sequences from 600 to 800 bp, which display numerous A/T-rich regions, but these replication initiation sites in *S. pombe* have a low level of similarity to the ARS of *S. cerevisiae*, and we have not been able to identify a consensus sequence within this region (Dubey et al., 1996; Zhu et al., 1994).

The presence of conserved sequences in *S. cerevisiae* was important for the isolation of the origin recognition complex (ORC; Bell & Stillman, 1992). The existence of this complex revealed that eukaryotes depend on the binding of specific proteins to identify and activate specific genomic regions to initiate replication. The ORC complex, which is composed of six closely related proteins, Orc1-6, is conserved through evolution and has been identified in all analyzed eukaryotic cells (reviewed in Bell, 2002). Before replication begins, numerous other

proteins form the pre-replicative complex (pre-RC), which signals permission for the initiation of replication (reviewed in Stillman, 2005). Recently, it was reported that the pre-RC complex is detected in initiation zones with low nucleosome occupancy (Lubelsky et al., 2010). Although the ORC composition is similar in eukaryotes, replication initiation in metazoans is a complex process, and the structure and mechanism of determining the selection of these initiation sites are not fully understood. The nature of the information required for activation of replication origins in multicellular organisms, where multiple initiation sites of replication occur in cells with differential gene expression, has made it difficult to associate function to a specific replication origin sequence. Analyses of replication intermediates using alternative methods indicated that the replication origin lacked a nucleotide consensus sequence (Biamonti et al., 2003; DePamphilis, 1999; Toledo et al., 1998; Tower, 2004). The activity of the initiation site for replication in metazoans, which lacks a consensus sequence, can be influenced by chromatin structure and/or selected by epigenetic factors (Anglana et al., 2003; Balani et al., 2010; Courbet et al., 2008; Debatisse et al., 2004; Fiorini et al., 2006a; Gimenes et al., 2009; Stehle et al., 2003). The presence of foci of replication (replication factories) that bring together numerous sites of replication initiation and the association of the nuclear matrix (S/MAR) demonstrates the importance of chromosome structure in DNA replication process (Anachkova et al., 2005; Courbet et al., 2008; Jackson, 2003; Newport & Yan, 1996). The analysis of DNA replication using microarrays of the complete genome has increased our understanding of this process and has led to the formulation of new questions about selection and activation of replication origins events in metazoans (MacAlpine & Bell, 2005). Thus, although a large amount of experimental data have been obtained in recent years, the question of whether there are sequences and/or structures conserved in the metazoan replication initiation regions has not been answered yet. The existence of important topological features in the replication initiation sites and/or in the segments that flank these sites could be essential for the selection and activation of accurate DNA replication in both prokaryotic and eukaryotic systems. The bacterial DnaA protein interacts with the sequences called DnaA boxes, and although this interaction is highly conserved among different species, the numbers and sequences of these sites are not conserved (Gimenes et al., 2008a; Mackiewicz et al., 2004). How then is DnaA capable of interacting with these sites, even within different regions of the DnaA box? The same question can be formulated for the initiation sites in yeast; although ARS regions are conserved, the elements A, B1 and B2 exhibit alterations in their sequences. Thus, how then is the specificity of the protein components of the ORC complex maintained in the ARS sites? The analysis of the molecular structure of DnaA and the ORC complex in *Saccharomyces cerevisiae* and *Drosophila melanogaster* revealed that these proteins belong to the superfamily of ATPases with AAA⁺ domains and exhibit increased affinity when exposed to negatively supercoiled DNA (Clarey et al., 2006; Erzberger et al., 2006; Remus et al., 2004; Speck et al., 2005). The structure of negatively supercoiled DNA in replication initiation regions implies that repression of replication activity at these sites could be due to the loss of this supercoiling. For bacteria, the protein SEQA, which has repressor activity in DNA replication, has been reported to promote changes in the helical structure of DNA, causing positive supercoiling of the segment (Kjellesvik & Skarstad, 2004). In eukaryotes, the implication of the supercoiling changes in DNA replication seems to be more complex. The protein geminin, which regulates DNA replication by interacting with the Cdt1 protein, inhibits replication re-initiation in the same cell cycle (Pitulescu et al., 2005), but these proteins do not show DNA binding domains. The DEK protein, derived from a proto-oncogene,

was reported to modify the topology of replication initiation sites, but has not shown an affinity for DNA without the presence of histones (Alexiadis et al., 2000). Recently, studies have used genome-wide approaches to analyze the features associated with the choice of the replication origins and the nucleosome occupancy within these regions (review in Meisch & Prioleau, 2011).

The correlation between specific chromosomal regions and the topological features of DNA is an exciting area of research. Our laboratory has been devoted to mapping the presence of intrinsically bent DNA sites in regions of replication initiation in prokaryotes (Gimenes et al., 2008a), replication origins and promoter regions in gene amplified domains of eukaryotes (Balani et al., 2010; Fiorini et al., 2001, 2006a; Gimenes et al., 2009; Gouveia et al., 2008) and in recombination regions from the eukaryotes genome (Barbosa et al., 2008). Here we discuss the sequence and topological features of intrinsically bent DNA sites as well the methodology used to explore these sites in replication origin segments.

2. Determinants of DNA bending

The DNA molecule is composed of two nucleic acid polymer chains that are wound around one another to form a regular right-handed helix, which is similar to the canonical DNA conformation, called B-DNA (Marko & Cocco, 2003). There are two other well-known forms of DNA, A-DNA and Z-DNA, which are a right-handed helix and a left-handed helix, respectively. It has been found alternative genomic DNA conformations that are different from the canonical B-DNA helix (Potaman & Sinden, 2005; Ohyama, 2005). These alternative DNA structures are very important for certain biological functions, such as the DNA-protein binding involved in replication, gene expression and recombination. Alternative conformations may also participate in the formation of nucleosomes and other supramolecular structures involving DNA (Lu et al., 2003; Meisch & Prioleau, 2011; Richmond & Davey, 2003).

The DNA double helix is a highly dynamic structure; consequently, the curvature of DNA cannot be construed as a static and rigid alteration (Hagerman, 1990). The flexibility of DNA can be described by the alterations in the twist angles between adjacent base pairs, called torsional flexibility, and by deviations along the axis of the double helix from a straight trajectory, characterizing the bending flexibility (Travers, 2004). This bending flexibility depends primarily on the physico-chemical properties of individual base steps and secondarily on the DNA sequence context. The number of hydrogen bonds in a base pair, the stacking energy of a given base step and the occupation of both the major and minor grooves by nitrogenous bases are the major determinants of DNA (Calladine et al., 2004; Travers, 2004).

The bending of DNA is one of the most important deformations of the DNA structure, which is universal in biology, for both storage and retrieval of information encoded in the base-pair sequence (Gimenes et al., 2008b). Many base sequences can conform to a systematic curvature in DNA; however, a series of A_{5-6} (A-tracts) repeats spaced in phase with the DNA helical repeat (10-11 bp intervals) are the most important causative sequences for the presence of intrinsically bent DNA sites. This discovery was made by Trifonov & Sussman (1980), who demonstrated the periodicity of A-tracts in genomic DNA and suggested that this observation was due to the DNA packing in chromatin. Particularly, they suggested that the AA/TT dimer has an intrinsic wedge-like shape, which would introduce intrinsic bending in DNA when repeated periodically. The model was called a

wedge model and highlighted that a series of small alterations in the roll or tilt angles between adjacent base-pair planes could be generating the bending of DNA (Figure 1). Alternatively, Wu & Crothers (1984) introduced their junction model, which assumed that A-tracts adopt an alternative non-B-DNA structure, where the cause of curvature is the deflection of the global axis of the A-tract structure from that of the adjoining B-DNA region (Figure 1). The main difference between the wedge and junction models is that the former is a nearest-neighbor dimeric model, assuming that the curvature of any dimeric step is independent of the other adjacent A-tracts, whereas the latter suggests cooperative interactions along the DNA chain. However, other DNA sequences can display an anomalous gel mobility that is characteristic of curved DNA, even in absence of A-tracts. It has been observed that the GGGCCC segment may cause curvature toward the major groove of the DNA helix, with the presence of a positive roll angle (Brukner et al., 1993). Thus, we have observed that DNA bending follows a simple rule: the purine-pyrimidine (RY) and AA/TT dimers bend predominantly into the minor groove, whereas the pyrimidine-purine and GG/CC dimers bend more frequently toward the major groove (Ohyama, 2005). From gel electrophoresis experiments, it was possible to establish three important features of curved DNA (Diekmann, 1986; Hagerman, 1985; Koo & Crothers, 1987): periodically phased A-tracts are very important for “strong” DNA curvature; the orientation of the A-tract is important because the A₄T₄-induced bending differs from that of T₄A₄; and the magnitude of DNA curvature is influenced little by the flanking sequences.

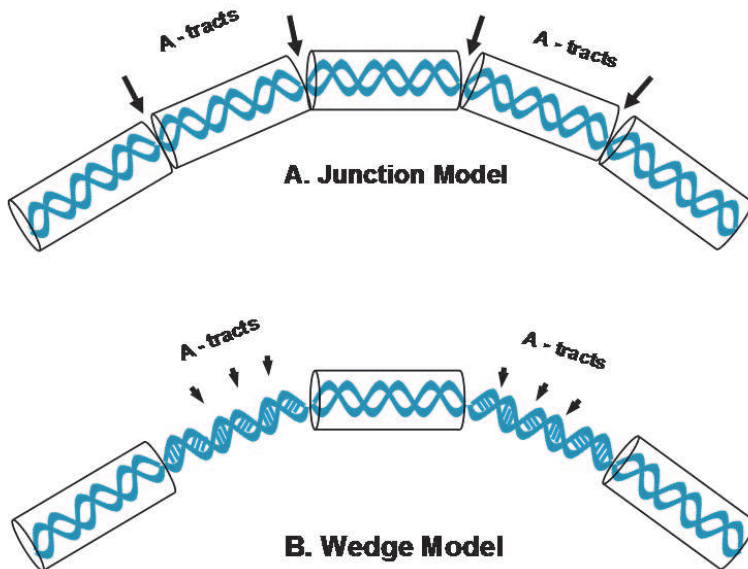


Fig. 1. Junction and Wedge models of an intrinsic DNA curvature.

3. Methods of analyzing intrinsically bent DNA

Many methods can be applied to localize intrinsically bent DNA sites, for example, circular permutation (Gimenes et al., 2008b; Wu & Crothers, 1984), computational analysis (Marilley

& Pasero, 1996; Pasero et al., 1993), and atomic force microscopy (Marilley et al., 2007). Here we present methods for analyzing bending DNA regions by computational simulation using algorithms for curvature and gel electrophoresis mobility assays, including the circular permutation strategy.

3.1 Curvature parameters calculations

The helical parameters reflect the structural characteristics of the DNA double helix, which was described initially as an ideal isotropic rod, with elastic properties independent of sequence. However, once it became clear that the conformation of DNA is dynamic, the initial isotropic representation was questioned, and the concept of anisotropic DNA was introduced. Therefore, there was a need to standardize the definitions and nomenclature of nucleic acid structure parameters. From the EMBO Workshop on DNA Curvature and Bending, the definitions of parameters used to describe the geometry of nucleic acid chains and helices and a common nomenclature for these parameters were introduced (Dickerson, 1989).

The values of helical parameters such as the ENDS ratio parameter and roll, tilt and twist rotational angles can be obtained using the computational program Map15a (Marilley & Pasero, 1996; Pasero et al., 1993), which was performed employing the algorithm of Eckdahl & Anderson (1987) and the helical parameters described by Bolshoy et al. (1991). The ENDS ratio parameter (the ratio of the axis outline of the helix to the smallest distance between the extremities of the fragment) reflects the probability of finding a bend at a determined site. Figure 2A shows an example of this application for a hypothetical segment of 800 bp, using a 120-bp window width and a 10-bp step. Values greater than or equal to 1.10 is indicative of the presence of intrinsically bent DNA sites (Milot et al., 1992).

The twist angle corresponds to a rotation around the local twist axis that runs vertically through, or near, the centers of any two neighboring base pairs. In the canonical B-DNA conformation, this angle is estimated to be approximately 32° and is shown in Figure 2B (Calladine et al., 2004; Dickerson, 1989). The roll angle (Figure 2B), described the rolling open of base pairs along their long axes. This movement compresses the major (positive roll) or minor (negative roll) grooves of the DNA helix (Calladine et al., 2004; Crothers et al., 1990). The tilt angle (Figure 2B) which is in the direction of hydrogen bonding (opening or closing towards the phosphate backbone) is the bending across the grooves, in which the rotational displacement causes a deviation of the DNA double helix (Hagerman, 1990; Ohyama, 2005; Travers, 2004).

3.2 2D Modeling

The 2D projections of the 3D trajectories from specific fragments can be obtained from computational programs such as 3D15m1 (Marilley & Pasero, 1996; Pasero et al., 1993). As described for the Map15a program, the algorithm for calculating a 3D trajectory from a nucleotide sequence was also developed by Eckdahl & Anderson (1987), and the three-dimensional coordinates of the helical axis are obtained using the parameters of the wedge model from Bolshoy et al. (1991). While using the program 3D15m1, it is possible to rotate the molecule in space in real time, and the user can obtain a 2D projection of the analyzed fragment. We can also use the server model.it, which creates 3D models of canonical or bent DNA starting from sequence data and presents the results in the form of a standard PDB file that can be viewed directly using programs such as Swiss-PDBviewer or RasMol

(Vlahovicek et al., 2003). Example 3D models of hypothetical sequences created by the model.it server are shown in Figure 3.

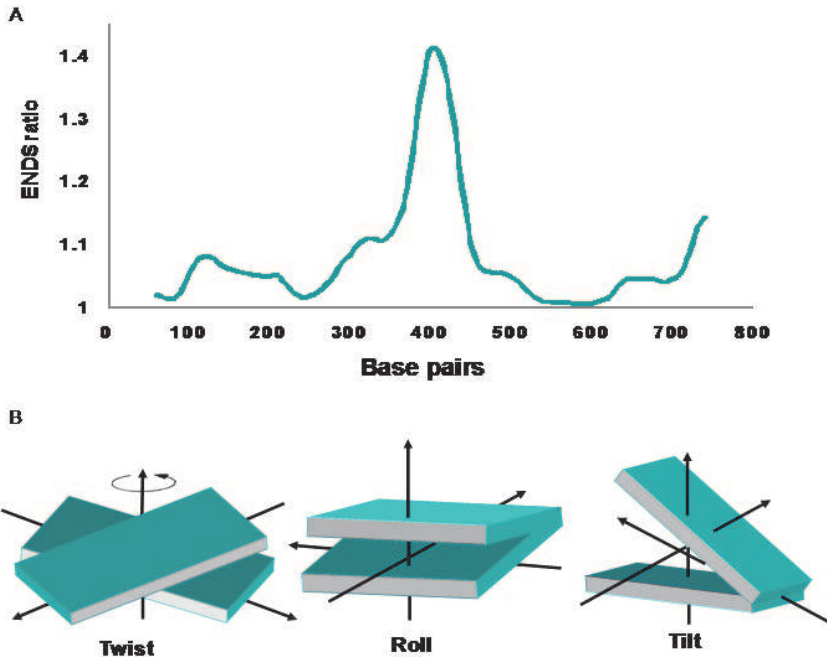


Fig. 2. Helical curvature parameters. **A.** ENDS ratio calculation graphic using the Map15a software. **B.** Rotational angles of DNA double helix, adapted from Dickerson (1989).

3.3 Experimental approaches

3.3.1 Isolation and cloning of intrinsically bent fragments

After mapping intrinsically bent DNA sites by computational analysis, regions containing bent DNA can be isolated using polymerase chain reaction (PCR). Nucleotide sequences of approximately 100 bp containing DNA bent sites are amplified from a DNA sample (approximately 50 ng/ μ l), and the primers used to amplify the DNA bent sites are designed with the restriction sites of *Xba*I and *Sal*I at the ends for posterior cloning into a circular permutation vector such as pBendBlue (Sperbeck & Wistow, 1998). The PCR amplified fragments can be cloned into any PCR cloning vector such as the pGEM-T Easy Vector System (Promega), pMOSBLUE (GE Healthcare), pTZ57R/T (Balani et al., 2010; Gimenes et al., 2009; Hägg et al., 2004; Rodríguez-Lecompte et al., 2001) or the TOPO® PCR Cloning vector (Invitrogen). The recombinant plasmids may be sent for sequencing to confirm the identity of the insert. Helpful aids for primer design include commercially available primer design software such as Oligo^R (National Biosciences, Plymouth, NC) and FastPCR© (4.0.27 versus) and online search web tools such as BLAST (NCBI, www.ncbi.nlm.nih.gov/BLAST/), IDT SciTools software OligoAnalyzer 3.0 and PrimerQuest.

SEQUENCE	NUCLEOTIDE SEQUENCE	SOURCE
A	AGGGCCCTAGAGGGGCCCTAG	Brukner et al.; 1993
B	CGGAGGGGCCCTAGAGGGGCCCTAGAGGGC CCCCCAAAAACCCCCAAAACCCCC	Brukner et al.; 1993
C	TCTCTAAAAATATATAAAAA	Bolshoy et al.; 1991
D	AAAAAAAAAAAAAAAAAAAAA	Bolshoy et al.; 1991
E	CATGTCACCGACGCATCACCG	Bolshoy, et al.; 1991
F	ATACACAGAAAATAGAAATGTCCTTAAAT TTTTATATTTTTACACTTTAAAGT	Balani et al.; 2010

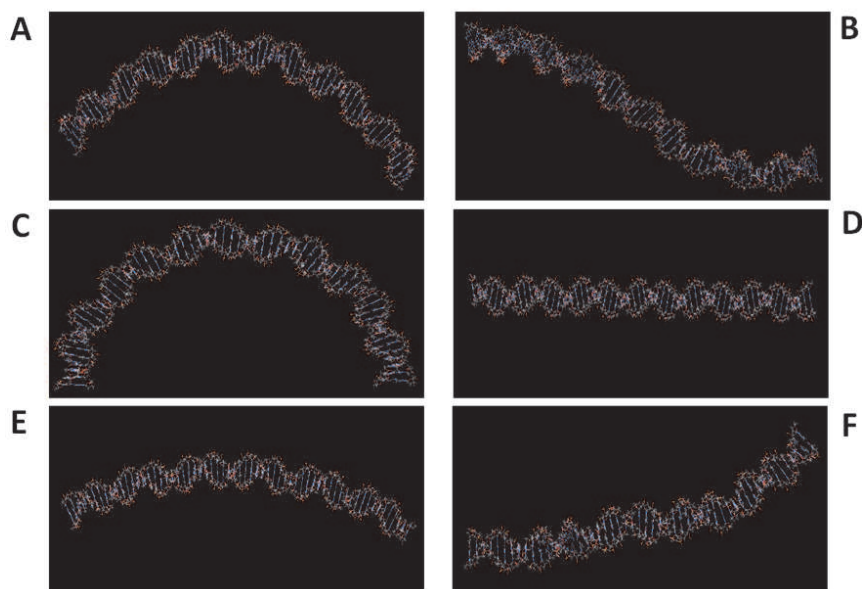


Fig. 3. A- F, 2D projection of the 3D trajectories of the DNA nucleotides sequences showed in the upper table.

3.3.2 Analysis of bending DNA fragments by gel electrophoresis

DNA fragments containing an intrinsic bent sequence can be analyzed by electrophoresis using a non-denaturing polyacrylamide gel run at a low temperature. This method contributes to the detection of alterations in the mobility of bent fragments.

Fragments isolated for restriction digestion of cloning plasmids or amplified by PCR are subjected to electrophoresis in a polyacrylamide gel. The gel concentration and the electrophoresis conditions vary depending on the fragment size. In general, for fragments of approximately 100 bp, a 12% polyacrylamide gel with 1X TBE running buffer (45 mM Tris-borate and 1 mM EDTA, pH 8.0) and a constant voltage of 5-10 V/cm at 4°C can be used. After running, the gel is stained with 1 µg/ml of ethidium bromide or another DNA intercalating agent and photographed under UV light. After running, the mobility pattern of each fragment can be obtained through the calculation of the R-value (ratio of apparent/real

fragment size) (de Souza & Ornstein, 1998; Fiorini et al., 2006b). The R-values indicate the degree of curvature for a given fragment. An R-value between 0.90 and 1.09 indicates no alterations in mobility, whereas those higher than or equal to 1.10 indicate reduced mobility, and those lower than 0.90 indicate fast mobility (Fiorini et al., 2006b; Milot et al., 1992). Fragments presenting significant R-values are then analyzed by circular permutation to determine the region responsible for the curvature.

3.3.3 Circular permutation assay

The circular permutation assay is used to determine whether bent DNA sites promote curvature in a distinct sequence context and to determine the bending-center sites on permuted fragments (Wu & Crothers, 1984).

To perform this assay, fragments containing curvature were chosen, isolated and cloned into the PCR cloning plasmids and were subcloned into the center of the duplicated polylinker of the pBendBlue vector (Sperbeck & Wistow, 1998) or other pBend plasmid such as pBend2 and pBend3 (Kim et al., 1989; Zwieb & Adhya, 1994). The pBendBlue vector contains two identical but inverted DNA segments with 17 repeated restriction sites spanning a central region containing *Sall* and *XbaI* cloning sites (Figure 4). Briefly, the insert is removed from the PCR cloning plasmid by digestion with *Sall* and *XbaI* restriction enzymes and subsequently subcloned between the *Sall* and *XbaI* sites of the pBendBlue plasmid. The generated recombinant pBendBlue plasmids are then introduced into host bacteria by means of DNA transformation and selected by a direct assay. The plasmid DNA is isolated by minipreparation and digested in independent reactions with suitable restriction enzymes, which recognize the duplicated pBendBlue polylinker, yielding a set of circularly permuted DNA fragments of identical size, but with a permuted bent position (Fiorini et al., 2006a; Gimenes et al., 2008b; Gimenes et al., 2009; Sperbeck & Wistow, 1998).

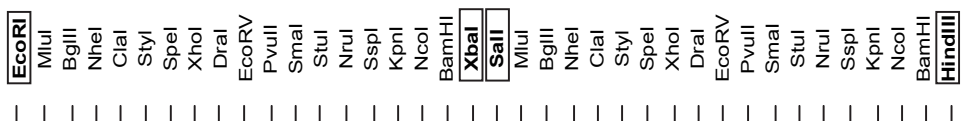


Fig. 4. pBendBlue polylinker.

3.3.4 Permuted fragment analysis by electrophoresis

Wu & Crothers (1984) have designed an elegant mobility gel electrophoresis assay to localize the bending locus of an intrinsically curved DNA fragment. De Santis et al. (1988; 1990) and Zuccheri et al. (2001) have proposed a theoretical model for DNA curvature and have shown that curvature dispersion is linearly correlated with gel electrophoretic retardation. The model has been experimentally verified and has been applied in analyzing several systems (Nair, 1998; Nair et al., 1994).

The permuted fragments have conformations that differ in the position of the bend relative to the molecular ends. Because the mobility of the fragments is a function of their intrinsic curvature, a bend near the middle of the molecule should encounter more difficulty traversing through the pores of a polyacrylamide gel than a more linear fragment. Curvature causes a slower migration compared with non-curved sequences of the same length. However, fragments containing intrinsic DNA structures near the ends migrate

faster in polyacrylamide gels (Drak & Crothers, 1991; Fiorini et al., 2001; Schroth et al., 1992) (Figure 5A).

In an experimental approach, the resulting circular permuted fragments are analyzed by electrophoresis mobility shift assay and resolved in a non-denaturing polyacrylamide gel following the electrophoresis conditions above described. After running, the relative mobility of each fragment is calculated by the ratio of the expected mobility according to the molecular weight of the fragment and the apparent mobility observed on the gel. The relative mobility of each permuted fragment is plotted against the distance (in base pairs) from the 5' end of the used restriction sites to the middle of the cloned fragment. Quantitative measurement of the relative gel mobility of a set of circularly permuted fragments allows extrapolating the position that would yield the maximum gel mobility and was therefore located at the center of the molecular bend (Wu & Crothers, 1984). The position at which maximal gel retardation should have occurred is extrapolated from the graph and is indicated by broken lines (Figure 5B).

The permuted fragments can be further analyzed theoretically to further understand their mobility behavior with the 2D path projection software, as described previously.

Transient interactions between intrinsically bent DNA sites and nucleic acids are widespread in nature. Almost all functions performed by DNA and RNA in the cellular context depend on the involvement of several associated proteins. Generally, interactions are divided into three main categories: structural, regulatory and enzymatic. Trials of DNA-protein interactions can be used to study specific regions of nucleic acids, such as curved regions (bent DNA) and linear, potentially flexible regions.

4. Possible roles of intrinsically bent DNA in replication

Statically curved DNA elements are known to be present at many replication origins, and it has been proposed that these sequence elements are important in initiating DNA replication. An analysis of the secondary structure of the replication origins of the prokaryotic organism *Xylella fastidiosa* 9a5c was previously described by our group and indicated that these replication origins display intrinsically bent DNA sites that induce a curvature in this segment (Gimenes et al., 2008a). In the same work, the *in silico* analysis of the replication origin segments from *X. fastidiosa* Temecula, *Bacillus subtilis* and *Escherichia coli* showed that all of the replication sites, with some variability in their helical parameters, displayed curved segments. In eukaryotes cells, our work was associated with gene amplified domains in developmental systems (Fiorini et al., 2001; 2006a; Gimenes et al., 2009) and induced gene amplified segments in mammalian culture cells (Balani et al., 2010). Using the developmental amplified models, we analyzed amplicons from the gene *BhC4-1* from *Bradysia hygida* (Fiorini et al., 2001), an amplified segment of the C3-22 gene from *Rhynchosciara americana* and in the segment DAFC-66D from chromosome 3 of *Drosophila melanogaster*, which contains the amplification control element ACE3 and the replication origin ori- β (Gimenes et al., 2009). In the dihydrofolate reductase (DHFR) amplicon, Altman & Fanning (2004) reported four elements that are required to initiate DNA replication at ori- β . One of them is an intrinsically bent DNA site, which provides the capacity to replicate the DNA locus ectopically. Taken together, these results indicate a relationship between secondary DNA structure and replication origins. Functional experiments and the relationship between proteins in the curved structure could be enabling us to establish a strong relationship between the DNA structure and the initiation of DNA replication in eukaryotes cells.

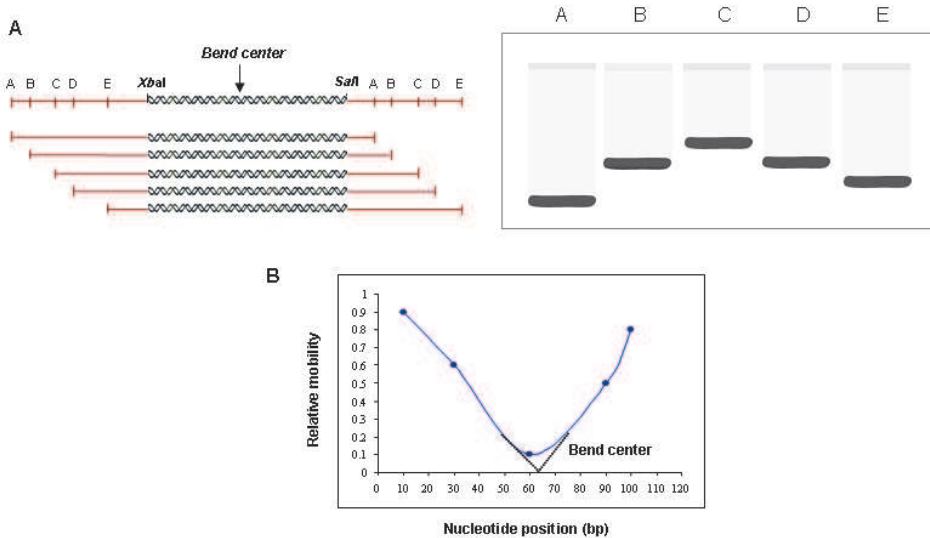


Fig. 5. **A.** Permuted fragments cloned into the duplicated polylinker of a pBend plasmid (on the left) and a schematic analysis of the electrophoresis mobility shift assay of the permuted fragments (on the right). **B.** Relative mobility versus nucleotide position for bent position determination.

5. Conclusion

Here we discussed reports in the literature concerning the presence of intrinsically bent DNA sites in regions of replication origins and the possible involvement of these sites in replication function in both prokaryotic and eukaryotic organisms. We presented *in silico* and *in vitro* structural analysis by specific software and electrophoresis mobility assays to detect and analyze intrinsically bent DNA sites in regions of replication initiation. Recent technologies for analyzing the relationship between and the binding of DNA and proteins and nucleosome occupancy in the replication domains could be used in the future in establishing the relationship among DNA secondary structure and sites of replication initiation in specialized cells.

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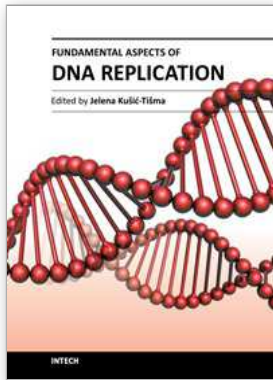
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DNA replication, the process of copying one double stranded DNA molecule to produce two identical copies, is at the heart of cell proliferation. This book highlights new insights into the replication process in eukaryotes, from the assembly of pre-replication complex and features of DNA replication origins, through polymerization mechanisms, to propagation of epigenetic states. It also covers cell cycle control of replication initiation and includes the latest on mechanisms of replication in prokaryotes. The association between genome replication and transcription is also addressed. We hope that readers will find this book interesting, helpful and inspiring.

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