

# Construction of Vector for Functional Analysis of the Intrinsically Bent DNA in the ACE3 Replicator from *Drosophila melanogaster*

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## Abstract

DNA replication is a crucial process for species survival, nevertheless it is not clear which factors define origin selection in multicellular eukaryotes. Developmental gene amplification systems, such as the one described during ovarian follicles development in *Drosophila melanogaster*, are useful tools for studying of DNA replication process in these organisms. We previously described that the well characterized third chromosome amplified domain of *D. melanogaster* displays three intrinsically bent DNA sites: *b1*, localized at an amplification control element (ACE3), *b2* and *b3*, both localized at the preferential origin *ori-β*. This proposal aimed to construct a *Drosophila* transformation vector, which contains a short deletion at the ACE3, in order to reduce the intrinsically bent DNA site *b1*, and analyze the functional role of this site in the gene amplification process. Through a series of cloning steps, we obtained a Big Parent vector derivative, containing a deletion at the positions 176 - 180 bp, inside the ACE3. The generation of a *Drosophila* transformation vector displays a reduced intrinsically bent DNA site in the third chromosome amplified domain, it will allow the analysis of the functional role of this curvature in developmental gene amplification, providing new insights on replication initiation in *D. melanogaster* and the function of intrinsically bent DNA sites.

## Keywords

DNA Replication; Gene Amplification; ACE3; Intrinsically Bent DNA; *Drosophila melanogaster*

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## 1. Introduction

The efficiency of the DNA replication process is essential for species survival. Thus, the genome should be quickly and faithfully duplicated only once per cell division. In bacteria, usually there is only one single origin of replication in the bacterial chromosome called OriC, which presents multiple consensus sequences called DnaA box, which act as interaction sites for protein DnaA, directing the local opening of the double-stranded DNA and recruitment other proteins [1] [2]. In the yeast *Saccharomyces cerevisiae*, the sites of initiation of replication (Autonomously Replicating Sequences, ARS) include a short 10 to 11 bp consensus sequence rich in A/T called ACS (ARS consensus sequence) [3]. From this organism, during late M phase and early G1 phase of cell cycle, was isolated the ORC (Origin Recognition Complex), responsible for recognizing the sites of initiation of replication and nuclear components of pre-replicative complex (pre-RC), that are necessary to indicate permission for the initiation of replication in S phase [4]. In multicellular eukaryotes, although the machinery required for the replication process is well-known, the factors determining the selection and where is localized the origins which will be fired in a genome segment are still poorly understood, and a limited number of DNA replication origins had been studied in detail. The use of different methodologies for the analysis of replication intermediates indicates no consensus sequences for these regions [5], as described only in *S. cerevisiae* and closely related species. Moreover, the selection process is influenced by the chromatin structure where the DNA replication origin is localized and the cell type [5]-[8]. The systems which are extensively used as a model for studying the replication origins of the multicellular eukaryotes are those where occurs the developmentally gene amplification. The best system studied in this research area is the *Drosophila* ovarian follicles cells and the genomic segments where the DNA puffs open in the larval Sciaridae salivary gland cells. The study of the DNA replication process is advantageous in both systems due to the high number of copies of DNA segments generated and the fact that the enzymatic machinery used is the same as described for regions not amplified [9]-[11].

In *Drosophila*, transgenic constructs, mediated via P-element transformation in ectopic location, preserve the tissue and temporal pattern of gene amplification [12]. To avoid some problems in the chromosomal position, which can affect the process in some cases, vector elements insulators can be used as the suppressor of Hairy-wing insulator protein binding site [su (Hw) BS; 13]. The better characterized *Drosophila* amplified segment is located on the third chromosome (DAFC-66D), where were initially identified the preferential ori- $\beta$  origin, an amplification control element (ACE3) located upstream of the chorionic gene S18 (CG6517), and four regions amplification enhancers, AER-a, b, c and d [14]. The elements ACE3 and ori- $\beta$  are necessary and sufficient to promote amplification of transgenic constructs with insulator element, and these elements interact in cis [15]. To identify the sequences required for amplification of this region, was analyzed transgenic lines containing deletions in different regions of ACE3, on the gene S18 and ori- $\beta$  DNA replication origin, resulting in quantitative effects on amplification levels [16]. Recently, were identified three intrinsically DNA bent sites on segment DAFC-66D. One of them was localized at ACE3 (*b1*) region and the others two in the  $\beta$ -ori origin (*b2* and *b3*) [17]. However, the functional role of these DNA bent sites remains unclear. Thus, the aim of this work was to construct a vector transformation in *Drosophila* with a short deletion in ACE3 able to disrupt the *b1* DNA bent curvature, in order to analysis the importance of this site in the gene amplification process.

## 2. Method

### 2.1. Computational Analysis

Map15a software was used for the analysis of ENDS ratio helical parameter (ratio of the length of the curve by the distances between the ends of a fragment) and the software 3D15m1 to visualize the two-dimensional projection of the three-dimensional fragment trajectory. Both the software is based on Eckdahl and Anderson algorithm and Bolshoy *et al.* [18]-[21]. The DAFC-66D 2348 bp fragment was analyzed, which includes the 320 bp from ACE3 region, a 1200 bp from S18 gene and 840 bp of the ori- $\beta$  DNA replication origin segment.

### 2.2. Transformation Vector Construction Strategy

First, the 320 bp ACE3 segment was cloned into the vector pBluescript KS<sup>+</sup> (Stratagene) in *KpnI* and *BamHI* restriction enzymes sites. The strategy to obtain a mutated sequence from ACE3 start with a special construction of complementary forward and reverse primers sequences:

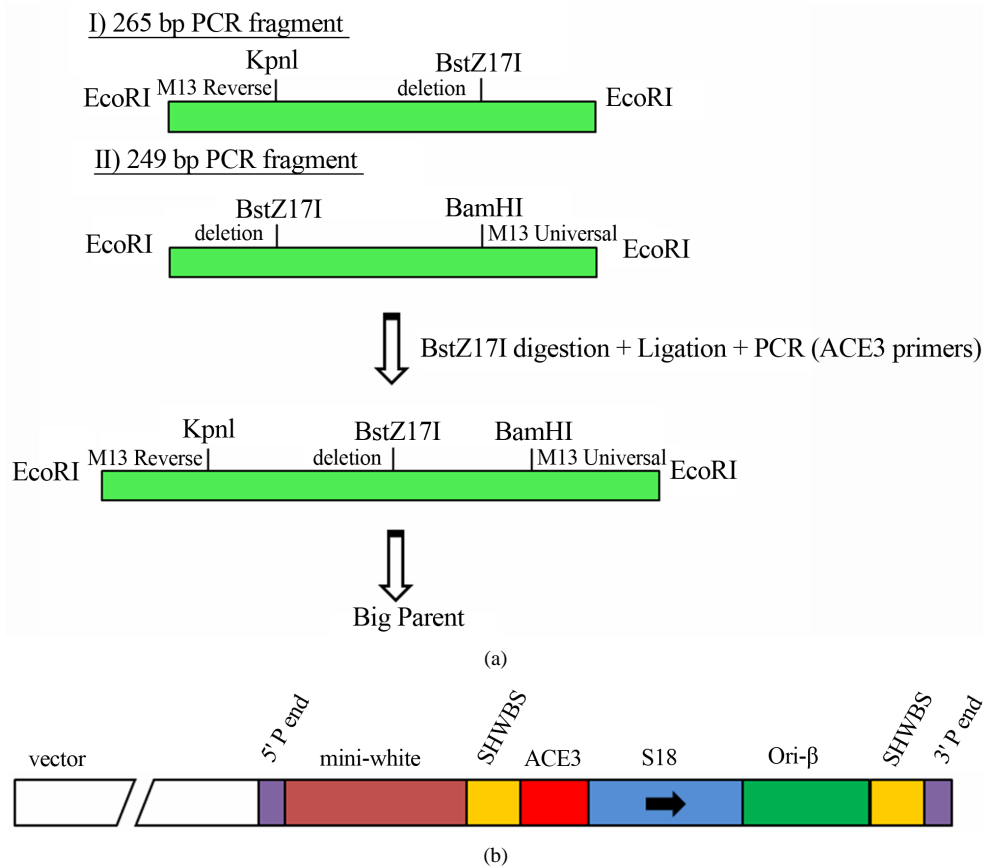
5'-GTAATTTTATCTCATTTCGTTTTGTATACAAATTC-3' and 5'-GAATTTGTATACAAAAGCAAAATGAGATAAAATTAC-3', **Figure 1**, underlined. The 5 bp deletion in ACE3 was designed in the positions 176 - 180 bp from the original ACE3 sequence, which is able to disrupt the intrinsically curvature *b1* (**Figure 1**, bold).

The substitution of a C nucleotide base for a T nucleotide in the 196 bp position was capable to promote the sequence of a new enzyme site, the *BstZ171*. For this construction, was necessary two individually PCR reactions, one using the plasmid M13 universal primer and the primer containing the deletion and another using the plasmid reverse primer plasmid and the reverse primer from the plasmid deletion. **Figure 2(a)** shows the details from these reactions.

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GGTACCCCTGAGCCTGGCCAACATCTAAATAATTTATTATTCAATTATTAAT
GTTTGCGCCCGCCATAAGCCATTACAATTTGTGTAGCGCCAATTGAATG
TTATAAAAAGCTTAGTGCGGCAGTTTGGAAAGTGGAAACGGTTGTGTTATA
ATTTTATTGTAATTTTATCTCAATTTTTTTTGCTTTTGTATAAAATTCTA
CCAACGCAGCAGAATTTTCAGGCCACTGCCTTGACTTCACTGTGTCACTGA
AAAATCGGTGTCAAGCTCTCGGCACCGTGGGGCAAAGCAACTGCAATACTG
ATCGAAACTATGCGGATCC
    
```

**Figure 1.** *Drosophila* ACE3 vector cloning strategy. This figure display the 320 pb from *Drosophila* ACE3 and the annealing primers region are underlined. The five nucleotides in bold (ATTTT) at the positions 176 - 180 bp was deleted to abolished the intrinsically DNA bent site *b1*. The nucleotide T is replaced by C (196 bp position), to created the restriction site enzyme *BstZ171*, is shadowed in gray.



**Figure 2.** (a) Cloning strategy to ensure a final vector with intrinsically DNA bent site *b1* abolished and a creation of a new restriction site enzyme *BstZ171*; (b) Scheme of the Big Parent vector, showing ACE3, S18 gene and ori-β flanked by the insulator element SHWBS.

The products were cloned separately into the vector pGEM (Promega). Both clones were cleaved with the enzyme and *Bst*Z17I and ligated together. The product was subjected to a PCR reaction with ACE3 primers (forward 5'-GGTACCCTGAGCCTGGCCAAC-3' and reverse 5'-GGATCCGCATAGTTTCGATC-3') with the objective of generating a fragment of 320 containing the 5bp deletion and a new *Bst*Z17I restriction site. This product was cloned into the pGEM vector, and then was moved to the pBluescript KS<sup>+</sup> vector containing a fragment of 2.8 kb vector Big Parent via the *Kpn*I and *Bam*HI sites. Then, the original 2.8 kb segment of Big Parent ACE3 without the deletion was replaced by the fragment containing the deletion via *Xho*I and *Not*I sites.

The products resulting from each step of the construction were subjected to sequencing reactions with DYE-namic<sup>TM</sup> ET Terminator kit (Amersham Biosciences) and the products were analyzed on MegaBACE 1000 sequencing system, in order to confirm the *b1* site deletion and the new site *Bst*Z17I.

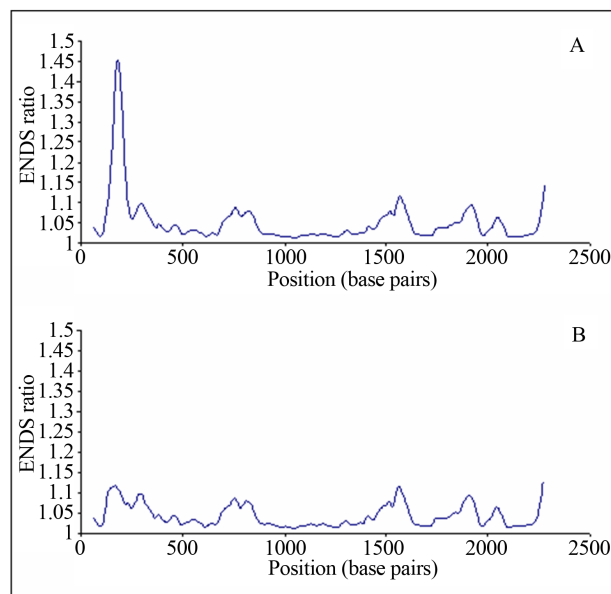
The *Drosophila* Big Parent transforming vector was kindly provided by Dr. John Tower (University of Southern California, USA). This vector contains the ACE3, the S18 gene and ori- $\beta$  element flanked by insulator SHWBS (Figure 2(b)).

### 3. Results

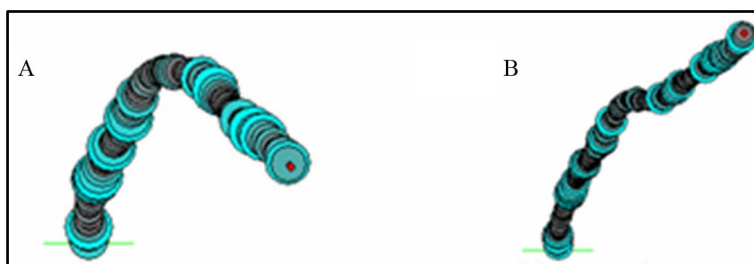
The computational analysis of the 2348 bp fragment from *Drosophila* DAFC-66D with the software Map15a and 3D15m1 revealed that deletion of the five nucleotides, ATTTT, from positions 176 - 180 bp within ACE3 region cause a significant reduction in the curvature of the intrinsically DNA bent site *b1* (Figures 3 and 4).

Thus, the first step in the strategy to generate a transforming vector taking this unstructured intrinsically bent DNA site *b1*, was to construct a primer pair complementary introducing a deletion of 5 bp. The last step of the construction was to replace the original fragment of 2.8 kb within the Big Parent vector for a similar fragment containing the deletion. At the end, it has been isolated 12 positive clones and for all of them was extracted the plasmid DNA, which were subjected to restriction analysis, which selected six clones for sequencing.

The results of the sequencing reaction confirmed the presence of deletion and a new site for *Bst*Z17I restriction enzyme in all clones (Figure 5). Thus, one of the clones will be used to obtain transgenic *Drosophila* lines for subsequent functional analysis of intrinsically bent DNA site *b1* curvature on amplification processes in the segment analyzed.



**Figure 3.** ACE3 curvature analysis. The ENDS ratio analysis by Map15a software from the 2348 pb sequence that contains the 320 pb ACE3, ~1200 pb from S18 corionic gene and 840 pb from the ori- $\beta$  replication origin. (A) original sequence; (B) deleted sequence, that shows a significative reduction from the intrinsically DNA bent site *b1* ENDS ratio value (arrow).



**Figure 4.** Two-dimensional (2D) projection of the 3D trajectories from ACE3 320 bp sequence by 3D15m1 software. (A) ACE3 bending curved structure; (B) straight ACE3 sequence with 176 - 180 bp deletion.

C1	-----ACTTAATA--TTATTATTCATTATATA--TGTTTGCGCC	35
C2	-----CTTAATA--TTATTATTCATTATATA--TGTTTGCGCC	34
C3	-----CTTAATA--TTATTAT--CATTATATA--TGTTTGCGCC	33
C4	-----CTTAATA--TTATTAT--CATTATATA--TGTTTGCGCC	33
C5	-----CTTAATA--TTATTAT--CATTATATA--TGTTTGCGCC	33
C6	-----ACTAATA--TTATTATTCATTATATA--TGTTTGCGCC	34
ACE3	GGTACCCCTGAGCCTGGCCAAACATCTAAATAATTATTATTCATTATATAATGTTTGCGCC	60
	**** *	
C1	CACCCATAAGCCATTCACAATTTGTGTAGCGCCAATTGAATGTTATAAAAAGCTTAGTG	95
C2	CACCCATAAGCCATTCACAATTTGTGTAGCGCCAATTGAATGTTATAAAAAGCTTAGTGC	94
C3	CACCCATAAGCCATTCACAATTTGTGTAGCGCCAATTGAATGTTATAAAAAGCTTAGTGC	93
C4	CACCCATAAGCCATTCACAATTTGTGTAGCGCCAATTGAATGTTATAAAAAGCTTAGTGC	93
C5	CACCCATAAGCCATTCACAATTTGTGTAGCGCCAATTGAATGTTATAAAAAGCTTAGTGC	93
C6	CACCCATAAGCCATTCACAATTTGTGTAGCGCCAATTGAATGTTATAAAAAGCTTAGTGC	94
ACE3	CGCCATAAGCCATTCACAATTTGTGTAGCGCCAATTGAATGTTATAAAAAGCTTAGTGC	120
	* *****	
C1	CGGGCAGTT--GGGAAAGTGGAACGGTGTGTATTATAATTTTATTGTAATTTTATCTCA--	152
C2	--GGCAGTT--GGGAAAGTGGAACGGTGTGTATTATAATTTTATTGTAATTTTATCTCA--	149
C3	--GGCAGTT--GGGAAAGTGGAACGGTGTGTATTATAATTTTATTGTAATTTTATCTCA--	148
C4	--GGCAGTTTGGGAAAGTGGAACGGTGTGTATTATAATTTTATTGTAATTTTATCTCA--	149
C5	--GGCAGTTTGGGAAAGTGGAACGGTGTGTATTATAATTTTATTGTAATTTTATCTCA--	149
C6	--GGCAGTT--TGGAAAGTGGAACGGTGTGTATTATAATTTTATTGTAATTTTATCTCA--	149
ACE3	--GGCAGTT--TGGAAAGTGGAACGGTGTGTATTATAATTTTATTGTAATTTTATCTCAAT	177
	*****	
C1	---TTTTGCTTTTGTATACAAATTTCTACCAACGCAGCAGAATTTTCAGGCCACTGCCCTTG	209
C2	---TTTTGCTTTTGTATACAAATTTCTACCAACGCAGCAGAATTTTCAGGCCACTGCCCTTG	206
C3	---TTTTGCTTTTGTATACAAATTTCTACCAACGCAGCAGAATTTTCAGGCCACTGCCCTTG	205
C4	---TTTTGCTTTTGTATACAAATTTCTACCAACGCAGCAGAATTTTCAGGCCACTGCCCTTG	206
C5	---TTTTGCTTTTGTATACAAATTTCTACCAACGCAGCAGAATTTTCAGGCCACTGCCCTTG	206
C6	---TTTTGCTTTTGTATACAAATTTCTACCAACGCAGCAGAATTTTCAGGCCACTGCCCTTG	206
ACE3	<u>TTTTTTGCTTTTGTATATAAATTTCTACCAACGCAGCAGAATTTTCAGGCCACTGCCCTTG</u>	237
	*****	
C1	ACTTCACTGCGTCACTGAAAAATCGGTGTCAAGCTCTCGGCACCCTGGGCAAAGCAACT	269
C2	ACTTCACTGCGTCACTGAAAAATCGGTGTCAAGCTCTCGGCACCCTGGGCAAAGCAACT	266
C3	ACTTCACTGCGTCACTGAAAAATCGGTGTCAAGCTCTCGGCACCCTGGGCAAAGCAACT	265
C4	ACTTCACTGCGTCACTGAAAAATCGGTGTCAAGCTCTCGGCACCCTGGGCAAAGCAACT	266
C5	ACTTCACTGCGTCACTGAAAAATCGGTGTCAAGCTCTCGGCACCCTGGGCAAAGCAACT	266
C6	ACTTCACTGCGTCACTGAAAAATCGGTGTCAAGCTCTCGGCACCCTGGGCAAAGCAACT	266
ACE3	ACTTCACTGTGTCACTGAAAAATCGGTGTCAAGCTCTCGGCACCCTGGGCAAAGCAACT	297
	*****	
C1	GCAATACTGATCGAAACTATGCG-----	292
C2	GCAATACTGATCG-----	279
C3	GCAATACTGATCGAAAC-----	282
C4	GCAATACTGATCGAAACTATGCG-----	290
C5	GCAATACTGATCGAAACTATGCG-----	289
C6	GCAATACTGATCGAAACTATGCG-----	290
ACE3	GCAATACTGATCGAAACTATGCGGATCC	325
	*****	

**Figure 5.** ACE3 sequence alignment. The sequence of the six modified ACE3 clones (C1-6), with the original ACE3 sequence is showed. The 5 bp deletion is underlined and the new restriction site *BstZ17I* is shaded.

## 4. Discussion

Intrinsic curvature of DNA can be found in promoter regions of prokaryotes and eukaryotes replication origins and transcription regions (reviewed in [22]). Bramhill and Kornberg [23] proposed a model involving both discrete sites of intrinsically DNA bent in replication origins of prokaryotes, which can act at the opening of double-stranded DNA mediated by protein DnaA. *Saccharomyces cerevisiae* intrinsically DNA bent sites, occurs naturally in the initiation of replication sites (ARS), and can be bent functionally replaced by synthetic DNA [24]. A study in the amplified dihydrofolate reductase (DHFR) locus in Chinese hamster cultured cells identified a site of intrinsically bent DNA as one of the four elements required for the activity of ori- $\beta$  [25].

Such sites can make easier or help the unwinding of the double helix target and the interaction of proteins in the processes of transcription and DNA replication, since DNA usually intrinsically curvatures are formed in regions rich in A/T [26], and some proteins such as HMGs, which bind, preferentially, an unusual DNA structures [27]. However, the function of these sites is still unclear, and the analysis of bends present in the amplified locus on *Drosophila melanogaster* chromosome 3 using the methodology of P-element mediated transformation, may contribute to a better understanding of the functional role of intrinsic curvatures DNA.

The vector constructed can be used to obtain a transgenic line of *Drosophila* in order to assess the level of amplification of the element containing the deletion in ACE3 and determine whether the curvature present in this region shows any role in the activation of preferential ori- $\beta$  origin. The absence of this region drastically reduces element ACE3 amplification levels [28], and it was observed that this element interacts in cis with this DNA replication origin [15]. Austin *et al.* [29] observed that the ACE3 is sufficient for localization of ORC in vivo, making compelling analysis in the role of the b1 curvature in the association of this region with the ORC from the methodology for chromatin immunoprecipitation. Remus *et al.* [30] showed an increased affinity to DmORC ACE3 and ori- $\beta$  segments with negative supercoiling *in vitro*, demonstrating the possible influence of topological properties in the process of DNA replication initiation.

In addition, our work could be also evaluated the b1 binding curvature with Myb complex proteins, which presents role in the nucleation of pre-RC complex in *Drosophila*. Members of this complex specifically bind to the ori- $\beta$  and ACE3 [31] [32], and consensus sequences for the association of proteins Dm-myb and Mip120 are present at the ends of curvature of ACE3. It is possible to formulate the hypothesis that a conformational change, resultant from the interaction between the complex and the Myb site, may increase the affinity of the complex pre-RC for the region. It may also be investigated the role of proteins that exhibit preferential binding to unusual DNA structures like the HMGs, since it was observed that HMGB1 protein may have a regulatory role in the process of DNA replication [33].

The construction of a *Drosophila* transformation vector containing a region of an unstructured intrinsically DNA curvature at an amplified locus during development to allow analysis of the functional role of this curvature in the process of gene amplification in order to contribute to a better understanding of the process initiation of replication in multicellular eukaryotes and the functional role of intrinsic DNA bends.

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